G.

School of Biomedical Sciences

STRUCTURE AND FUNCTION OF THE INSULIN RECEPTOR: ITS ROLE DURING LACTATION AND FOETAL DEVELOPMENT

Domenica Deleo BSc(Hons) (Curtin)

This thesis is presented as part
of the requirements for the award of the
Degree of Doctor of Philosophy
of
Curtin University of Technology

July 1994

I would like to dedicate this thesis to my parents,
who were my first teachers.

ACKNOWLEDGMENTS

I wish to sincerely thank my supervisor, Associate Professor Erik Helmerhorst, who has supported and encouraged me throughout this project. His guidance, knowledge and friendship have been invaluable over the past years.

I would like to thank the Head of the School of Biomedical Sciences, Associate Professor John Wetherall for his support and allowing me to carry out this study within the School. I also wish to acknowledge the financial support of the Australian government through the Australian Post-graduate Research Award Scheme.

I would like to thank the staff and students of the School of Biomedical Sciences for their support, technical assistance, advice and friendship afforded to me during this study. In particular I would like to thank Steve Dawson, master purveyor of fine chemicals and equipment, and Associate Professor Ian Lee for his constant encouragement, particularly during my undergraduate years here at Curtin. My sanity (or lack thereof) at this particular time, has been helped along by my fellow post-graduate colleagues, who have been a constant source of entertainment and support - and for which, they are duly thanked.

I thank my seemingly endless and constantly overwhelming network of family and some very dear friends for their constant faith in me and their well-timed diversionary tactics.

Finally, I wish to sincerely thank my parents, Gaetano and Marina Deleo, and Peter, Josie, Tiziana and Mason - my family, for their love and unfailing support.

CONTENTS

	Page
List of figures	i
List of tables	iii
Abbreviations	iv
Summary	vi

CHAPTER ONE LITERATURE REVIEW

1.1 Insulin and the Insulin Receptor

1.1.1	The insulin gene	1
1.1.2	Insulin synthesis	2
1.1.3	Insulin secretion	3
1.1.4	The action of insulin	4
1.1.5	Insulin and diabetes mellitus	6
1.1.6	The insulin receptor	7
1.1.7	The insulin receptor gene	8
1.1.8	Regulation of insulin receptor gene transcription	8
1.1.9	The insulin receptor signal peptide	12
1.1.10	The insulin receptor α-subunit	12
1.1.11	Alternative splicing of the α-subunit of the insulin receptor	12
1.1.12	The insulin receptor β-subunit	16
1.1.13	Post-translational processing of the insulin receptor	18
1.1.14	The heterotetrameric complex of the functional insulin receptor	20
1.1.15	The insulin-insulin receptor interaction	21
1.1.16	Insulin-stimulated signal transduction	21
1.1.17	Guanosine-triphosphate binding proteins in insulin signal transduction	27
1.1.18	The role of protein phosphatases in the regulation of the insulin receptor tyrosine kinase activity and post-receptor signalling	28

1.2 Insulin Action during Foetal Development, Pregnancy and Lactation

1.2.1	Mammary gland morphology	29
1.2.2	Maternal metabolism during pregnancy and lactation	30
1.2.3	Insulin resistance during pregnancy	31
1.2.4	Insulin action during lactation	32
1.2.5	Insulin action in the foetus	33

1.3 The Present Study 35

Page

CHAPTER TWO PURIFICATION OF A14-TYROSYL[^{125}I]-IODO-INSULIN USING C18 REVERSE PHASE CARTRIDGES

2.1 Introduction 36
2.2 Experimental Procedures
2.2.1 Preparation of zinc-free insulin 37
2.2.2 Iodination of zinc-free insulin 38
2.2.3 Purification of A14-tyrosyl[^{125}I]iodoinsulin: a novel procedure 38
2.2.4 High performance liquid chromatography analysis 39
2.2.5 N-terminal amino acid sequencing of purified A14-tyrosyl[^{125}I]iodoinsulin 39
2.2.6 Preparation of human placental microsomal membranes 40
2.2.7 Competitive displacement binding studies 41
2.2.8 Analysis of binding data 41
2.3 Results 42
2.4 Discussion 51

CHAPTER THREE STRUCTURE OF THE INSULIN RECEPTOR IN MAMMARY AND LIVER TISSUES

3.1 Introduction 55
3.2 Experimental Procedures
3.2.1 Animals 56
3.2.2 Specialised reagents 56
3.2.3 Radioiodination of $N^{\epsilon B29}$-mono[azidobenzoyl]insulin 56
3.2.4 Preparation of liver plasma membranes 57
3.2.5 Preparation of mammary membranes 58
3.2.6 Estimation of protein concentration 58
3.2.7 Measurement of 5'-nucleotidase activity 59
3.2.8 Photoaffinity labelling of plasma membranes 59
3.2.9 Neuraminidase and glycopeptidase F treatment of the eluted 125 kDa/130 kDa proteins from mammary and liver tissues 60
3.3 Results 61
3.4 Discussion 69

Page

CHAPTER FOUR ALTERNATIVE SPLICING OF THE INSULIN RECEPTOR MESSENGER RNA

4.1 Introduction 72

4.2 Experimental Procedures

4.2.1 Animals 73

4.2.2 RNA extraction 74

4.2.3 Oligonucleotide primers 74

4.2.4 Complimentary DNA synthesis 75

4.2.5 Quantitation of the cDNA yield 75

4.2.6 PCR amplification of insulin receptor cDNA 76

4.2.7 PCR positive control 77

4.2.8 Structural analysis of the exon 11 encoded peptide of the rat insulin receptor 77

4.3 Results

4.3.1 Evaluation of RNA integrity 78

4.3.2 Estimation of RNA concentrations 78

4.3.3 Assessment of primer integrity 81

4.3.4 Optimisation of the PCR protocol for the amplification of rat insulin receptor cDNA

4.3.4.1 PCR cycle number 81

4.3.4.2 PCR primer concentration 81

4.3.4.3 Characterisation of annealing temperature 84

4.3.4.4 Incorporation of [α-^{32}P]dCTP into the PCR product 84

4.3.4.5 Evaluation of the relative rates of amplification of the PCR products which encode the IR-A and IR-B insulin receptor isoforms 86

4.3.5 Distribution of IR-A and IR-B in mammary and liver tissues from adult, pregnant and lactating animals 86

4.3.6 Distribution of IR-A and IR-B during development in rat tissues 90

4.3.7 Effect of streptozotocin-induced diabetes on the IR-A and IR-B isoform distribution in adult female rats 92

4.4 Discussion

4.4.1 Development of the reverse transcriptase-polymerase chain reaction 94

4.4.2 Distribution of IR-A and IR-B in rat tissues 96

Page

CHAPTER FIVE INSULIN-INSULIN RECEPTOR BINDING IN MAMMARY AND LIVER TISSUES

5.1 Introduction 105

5.2 Experimental Procedures

5.2.1 Animals 106

5.2.2 Preparation of membranes from rat mammary and liver tissues 106

5.2.3 Insulin binding to mammary and liver membranes 106

5.2.4 Derivation of ligand affinity constants and receptor numbers using Scatchard analysis 107

5.2.5 Derivation of the concentration of hormone-receptor complexes 108

5.2.6 Analysis of binding data 109

5.2.7 A14-tyrosyl[^{125}I]iodoinsulin degradation 109

5.2.8 Measurement of serum insulin concentrations 109

5.3 Results

5.3.1 Enrichment of plasma membranes 110

5.3.2 Validation of hormone-receptor binding experiments

5.3.2.1 Time course of insulin binding 110

5.3.2.2 Assessment of insulin degradation 113

5.3.3 Insulin binding in purified mammary and liver membranes 113

5.3.4 Estimation of the insulin binding affinity of liver plasma membranes from nulliparous rats 116

5.4 Discussion 121

CHAPTER SIX CONCLUDING COMMENTS 128

REFERENCES 133

APPENDIX A Deleo D.T., Helmerhorst E. (1992) Purification of A14-tyrosyl [^{125}I] iodoinsulin using C18 reverse phase cartridges. Analytical Biochemistry 206: 207-210

APPENDIX B Abstract (SP57) "The structure of the mammary insulin receptor" from the Proceedings of the 35th Annual Conference of the Australian Society for Biochemistry and Molecular Biology, Canberra 1991

LIST OF FIGURES

		Page
CHAPTER ONE		
Figure 1.1	Organisation of the human insulin receptor gene	9
Figure 1.2	Transcription and translation of the human insulin receptor gene	13
Figure 1.3	Two hypothetical mechanisms for insulin signal transduction	22
CHAPTER TWO		
Figure 2.1	High performance liquid chromatography of an insulin iodination mixture	43
Figure 2.2	Step-wise elution of iodoinsulin derivatives on a C18 cartridge	44
Figure 2.3	Rechromatography of the A14-tyrosyl[^{125}I]iodoinsulin purified using a C18 cartridge	45
Figure 2.4	N-terminal amino acid sequencing of the purified iodoinsulin derivatives	47
Figure 2.5	Comparison of C18 cartridge-purified A14-tyrosyl[^{127}I]-iodoinsulin and native insulin binding affinity	48
Figure 2.6	Stability of the C18 cartridge-purified A14-tyrosyl[^{125}I]-iodoinsulin over a 60 day period	50
CHAPTER THREE		
Figure 3.1	A typical protein molecular weight marker standard curve	62
Figure 3.2	The effect of increasing native insulin concentrations on the specificity of [^{125}I]-N$^{\varepsilon B29}$-MABI labelling	63
Figure 3.3	Photoaffinity labelled α-subunits of the rat mammary and liver insulin receptors	64
Figure 3.4	The effect of neuraminidase on the mobility of the photoaffinity labelled mammary and liver insulin receptors	65
Figure 3.5	The effect of glycopeptidase F on the 125 kDa photoaffinity labelled protein band from rat mammary membranes	67
Figure 3.6	The effect of endogenous mammary tissue proteases on liver plasma membranes	68

Page

CHAPTER FOUR

Figure 4.1	RNA isolated from mammary and liver tissues from day 7 lactating Wistar rats	79
Figure 4.2	A typical standard curve from the fluorimetric measurement of RNA concentration	80
Figure 4.3	Assessment of primer quality	82
Figure 4.4	The effect of PCR cycle number on the amplification of IR-A and IR-B cDNA	83
Figure 4.5	The effect of annealing temperature on the amplification of IR-A and IR-B cDNA	85
Figure 4.6	Assessment of the relative rates of IR-A and IR-B cDNA amplification	87
Figure 4.7	Distribution of IR-A and IR-B PCR products in mammary and liver tissues during pregnancy and lactation	88
Figure 4.8	The relative expression of IR-B mRNA in mammary and liver tissue during pregnancy and lactation	89
Figure 4.9	The relative expression of IR-A mRNA in foetal and neonatal rat tissues	91
Figure 4.10	The relative expression of IR-B mRNA in tissues from streptozotocin-treated rats	93
Figure 4.11	Insulin receptor isoform expression and circulating corticosteroid concentrations during foetal ontogeny and lactation	99
Figure 4.12	Predictions of the hydrophilicity, antigenic probability and secondary structure within the twelve amino acid sequence encoded by exon 11 of the rat insulin receptor gene	102

CHAPTER FIVE

Figure 5.1	Binding of A14-tyrosyl[^{125}I]iodoinsulin to liver membranes as a function of time	112
Figure 5.2	Scatchard plots from mammary and liver membranes from lactating Wistar rats (4°C)	114
Figure 5.3	Scatchard plots from mammary and liver membranes from lactating Wistar rats (37°C)	117
Figure 5.4	Scatchard plots from liver plasma membranes from 8 week old, nulliparous Wistar rats (4°C)	119

LIST OF TABLES

Page

CHAPTER ONE

Table 1.1	The effect of insulin on specific enzymes involved in metabolism	5
Table 1.2	Distribution of the IR-A and IR-B insulin receptor isoforms in human and rat tissues	15
Table 1.3	Potential substrates for the insulin receptor tyrosine kinase	24

CHAPTER FIVE

Table 5.1	Mammary and liver membrane isolation	111
Table 5.2	Data from the analysis of insulin binding at 4°C in mammary and liver membranes from day seven lactating Wistar rats	115
Table 5.3	Data from the analysis of insulin binding at 37°C in mammary and liver membranes from day seven lactating Wistar rats	118
Table 5.4	Data from the analysis of insulin binding at 4°C to liver plasma membranes from 8 week old, nulliparous Wistar rats	120

ABBREVIATIONS

The following is a list of abbreviations used throughout this thesis. All abbreviations are defined on first usage within the text.

ATP	adenosine triphosphate
bp	base pairs (of nucleotides)
cAMP	cyclic adenosine monophosphate
chloramine-T	N-chloro-p-toluene sulphonamide (sodium salt)
CHO	Chinese hamster ovary
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEPC	diethyl pyrocarbonate
dGTP	deoxyguanosine triphosphate
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
Glut	glucose transporter(s)
G-protein	guanosine triphosphate binding protein
GRE	glucocorticoid response element
HEPES	4-(2-hydroxy ethyl)-1-piperazine ethane-sulphonic acid
Ho	total hormone concentration (M)
HPLC	high performance liquid chromatography
IC_{50}	concentration required to result in 50 % inhibition
IDDM	insulin-dependent diabetes mellitus
IGF-I	insulin-like growth factor 1
IGF-II	insulin-like growth factor 2
IR-A	insulin receptor A (excluding the exon 11 encoded region)
IR-B	insulin receptor B (including the exon 11 encoded region)
IRNF-I	insulin receptor nuclear factor 1
IRNF-II	insulin receptor nuclear factor 2

IRS-1	insulin receptor substrate 1
Ka	association constant
kb	kilo bases
Kd	dissociation constant
kDa	kilo daltons
MAP kinase	mitogen-activated protein
Mr	relative molecular mass
$N^{\varepsilon B29}$-MABI	$N^{\varepsilon B29}$-mono(azidobenzoyl)insulin
NIDDM	non-insulin-dependent diabetes mellitus
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDH	pyruvate dehydrogenase
PI-3'-kinase	phosphatidylinositol-3' kinase
PITC	phenylisothiocyanate
PMSF	phenylmethylsulphonyl fluoride
RNAsin	RNase inhibitor
Ro	total receptor concentration (M)
RT	reverse transcriptase
SDS	sodium dodecyl sulphate
SH2	*src* -homology 2
TCA	trichloroacetic acid
TEA	triethylamine
TFA	trifluoroacetic acid
Tris	tris(hydroxymethyl)amino methane
(v/v)	volume per volume
WHO	World Health Organisation
(w/v)	weight per volume

SUMMARY

Prior to the commencement of this study in 1990, a number of reports had appeared in the literature describing the importance of insulin action during lactation in mammals (see Chapter 1). These studies investigated the changes in circulating insulin and glucagon concentrations during lactation, the relative numbers of insulin receptors in insulin-sensitive tissues, and glucose utilisation by these tissues. However, at that time, no information was available on the structure of the mammary insulin receptor. The rationale for undertaking this study was to characterise the structure of the rat mammary insulin receptor as a means of furthering our understanding of the role insulin plays during lactation.

An initial requirement of this study was the development of a method for the convenient and inexpensive preparation of A14-tyrosyl[^{125}I]iodoinsulin. A14-tyrosyl[^{125}I]iodoinsulin displays binding characteristics which are virtually indistinguishable from the native hormone, which is a necessary requirement for tracers which are to be used in binding studies. In Chapter 2, I describe a method for the purification of A14-tyrosyl[^{125}I]iodoinsulin from a mixture of iodinated insulin molecules which are produced following oxidation by chloramine-T in the presence of Na^{125}iodine. In this method I employed disposable cartridges packed with a C18 support matrix to which the iodinated insulin molecules are readily adsorbed when in an aqueous solution. A14-tyrosyl[^{125}I]iodoinsulin absorbed most strongly to the C18 matrix and unwanted products were removed through a sequence of washes prior to the specific elution of the A14-tyrosyl[^{125}I]iodoinsulin derivative using a buffer containing 50% (v/v) acetonitrile. This product was unambiguously shown to be A14-tyrosyl[^{125}I]iodoinsulin by N-terminal amino acid sequencing. The quality of this radiolabel compared favourably with commercially available A14-tyrosyl[^{125}I]iodoinsulin preparations both in terms of specific activity and stability

upon storage at -20°C. Furthermore, a modified method based on this protocol has been used in our and other laboratories for the isolation of other iodinated peptides with highly satisfactory results.

I have established that the size of the α-subunit of the rat mammary insulin receptor is significantly diminished compared with the liver insulin receptor (125 kDa versus 130 kDa). This difference in size was present throughout all stages of lactation and was not due to proteolysis of a larger form. Furthermore, I demonstrated that both the mammary and liver insulin receptor α-subunits migrated equally on PAGE following treatment with neuraminidase, indicating that the apparent size difference may be accounted for by a variation in the extent of receptor sialation. Treatment of the mammary insulin receptor α-subunit with glycopeptidase F demonstrated that the size of the aglycoreceptor (100 kDa) was similar to that described for insulin receptors from other insulin-sensitive tissues.

I characterised the distribution of mRNA encoding the two, naturally-occurring insulin receptor isoforms in mammary tissue throughout all stages of pregnancy and lactation. These insulin receptor isoforms differ due to the absence (IR-A) or presence (IR-B) of a 12 amino acid peptide, encoded by exon 11 of the insulin receptor gene, and located near the C-terminus of the insulin receptor α-subunit. Mammary tissue predominantly expressed IR-A mRNA in contrast to liver tissue, which almost exclusively expressed IR-B mRNA. Furthermore, the ratio of IR-A to IR-B mRNA in mammary tissue changed significantly during the first week post-partum whilst the distribution of IR-A and IR-B mRNA in the liver remained constant throughout pregnancy and lactation. This difference in insulin receptor isoform expression between mammary and liver tissue also contributed to the estimated size difference between the insulin receptor α-subunits from these two tissues. In addition, I characterised the expression of IR-A and IR-B mRNA in several different tissues obtained from rats on day 14 of gestation through to 7 days post partum. I established that the splicing mechanism is functional at least as early as day 14 of gestation,

suggesting a possible role for the preferential expression of a particular insulin receptor isoform during organogenesis. I observed that IR-A mRNA was the predominant isoform in all foetal tissue studied, and the proportion of this isoform declined as the animal matured. These changes were significant in cardiac muscle, kidney and most dramatic in the liver where the expression of IR-A mRNA changed from 53% in the 21 day old foetus (the day before parturition) to 13% in the 1 day old neonate. These results suggest that the splicing mechanism which generates the receptor isoforms is subject to acute hormonal and/or metabolic control.

The current literature suggests that the carbohydrate moieties of the insulin receptor affects its affinity for insulin. Furthermore, the IR-A and IR-B isoforms have been shown to display a 2-fold difference in their insulin binding affinity when expressed in heterologous cell lines such at CHO cells or Rat-1 fibroblasts. Since both glycosylational and isoform distribution differences were evident between mammary and liver tissues, the insulin binding affinities of these receptors were compared. Estimates of the binding affinity parameters were performed at both 4°C and 37°C. At both temperatures the equilibrium binding constants for mammary and liver tissues were not significantly different suggesting that structural variations of the mammary insulin receptor had no effect on the insulin binding affinity under the conditions described in this study. Comparison of the 4°C and 37°C binding data showed that the mammary insulin receptor exhibited complex, temperature-dependent binding characteristics, similar to those previously described for the liver insulin receptor, and entirely consistent with the presence of a temperature-dependent regulatory protein that affects insulin binding.

CHAPTER ONE

LITERATURE REVIEW

This review will be presented under two broad categories; (i) Insulin and the insulin receptor and, (ii) Foetal development, pregnancy and lactation. Information will be presented, in the first part, as a summary of the current knowledge of the insulin and insulin receptor genes, and the structure and functions of the proteins they encode. Secondly, the role of insulin during pregnancy and lactation in maternal tissues, and during foetal ontogeny will be discussed.

1.1 INSULIN AND THE INSULIN RECEPTOR

1.1.1 The insulin gene

Human insulin is encoded by a 1430 base pairs (bp) region of DNA located on chromosome 11 and consisting of 3 exons (reviewed by Espinal, 1989). In rodents, the insulin gene has been duplicated with the second intron absent from the additional gene. Exon 1 begins with a CAP site and encodes a region that is transcribed but not translated. Exon 2 encodes the prepeptide, the B-chain of insulin and part of the C-peptide. Exon 3 encodes the remaining part of the C-peptide, the A-chain and an untranslated region. Gene transcription is regulated by the TATA box located 24 bp upstream, and a CAAT box found 79 bp upstream from the CAP site. Two control elements, a promoter and an enhancer appear to be involved in the regulation of insulin gene transcription in the pancreas (Walker, 1983; Nir, 1986).

Insulin homologues have been identified in bird, reptiles, teleost and elasmobranch fish as well as mammals (reviewed by Pickup, 1991). Although their is variability in the primary amino acid sequences of these insulin molecules, structurally important residues are highly conserved indicating these molecules have

very similar tertiary structures. The conservation between the various mammalian insulin molecules is high. For example, human insulin differs from porcine insulin by one amino acid residue (position 30 in the B-chain) and from bovine insulin by two residues (positions 8 and 10 in the A-chain) and in each instance the substitutions are highly conserved (Dayhoff, 1969).

1.1.2 Insulin synthesis

Insulin is produced in the β-cells of the Islets of Langerhans in the pancreas (review Howell, 1989). The insulin gene is translated into a 11.5 kilodalton (kDa) single chain precursor, preproinsulin. Preproinsulin consists of the A, B and C regions plus a 23 amino acid, signal peptide which targets the transport of preproinsulin into the endoplasmic reticulum. During translation, or shortly thereafter, preproinsulin folds allowing the formation of specific disulphide bonds. The signal peptide is removed by an endopeptidase in the cisternae of the rough endoplasmic reticulum resulting in the synthesis of proinsulin. Proinsulin comprises the 21 amino acid A chain and 30 amino acid B chain of insulin which are connected by the 30-35 amino acid C-peptide. The C-peptide assists in the formation of the correct secondary structure and alignment of the disulfide bridges prior to cleavage. Furthermore, the presence of the C-peptide in the proinsulin molecule confers resistance to proteolytic degradation and makes the proinsulin molecule more soluble and amenable to transport (Steiner, 1972). Proinsulin is transported to the Golgi apparatus by an energy dependent process where it is packed into secretory vesicles surrounded by a membrane containing an adenosine triphosphate (ATP) dependent proton pump (Howell, 1972). Proinsulin is converted to insulin within these vesicles by the action of pH-dependent enzymes which have both tryptic- and carboxypeptidase B-like activities (Orci, 1986). Hydrogen ions are transported into the vesicle as the vesicle matures. This results in the lowering of the intra-vesicle pH and increases the activity of the proteases. Protease action leads to the release of the insulin and C-peptide within the vesicle. Insulin, which is less soluble than

proinsulin, self-associates in solution as zinc-coordinated hexamers and eventually precipitates within the vesicle as crystalline aggregates. Insulin and C-peptide are stored in the secretory vesicles until required (reviewed by Howell, 1989).

The synthesis of proinsulin is regulated by the concentration of glucose, as well as several other carbohydrates and their metabolites. Studies suggest that the regulation of proinsulin synthesis by these carbohydrates occurs during preproinsulin mRNA translation (Itoh, 1980; Steiner, 1972; Ashcroft, 1978).

1.1.3 Insulin secretion

Exocytosis of the secretory granules of the β-cells results in insulin and the C-peptide being secreted in equimolar amounts with approximately 3-5% being proinsulin (Gold, 1989). Insulin secretion is regulated predominantly by the concentration of circulating glucose and the amino acids leucine and arginine. Certain compounds such as glucagon and phosphodiesterase inhibitors act as potentiators, increasing the amount of insulin released in response to stimulation by glucose and amino acids (reviewed by Howell, 1989). Other factors involved in the regulation of insulin secretion include calcium uptake, intracellular ATP concentrations, the levels of reduced pyridine nucleotides and specific neurotransmitters. Furthermore, their is a complex inter-relationship between the pancreatic hormones which controls their secretion. Glucagon acts as an enhancer of insulin release following the initial stimulation, whereas insulin inhibits glucagon release (reviewed by Pickup, 1991). In contrast, somatostatin significantly inhibits both insulin and glucagon secretion. The mechanisms for these interactions are not fully understood, however it has been suggested that they may function through either a classical endocrine mechanism, a paracrine action, or both (Philippe, 1989). Stimulation of the vagus nerve also results in insulin release whilst insulin secretion is inhibited following stimulation of the sympathetic nervous system. Possible second messenger systems that potentiate insulin release include Ca^{2+}, cyclic adenosine monophosphate (cAMP) and

phosphoinositol metabolites (Montague, 1974; Wollheim, 1981; Rorsman, 1984; Turk. 1986; Howell, 1988).

1.1.4 The action of insulin

The biological effects of insulin on the integration of fuel metabolism are primarily to promote the storage of glucose as glycogen, amino acids as proteins and fats as triglycerides. Insulin promotes fuel storage via a number of mechanisms. Firstly, insulin increases blood flow to tissues thereby facilitating the disposal of substrates into the target tissues (Laasko, 1990). Secondly, in peripheral insulin-sensitive tissues, insulin promotes glucose uptake via the translocation and activation of specific glucose transporter (Glut) proteins to the cell membrane (Shigeta, 1991). In skeletal muscle, cardiac muscle and adipocytes, Glut 4, a specific insulin-sensitive glucose transporter, augments glucose uptake into these cells (Bell, 1990). Thirdly, and possibly the most important mechanism by which insulin regulates fuel metabolism, is via the indirect activation or inhibition of rate limiting enzymes involved in the various metabolic pathways (table 1.1).

In addition to playing a role in the regulation of fuel homeostasis by controlling the activity of certain enzymes, insulin has an effect on various cellular processes by modulating the synthesis of specific proteins. Insulin regulates the rate of protein synthesis at the translational level and has both positive and negative effects on specific gene transcription (reviewed by O'Brien, 1991). Insulin regulates the synthesis of various proteins which have diverse functions including specific intracellular enzymes, integral membrane proteins, hormones and several transcription factors (reviewed by O'Brien, 1991).

The diverse effects of insulin show considerable variations both in their time course and in the concentration of hormone required to elicit the response (reviewed Rosen, 1988). Whilst glucose transporter translocation occurs almost immediately following insulin stimulation, the effect on DNA synthesis is only apparent several

Increased activity	Decreased activity
Glycolytic enzymes: Phosphofructokinase Pyruvate kinase Glucokinase	**Gluconeogenic enzymes :** Glucose-6-phosphatase Fructose-1,6-bisphosphatase Pyruvate carboxylase Phosphoenolpyruvate carboxykinase
Glycogenic enzymes : Glycogen synthase	**Glycogenolytic enzymes :** Glycogen phosphorylase
Lipogenic enzymes : Acetyl CoA carboxylase Pyruvate dehydrogenase Lipoprotein lipase ATP citrate lyase Fatty acid synthetase Hydroxymethylglutaryl CoA reductase	**Lipolytic enzymes :** Triglyceride lipase
Others : Tyrosine aminotransferase Na^+, K^+-ATPase Phosphodiesterase	**Others :** Adenylate cyclase

Table 1.1 The effect of insulin on specific enzymes involved in metabolism. These effects may be due to changes in the rate of synthesis of the enzyme, covalent modifications or other mechanisms.

hours later. The action of insulin on many cytoplasmic enzymes occurs within an intermediate time frame.

1.1.5 Insulin and diabetes mellitus

The vital importance of insulin in the maintenance of metabolic homeostasis is most evident in diabetes mellitus where insulin activity is diminished or absent (reviewed by Pickup, 1991). Currently, the World Health Organisation (WHO) classifications of diabetes mellitus and associated syndromes of glucose intolerance come under six major groups, of which most cases are classified as either insulin-dependent diabetes mellitus (IDDM) or non-insulin-dependent diabetes mellitus (NIDDM) (WHO Expert Committee on Diabetes, 1980; WHO Study Group Report, 1985).

IDDM identifies patients who require insulin replacement therapy for survival and most often presents in children or young adults. The clinical symptoms of IDDM are the result of chronic autoimmune destruction of the β-cells of the pancreas. Both genetic and environmental factors are implicated in the disease aetiology (Sheldon, 1988; Fletcher, 1987). IDDM patients classically present with hyperglycaemia, polyuria and polydipsia. The long-term effects of IDDM may include microvascular complications, coronary heart disease, nephropathy and peripheral vascular disease with the rate of mortality increased some 4-7 fold compared with the non-diabetic population (reviewed by Pickup, 1991).

NIDDM individuals survive without the administration of insulin through strict dietary control, although insulin may be given to improve their carbohydrate homeostasis. These individuals are generally greater than 50 years of age, obese, and present with hyperglycaemic symptoms. NIDDM appears to be a culmination of both impaired insulin secretion and insulin resistance in the insulin target organs. Furthermore, their is a strong familial predisposition to the disease. Although the

long-term complications of the disease are less frequent than seen with IDDM, NIDDM is associated with a high risk of atherosclerosis, and myocardial infarction accounts for 60% of mortality (reviewed by Pickup, 1991).

The World Health Organisation (WHO) statistics indicate that in 1991 more than 50 million people in the world suffered from diabetes. Moreover, the prevalence of the disease is increasing in both developed and developing countries (reviewed by Hoet, 1991).

1.1.6 The insulin receptor

Insulin receptors were first identified in 1971 following studies by several independent groups who established the specific, saturable binding of [I^{125}]insulin to hepatocyte and adipocyte cell surfaces (reviewed by Haring, 1991). In the following year Cuatrecasas developed a protocol for the isolation of insulin receptors from hepatocyte membranes, thereby enabling the first specific studies into their structure and function (Cuatrecasas, 1972).

Over the past fifteen years, many research groups using various physical and biochemical techniques have laboured to define what is the current model for the structure of the insulin receptor, and to elucidate the mechanisms by which this protein transmits the insulin signal. Some of these techniques include gene cloning, photoaffinity-labelling, the use of anti-receptor antibodies and bifunctional cross-linking compounds, and the synthesis of receptor chimeras (reviewed in Haring, 1991 and Kahn, 1984). Together, these studies have brought us to our current understanding of the three-dimensional structure of the mature insulin receptor, and much of this work is continuing to fully elucidate the *in vivo* structure of the insulin receptor and the mechanism by which insulin elicits a cellular response.

1.1.7 The insulin receptor gene

Cloning and sequencing of the human insulin receptor cDNA (Ebina, 1985; Ullrich, 1985) enabled the deduction of the entire amino acid sequence of the receptor and provided invaluable insights into both the receptor structure and function[1]. The insulin receptor cDNA sequence predicts a protein of 1355 amino acids with a relative molecular mass (Mr) of 153,917. The insulin receptor precursor is encoded by a single mRNA and includes both the α and β subunits. In comparison, cloning and sequencing of the rat insulin receptor gene revealed that the rat insulin proreceptor contains 1357 amino acids and displays 95.2% identity with its human counterpart (Goldstein, 1990).

The insulin receptor is ubiquitous in vertebrate organisms although receptor numbers vary in a tissue-specific manner (reviewed by Gammeltoft, 1984). The human insulin receptor gene is located on chromosome 19 (Ebina, 1985). The use of southern blot analysis indicates the presence of only one insulin receptor gene in the haploid human genome (Ullrich, 1985). The human insulin receptor is encoded by a region of DNA approximately 120 kilobases (kb) in size and consisting of 22 exons (Yang-Feng, 1985; Seino, 1989a) (figure 1.1). Exons encoding the α-subunit of the insulin receptor are located over more than 90 kb whilst exons encoding the β-subunit are located over approximately 30 kb (Seino, 1989a). Syntenic regions of the human insulin receptor gene have been described in both the mouse and rat (Ludwig, 1988; Goldstein, 1990).

1.1.8 Regulation of insulin receptor gene transcription

Transcription of the human insulin receptor gene results in the generation of multiple mRNA species which are 1.5 to 5.4 kb larger than the cDNA coding region (Ullrich, 1985; Ebina, 1985; Tewari, 1989). The variation in size of these transcripts

---

[1] All insulin receptor residue numbers cited in this thesis correspond to those reported by Ebina *et al.* 1985

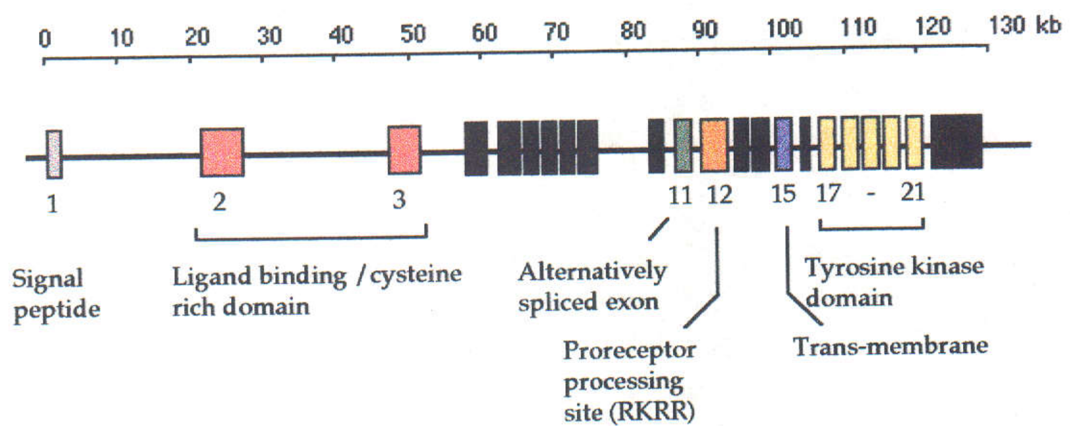

Figure 1.1 Organisation of the human insulin receptor gene. The function of protein domains encoded by specific exons are highlighted. Note that exon 11 (green) is a 36 bp exon located proximal to the insulin proreceptor processing site. The proreceptor cleavage site is shown by the single-letter amino acid code (this figure is adapted from Seino *et al.* 1989a).

is a result of termination (3') variations due to extensive regions of untranslated sequence (Tewari, 1989).

The activation of human insulin receptor gene transcription occurs in a 40 bp region 578 bp upstream from the translation initiation site (Levy, 1993). Characterisation of the insulin receptor gene promoter region has identified homologous sequences with the promoter sequences from specific housekeeping genes (Araki, 1987). These include human 3-phosphoglycerate kinase (Singer-Sam, 1984), human epidermal growth factor receptor (Ishii, 1985), human adenosine deaminase (Valerio, 1985), human hypoxanthine phosphoribosyltransferase (Kim, 1986), and mouse hypoxanthine phosphoribosyltransferase (Melton, 1984). Common features to these promoter sequences is the absence of both a TATA box and a CAAT box, high G/C content upstream of the cap site and the presence of potential Sp1 binding sites. As with other housekeeping genes which lack the TATA box, multiple initiation sites for transcription have been identified (Seino, 1989a; Mamula, 1988). Housekeeping promoters act to maintain a basal level of transcription in all cells. However, Seino *et al.* (1989a) established that in the HepG2 cell line the region of the insulin receptor promoter and the first exon which contain all the transcription initiation sites is more active when compared with the CV1 cell line. This directly correlates with insulin receptor gene expression in these two cell lines, thereby indicating the possible presence of unique insulin receptor gene promoter sequences which bind transcription factors resulting in tissue-specific regulation of insulin receptor mRNA transcription (Tewari, 1989).

Recent studies have identified multiple *trans*-acting factors which regulate expression of the human insulin receptor gene. These have included Sp1, the glucocorticoid receptor and two novel factors, insulin receptor nuclear factor-I (IRNF-I) and insulin receptor nuclear factor-II (IRNF-II). Seven repeats of the potential Sp1 binding site have been identified within the G/C-rich 5' flanking region of the human insulin receptor gene (Dynan, 1983). Sp1 was initially characterised as a mammalian

transcription factor which stimulates gene transcription by binding to GC box sequences in specific cellular promoters (Dynan, 1983). More recent studies have demonstrated that Sp1 also binds to other recognition sequences including the sequence GGGAGG (Dawson, 1988). Lee *et al.* (1992) established that Sp1 binds to a cluster of GC boxes in the human insulin receptor gene promoter and have implicated Sp1 binding to the G/A recognition sequence in the regulation of insulin receptor promoter activity.

The role of the glucocorticoid receptor in transcriptional regulation has been well documented (reviewed by Diamond, 1990). Upon binding of the hormone, the glucocorticoid receptor binds to specific DNA sequences known as glucocorticoid response elements (GRE) situated close to the promoter region of specific genes, thereby selectively regulating gene transcription. In the insulin receptor gene, the binding of the glucocorticoid receptor to the GRE is thought to control transcription by enhancing the recognition of the promoter by other transcriptional factors (Klein-Hitpass, 1990; Horikoshi, 1988; Sawadogo, 1985).

Footprint analysis has been used to identify two sequences within the region -550 to -502 as putative transcription factor binding sites, IRNF-I and INRF-II. These sites were shown to be highly conserved between the human and the mouse (Lee, 1992). Furthermore, IRNF-I and IRNF-II are differentially distributed in the various cell lines studied suggesting that these two transcription factors may have different roles in different cell types (only one of these transcription factors (IRNF-I) was essential for insulin receptor gene transcription in HepG2 cells) (Lee, 1992).

Differential control of insulin receptor gene transcription could be a result of the interaction of a combination of these *trans*-acting factors. The model of the mouse proliferin gene has established precedence that a single DNA element can evoke opposite regulatory effects on a gene depending on the physiological context (in this instance the binding of the glucocorticoid receptor, c-Jun and c-Fos to the 'composite'

GRE evoked either positive or negative effect on transcription depending on the combination of factors binding) (Diamond, 1990).

1.1.9 The insulin receptor signal peptide

A 27 amino acid region beginning with methionine, and which is predominantly hydrophobic, precedes the N-terminal amino acid sequence of the insulin receptor α-subunit (Ullrich, 1985; Ebina, 1985) (figure 1.2). This is characteristic of the signal peptide which precedes many secretory and membrane proteins (Sabatini, 1982). It also is presumed to be the initiation site for translation, however, a sequence commencing with a methionine residue at position -47 presents another possible translation initiation site.

1.1.10 The insulin receptor α-subunit

The α-subunit of the insulin receptor (residues 1-735) is characterised by a cysteine-rich domain consisting of 158 amino acids from positions 155 to 312. This region is comprised of 16.5% cysteine residues, 72% hydrophilic residues including 21% charged amino acids and 15% proline and glycine residues. Within this domain a region of 56 amino acids between Asn-230 and Ile-285 has been shown to contain the major determinants of hormone binding specificity (Gustafson, 1990) although more C-terminal regions of the insulin receptor are thought to be involved in attaining maximal hormone binding affinity (Yip, 1988; Prigent, 1990). The C-terminal end of the α-subunit contains an eight residue sequence (Pro-Arg-Pro-Ser-Arg-Lys-Arg-Arg 728-735) which serves as an enzyme(s) cleavage site which results in the generation of the α- and β-subunits (figure 1.2).

1.1.11 Alternative splicing of the α-subunit of the insulin receptor

The original cloning and sequencing of human insulin receptor cDNA by two independent groups resulted in the identification of two amino acid sequences that differed due to the presence (Ebina, 1985) or absence (Ullrich, 1985) of a 12 amino

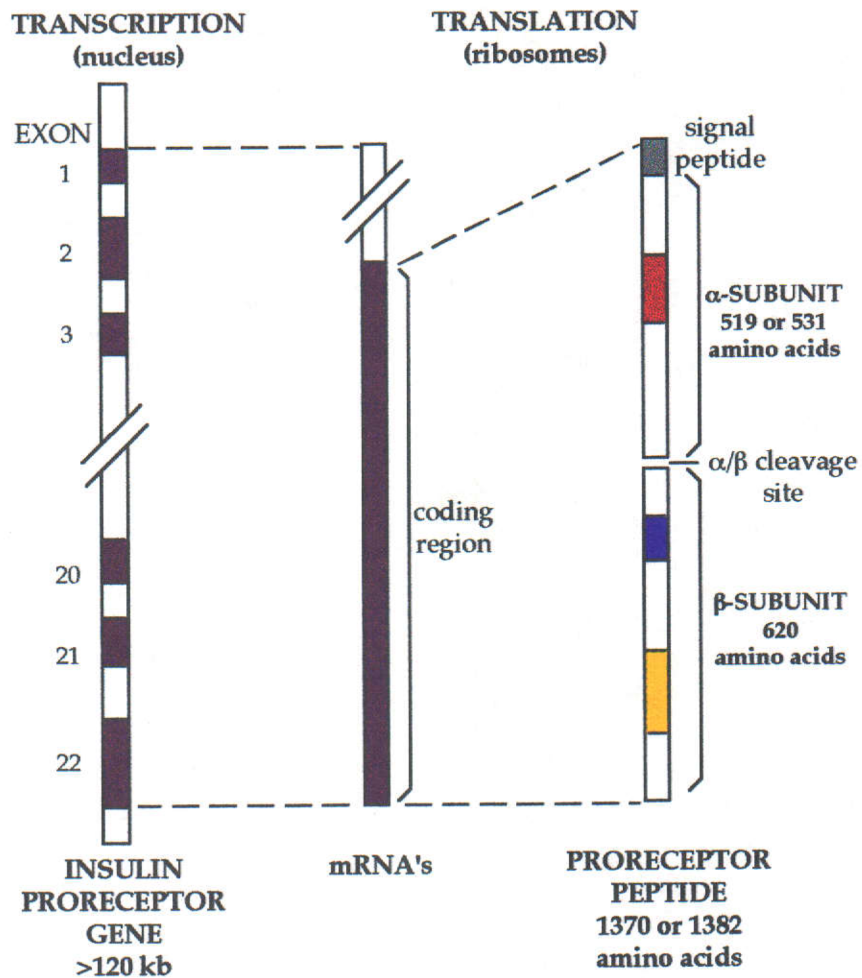

Figure 1.2 Transcription and translation of the human insulin receptor gene. The insulin-binding region of the insulin receptor is located in the cysteine-rich region of the α-subunit (red). The transmembrane region (blue) and tyrosine kinase domain (yellow) are located on the insulin receptor β-subunit.

acid sequence near the C-terminal region of the α-subunit. Investigation of the intron-exon organisation of the insulin receptor gene identified that this region was coded by the 36 bp cassette exon 11 (Seino, 1989b) (figure 1.1).

The alternative splicing of exon 11 encoded mRNA in the insulin receptor transcripts gives rise to two, functionally distinct insulin receptor isoforms (Seino, 1989b; Mosthaf, 1990; Yamaguchi, 1991; McClain, 1991; Vogt, 1991; Kellerer, 1992b; Yamaguchi, 1993). In humans and rats, the distribution of the insulin receptor isoform excluding the exon 11 encoded region (IR-A), and the insulin receptor isoform including the exon 11 encoded region (IR-B), occurs in a tissue-specific manner (Seino, 1989b; Moller, 1989; Goldstein, 1990) (table 1.2). This may enable the fine modulation of insulin-responsiveness in the various insulin-sensitive tissues.

Cell lines transfected with either IR-A or IR-B have been used as a means of isolating the functional properties of these isoforms. Initially, Mosthaf *et al.* (1990) demonstrated that the IR-A isoform has a 1.5-2.0 fold higher affinity for insulin than does the IR-B isoform. This observation has since been confirmed by a number of other studies (Yamaguchi, 1991; Yamaguchi, 1993; Kellerer, 1992b).

Isoform-specific differences in tyrosine kinase activity (Kellerer, 1992b), capacities for insulin-stimulated down-regulation (Vogt, 1991; Yamaguchi, 1993) and the ability of unlabelled insulin-like growth factor-1 (IGF-I) to displace radiolabelled insulin (Yamaguchi, 1993) have also been identified. Interestingly, preliminary studies indicate that IR-A and IR-B display similar capabilities for insulin-induced signal transduction, however, as yet only a limited number of these properties have been assessed (McClain, 1991; Yamaguchi, 1991; Carrascosa, 1991).

Several groups have investigated the tissue distribution of IR-A and IR-B in patients with various insulin resistant disorders (Moller, 1989; Mosthaf, 1991; Benecke, 1992; Mosthaf, 1992; Kellerer, 1992a; Anderson, 1993; Norgren, 1993;

Tissue	% IR-A	% IR-B	Reference
Placenta	50	50	Moller, 1989
	55	45	Seino, 1989b
	61 *	39	Benecke, 1992
	56 **	44	Goldstein, 1990
Skeletal Muscle	30	60	Moller, 1989
	~100	~0	Mosthaf, 1990
	55 *	45	Benecke, 1992
Skeletal Muscle-NIDDM	50	50	Mosthaf, 1991
	50 *	50	Benecke, 1992
Adipocytes	17	83	Moller, 1989
	38 *	62	Benecke, 1992
Fibroblasts	27	73	Moller, 1989
	~30	~60	Mosthaf, 1990
Liver	9	91	Moller, 1989
	13	87	Seino, 1989b
	~0	~100	Mosthaf, 1990
	24 *	76	Benecke, 1992
	15 **	85	Goldstein, 1990
Kidney	40	60	Seino, 1989b
	34 **	66	Goldstein, 1990
Brain	100	0	Seino, 1989b
	83 **	17	Goldstein, 1990
Spleen	100	0	Seino, 1989b
	~100	~0	Mosthaf, 1990
	94 **	6	Goldstein, 1990

Table 1.2 Distribution of the IR-A and IR-B insulin receptor isoforms in human and rat tissues. Proportion of insulin receptor isoforms is based on the expression of mRNA as determined using the PCR. Skeletal muscle tissue samples were obtained from the gastrocnemius from amputated limbs (Mosthaf, 1991) and biopsies from the vastus lateralis and rectus abdominus muscles (Benecke, 1992). NIDDM refers to insulin receptor isoform distribution in skeletal muscle samples obtained from individuals displaying the clinical symptoms of non insulin-dependent diabetes mellitus. * Proportion of insulin receptor isoform distribution is based upon the protein levels of IR-A and IR-B. ** Insulin isoform expression levels in tissues from 7-8 week old male Sprague-Dawley rats.

Mosthaf, 1993; Hansen, 1993). Initial work by Mosthaf *et al.* (1991) identified altered expression of IR-A and IR-B in the skeletal muscle of NIDDM patients (table 1.2). They demonstrated that whilst the skeletal muscle from non-insulin resistant individuals contains exclusively the IR-A encoding mRNA, IR-B and IR-A mRNA are expressed at comparable levels in the NIDDM patient (Mosthaf, 1991). This observation was confirmed by the subsequent studies of Mosthaf *et al.* (1992; 1993), Kellerer *et al.* (1992a; 1993) and Norgren *et al.* (1993). Moreover, Mosthaf *et al.* (1993), have suggested that the altered expression of the insulin receptor mRNA isoforms may be a cause rather than a consequence of the lower insulin responsiveness evident in patients with NIDDM. In their study, IR-A and IR-B expression in skeletal muscle biopsies from non-diabetic, first degree relatives of NIDDM patients were examined. PCR analysis indicated the abnormally increased expression of the IR-B mRNA isoform in these samples, suggesting that this alteration in insulin receptor isoform expression precedes the clinical onset of this insulin resistant state. In contrast, Benecke (1992), Anderson (1993) and Hansen (1993) have reported that their is no significant difference in IR-A and IR-B in tissues from normal and NIDDM patients. Possible reasons for these conflicting results may be the severity of the NIDDM in the affected individual, the effects of vascular disease, the origin and quality of the muscle biopsy (eg. Mosthaf *et al.* (1991) used gastrocnemius tissue samples obtained from limb amputations for vascular disease whereas Benecke *et al.* (1992) obtained muscle biopsies from the vastus lateralis and the rectus abdominus) or contamination by blood or other cell types.

1.1.12 The insulin receptor β-subunit

The insulin receptor β-subunit consists of 620 amino acids (residues 736-1355) and includes both an extracellular and intracellular domain (Ebina, 1985) (figure 1.2). Twenty three residues (929-952) which are uncharged and predominantly non-polar comprise the transmembrane domain of the receptor (Ebina, 1985). The intracellular portion of the β-subunit displays characteristics associated

with tyrosine kinase activity (Ebina, 1985; Ullrich, 1985). The tyrosine kinase domain of the insulin receptor (residues 1002-1257) displays a high degree of homology with a number of other tyrosine kinase receptors (reviewed by Hanks, 1988) and contains a consensus sequence for an ATP-binding site also observed in several other tyrosine and serine/threonine kinases (reviewed by Hunter, 1984).

Insulin-stimulated autophosphorylation of the insulin receptor β-subunit in both intact cells and cell-free systems was initially demonstrated by several groups in 1982 (Kasuga, 1982a; Kasuga, 1982b; Van Obberghen, 1982; Avruch, 1982; Petruzzelli, 1982). Insulin-stimulation of the insulin receptor increases the V_{max} for insulin receptor autophosphorylation *in vitro* by 20-fold with no effect on the K_m of the enzyme for ATP (White, 1984). Autophosphorylation of the insulin receptor was shown to activate the tyrosine kinase activity of the receptor (Rosen, 1983). Evidence suggests that activation of the insulin receptor tyrosine kinase is a key step in signal transduction by insulin (Rosen, 1987; Kahn, 1988). Insulin receptor autophosphorylation occurs as a sequential cascade involving multiple sites within the kinase domain and the C-terminus of the β-subunit (White, 1988; Tornqvist, 1987; Flores-Riveros, 1989; Tavare, 1988). Initially, phosphorylation of two tyrosine residues within the kinase domain occurs involving tyrosine-1158 and either tyrosine-1162 or tyrosine-1163. This is immediately followed by the modification of the third residue in addition to tyrosine-1328 and tyrosine-1334 in the C-terminal region (Tavare, 1988; Avruch, 1990). *Bis*-phosphorylation of the insulin receptor tyrosine kinase domain is associated with minimal activation of the kinase activity whilst *tris*-phosphorylation results in the full activation of the insulin receptor kinase towards exogenous substrates (White, 1988; Flores-Riveros, 1989). Furthermore, subsequent phosphorylation of the two closely associated β-subunits in the $\alpha_2\beta_2$ receptor complex may occur in a *cis* or *trans* mode (Shoelson, 1991). In addition to tyrosine phosphorylation, the β-subunit is also phosphorylated on serine and threonine residues (Kasuga, 1982a; Kasuga, 1982b).

Although tyrosine phosphorylation within the C-terminal region of the insulin receptor does not appear to be involved in the activation of the receptor tyrosine kinase activity, studies suggest that tyrosine phosphorylation in this region may have a role in the regulation of certain distal effects of insulin (Goren, 1987; Myers, 1991; Maegawa, 1988; Takata, 1991; Baron, 1991). Studies investigating the effect of anti-peptide antibodies against the insulin receptor sequence 1321-1338 (Baron, 1991) and using insulin receptors mutated at the two autophosphorylation sites, tyrosine-1328 and tyrosine-1334 (Takata, 1991), have demonstrated that blocking or mutation of these specific tyrosine residues resulted in an enhanced insulin-stimulated mitogenic signal whilst the capacity to transduct the metabolic signal was unaltered. This suggests that the C-terminal region of the insulin receptor may play a specific role in eliciting the mitogenic effects of insulin. It is interesting to note that the anti-peptide antibody does not enhance phosphorylation of ribosomal protein S6 which is a rapid effect of insulin but is considered to be part of the growth promoting effects of the hormone.

The 112 amino acid C-terminal region of the insulin receptor β-subunit has also been shown to confer stability to the subunit (Ellis, 1986). Deletion of this region, consisting of the last 14 amino acids in the tyrosine protein kinase domain and the 98 amino acid C-terminal tail of the insulin receptor, results in an unstable β-subunit which is susceptible to proteolytic degradation, loss of kinase activity and insulin-stimulated uptake of 2-deoxyglucose.

1.1.13 Post-translational processing of the insulin receptor

The insulin-binding subunit of the insulin receptor contains fourteen potential N-linked glycosylation sites of which Asn-16, Asn-397 and Asn-418 are known to be glycosylated (Ullrich, 1985; Ebina, 1985; Hayes, 1991). The tyrosine kinase subunit contains four potential sites for asparagine glycosylation including Asn-730 and Asn-881 which are glycosylated (Ullrich, 1985; Ebina, 1985; Hayes, 1991). O-linked

glycosylation has been demonstrated at the amino-terminal region of the insulin receptor tyrosine kinase subunit (Collier, 1991).

Post-translational glycosylation of the single-chain polypeptide insulin receptor precursor (Mr 153,917) results in the synthesis of the glycosylated proreceptor (Mr of α- plus β-subunit 210,000) which is subsequently disulfide-linked, cleaved and further glycosylated to form a heterotetrameric complex ($\alpha_2\beta_2$) (Ebina, 1985; Ronnet, 1984; Kahn, 1988). The amino acid sequences of the α- and β-subunits predicts a relative molecular mass for the mature, aglycosubunits of 84,214 and 69,703 respectively, compared with approximately 130,000 and 90,000 for the post-translationally modified subunits. These differences indicate that most of the potential sites for N-linked glycosylation are occupied by carbohydrate moieties (Ullrich, 1985).

Tissue-specific differences occur in the extent of post-translational glycosylation of the insulin receptor in both humans and rats. These variations have been described in brain, skeletal muscle, monocytes, erythrocytes and mammary tissues (Burant, 1986a; Heidenreich, 1983; Yip, 1980; McElduff, 1985; Burnol, 1990b). Receptor heterogeneity due to varied glycosylation patterns has been described for other cell-surface receptors including the IGF-I and insulin-like growth factor-II (IGF-II) receptors (McElduff, 1987). The specific function of these differences in insulin receptor structure remains to be elucidated. However, it has been demonstrated that N-linked glycosylation of the insulin receptor and the epidermal growth factor (EGF) receptor is necessary for the acquisition of ligand binding capabilities (Ronnet, 1984; Slieker, 1985) but this does not appear to be a prerequisite for all receptors as the acetyl choline receptor does not require glycosylation for ligand binding (Prives, 1983). The variations evident in receptor glycosylation of the insulin receptor may result from either minor variations in the primary sequence of the glycoprotein, resulting in either the inclusion or deletion of potential glycosylation sites, or due to subtle differences in the glycosylation

machinery within various cell types. Recently, Breiner *et al.* (1993) employed B29, B29-suberoyl-insulin, an insulin receptor partial agonist, to demonstrate heterogeneity in the insulin binding in various rat tissues. Interestingly, these tissue-specific differences appeared to be unrelated to the alternative splicing of the insulin receptor gene suggesting that differences may be attributed to variations in glycosylation or heterogeneity of disulfide bridge formation.

1.1.14 The heterotetrameric complex of the functional insulin receptor

The functional native insulin receptor exists in an $\alpha_2\beta_2$ configuration (Czech, 1982; Jacobs, 1981). Florke *et al.* (1990) observed that insulin promoted a decrease in the Stoke's radius and an increase in the sedimentation coefficient of the insulin receptor that coincided with an enhanced receptor tyrosine kinase activity. Insulin binding to the $\alpha\beta$ receptor, which does not exhibit tyrosine kinase activity, did not result in mass nor shape modifications (Florke, 1990; Bonischnetzler, 1986). This suggests that the interaction of two $\alpha\beta$ receptors are required for the conformational changes which are thought to result in receptor autophosphorylation. Recent studies using electron microscopy have identified that the native insulin forms a "T" or "Y" configuration with the α-subunit forming the top "arms" of the complex and the β-subunit forming the base (Christiansen, 1991; Schaefer, 1992). The micro-environment of the insulin receptor also affects receptor function. The phospholipid environment in the plasma membrane appears to have a role in the regulation of both insulin receptor autophosphorylation and endogenous substrate phosphorylation since phospholipase C treatment of the insulin receptor alters signal transduction (Zoppini, 1992).

The human insulin receptor has a half-life of between 8-12 hours which is shortened following ligand binding. Internalisation of the insulin-insulin receptor complex results in the down-regulation of cell surface receptor numbers and provides a mechanism for the regulation of cellular sensitivity to the ambient concentration of the ligand (Hedo, 1983; Deutsch, 1983).

1.1.15 The insulin-insulin receptor interaction

Insulin interacts with the insulin receptor with high affinity (K_d ~ 1.0nM) and high specificity. However, the insulin-insulin receptor interaction is complex and despite intensive research in this area no consensus has been reached as to a model which accurately describes the interaction. Initially, Gammeltoft and Gliemann (1973) proposed a model of a reversible, bimolecular and non-cooperative interaction between insulin and its receptor. Subsequently, heterogeneity and cooperativity between receptors was postulated (de Meyts, 1976; Sonne, 1980; de Meyts, 1973). However, the validity of ligand dissociation experiments for inferring negative-cooperative site-site interactions amongst insulin receptors has been challenged by numerous groups (Helmerhorst, 1987; Corin, 1982; Boeynaems, 1976). Current research suggests that there is a single class of homogeneous, non-interacting insulin receptors (Helmerhorst, 1993). Helmerhorst and Yip (1993) show a marked temperature dependence of the interaction between insulin and the insulin receptor which is consistent with a two-state model of the insulin receptor as initially proposed by Corin and Donner (1982). Similar two-state models have been described for the nerve growth-factor receptor and the human growth hormone receptor (Donner, 1980; Landreth, 1980). Furthermore, Helmerhorst and Yip (1993) proposed that a specific plasma membrane protein interacts with the insulin receptor in a temperature-dependent manner to modulate the affinity of insulin binding. This theory is supported by the observations of Kohanski, (1985), Baldini (1991), Ciaraldi (1989), and Davis (1990) (see section on G-proteins and the insulin receptor).

1.1.16 Insulin-stimulated signal transduction

The most probable scenario for the signalling of insulin action is that multiple pathways are activated following insulin-insulin receptor interaction. *A priori*, two mechanisms for signalling pathways by the insulin receptor kinase have been described (reviewed by Kasuga 1990) (figure 1.3). Firstly, and possibly the most widely accepted theory, is that insulin transmits its signal to the cell via the

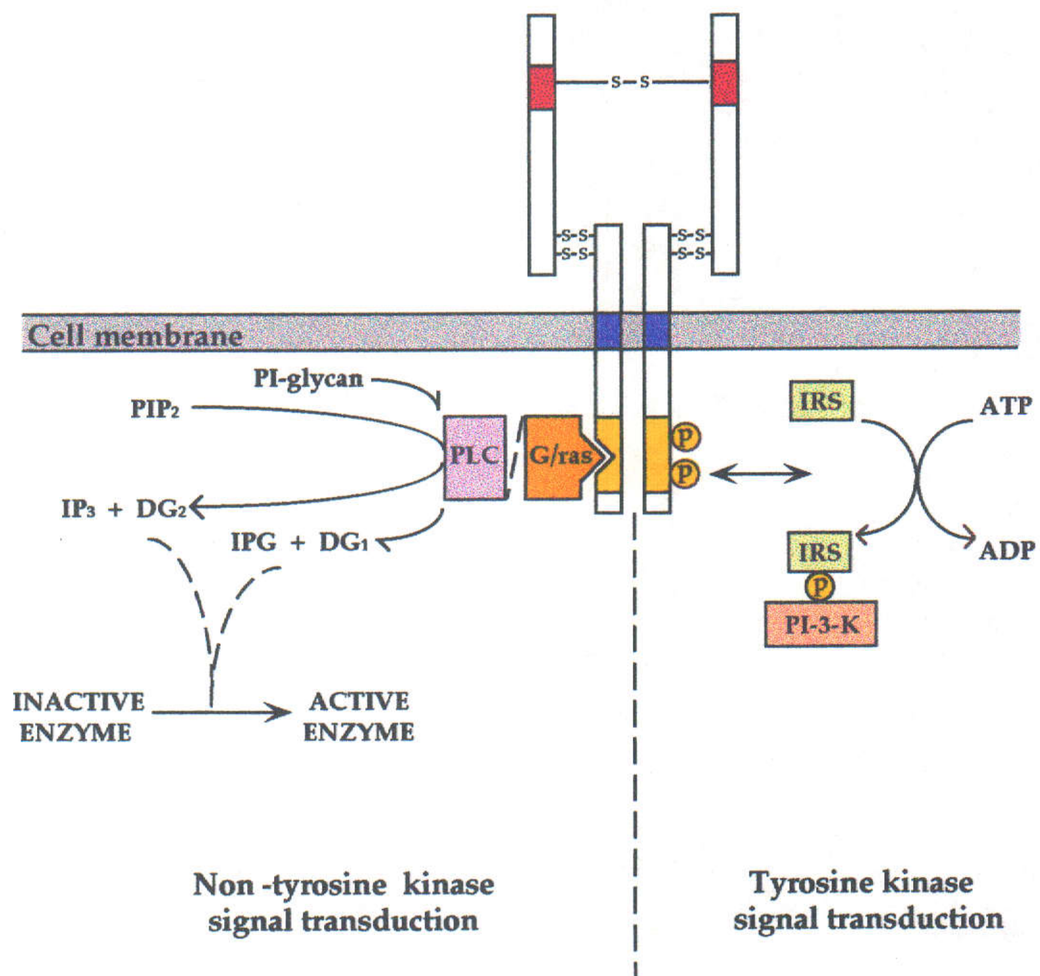

Figure 1.3 Two hypothetical mechanisms for insulin signal transduction. *Left,* non-covalent association with intracellular proteins and, *right,* tyrosine phosphorylation of intracellular substrates. **P**, represents tyrosine phosphorylation, and the yellow shaded area represents the tyrosine kinase domain of the insulin receptor. **IRS** insulin receptor substrate, **PI-3-K** phosphatidylinositol-3'-kinase, **G/ras** G protein or ras protein, **PI-glycan** phosphatidylinositol glycan, **PIP_2** phosphatidylinositol 4, 5-biphosphate, **PLC** phospholipase C, **IP_3** inositol 1,4,5-triphosphate, **DG_2** 1,2-diacylglycerol, **IPG** inositol phosphate glycan, **DG_1** diacylglycerol (adapted from Sung, 1992).

phosphorylation of specific substrates by the β-subunit tyrosine kinase. The requirement of the insulin-stimulated tyrosine kinase activity of the insulin receptor for signal transduction has been established by a number of independent studies. These have included the observation of diminished insulin receptor tyrosine kinase activity in certain forms of insulin resistance (Grunberger, 1984; Grigorescu, 1983; Kadowaki, 1984; Burant, 1986b; Okamoto, 1986), the demonstration of increased phosphorylation of ribosomal S6 protein in Xenopus oocytes injected with the insulin-stimulated, autophosphorylated insulin receptor (Maller, 1986) and that use of monoclonal antibodies which specifically inhibit the tyrosine kinase activity of the insulin receptor results in a diminished cellular response to ligand stimulation (Morgan, 1987). In addition, site-directed mutagenesis experiments of the insulin proreceptor show that in mutated proteins in which kinase activity is absent, the cellular response to insulin is decreased (Chou, 1987; Ebina, 1987; McClain, 1987).

In contrast to the autophosphorylation of tyrosine residues, serine and threonine phosphorylation of the β-subunit by two serine/threonine kinases, cAMP-dependent protein kinase (Roth, 1987), and protein kinase C (Bollag, 1986) have been shown to diminish receptor kinase activity. This suggests that some of the physiological actions of cAMP and activators of protein kinase C which are antagonistic to the actions of insulin may act via serine/threonine phosphorylation of the insulin receptor.

The actual identification of endogenous substrates for the insulin receptor tyrosine kinase has proved to be somewhat elusive. One of the major substrates for tyrosine phosphorylation in the cell is the insulin receptor itself (Kasuga, 1982a, Kasuga, 1982b; Van Obberghen, 1982; Avruch, 1982; Petruzzelli, 1982). Autophosphorylation may allow the insulin receptor to initiate a biochemical reaction by associating or disassociating with specific cellular protein(s). Recently, several other cellular proteins have been shown to be specifically phosphorylated on tyrosine residues in response to insulin-stimulation (table 1.3) (reviewed by Roth, 1992).

Substrate	Characteristics	M_r (kDa)	Reference
Fatty acid binding protein	Binds lipids and calcium	15	Bernier, 1987 Hresko, 1988
MAP-kinase	Serine/threonine kinase	42	Sturgill, 1991 Boulton, 1991 Seger, 1991
PI-3' kinase	Phosphorylates PI at position-3	85	Ruderman, 1990 Roth, 1991
Ecto ATPase	Displays ATPase activity	120	Margolis, 1990 Najjar, 1993
Insulin receptor substrate 1	Binds the type 1 PI kinase	185	Sun, 1991 Yonezawa, 1991

Table 1.3 Potential substrates for the insulin receptor tyrosine kinase. PI phosphatidylinositol, **PI-3' kinase** phosphatidylinositol-3' kinase, **MAP-kinase** mitogen-activated protein kinase.

The functions of the putative endogenous substrates of the insulin receptor tyrosine kinase following insulin-stimulated tyrosine phosphorylation have yet to be elucidated, however the more interesting of the potential substrates of the insulin receptor are phosphatidylinositol-3'-kinase (PI-3'-kinase) and the insulin receptor substrate 1(IRS-1). IRS-1 has multiple potential tyrosine phosphorylation sites, six of which are arranged in a Y-M-X-M motif which have been shown to define substrate specificity of the insulin receptor kinase (Sun, 1991; Shoelson, 1992). Whilst IRS-1 appears to have no intrinsic enzymatic activity, when phosphorylated it becomes physically associated with PI-3'-kinase. This binding occurs via the phosphorylated Y-M-X-M motif with *src* -homology 2 (SH2) domains in the noncatalytic subunit of PI-3'-kinase and results in the activation of the PI-3'-kinase enzyme activity (Cantley, 1991; Koch, 1991; Escobedo, 1991). Recent studies have demonstrated that PI-3'-kinase is necessary for insulin signalling of transcriptional events (Yamaguchi, 1993). These studies suggest that IRS-1 transmits the intracellular insulin signal by binding and regulating intracellular enzymes with SH2 domains. It is also interesting to note that previously, mitogen-activated protein kinase (MAP-kinase) was thought to be an important "switch" kinase which would switch the insulin receptor tyrosine kinase activity to the more common serine/threonine phosphorylations that occur in response to growth factors (Sturgill, 1991; Boulton, 1991). However, Seger *et al.* (1991) established that MAP-kinase was autophosphorylated and not phosphorylated via another endogenous tyrosine kinase (Seger, 1991).

Insulin also appears to indirectly modulate the activity of certain target proteins by the phosphorylation or dephosphorylation of serine and threonine residues on these proteins (reviewed by Rosen, 1987). Insulin induces the phosphorylation of ribosomal protein S6, ATP citrate lyase and acetyl coenzyme A carboxylase whilst proteins such as pyruvate dehydrogenase and glycogen synthase are dephosphorylated (Denton, 1981; Cohen, 1985).

The second theory for insulin signal transduction is that there are non-covalent interactions or coupling of the insulin-stimulated insulin receptor with specific cellular proteins (reviewed by Kasuga, 1990; Sung, 1992). Several studies using monoclonal and polyclonal antibodies and insulin receptor mutants suggest that non-tyrosine kinase dependent pathway for insulin signalling may also exist (reviewed by Sung, 1992). Conformational changes in the insulin receptor following ligand binding may allow the non-covalent interaction of the receptor with specific intracellular proteins. In this case, the insulin-stimulated conformational change of the insulin receptor β-subunit would enable the interaction of proteins such as IRS-1 or G/ras-proteins which would subsequently interact with PI-3'-kinase or phospholipase C, respectively (figure 1.3). In theory, phospholipase C may then act on a novel phosphatidylinositol-glycan or phosphatidylinositol 4,5-biphosphate to initiate a cascade of enzyme reactions resulting in insulin action. Other putative proteins which interact with the insulin receptor may include not only the closely located cytoskeletal constituents but also intracellular proteins. Ligand-stimulated internalisation may, in addition to regulating cell surface receptor numbers, deliver the activated β-subunit to an specific intracellular compartment. For example, following insulin binding, the insulin-receptor complex appears to undergo nuclear translocation (Podlecki, 1987).

Receptors interacting with receptor-like proteins have also been implicated in the activation of other tyrosine kinase receptors. Kadowaki *et al.* (1987) demonstrated that the EGF receptor tyrosine kinase phosphorylated the product of the human c-*erb* 2 gene at tyrosine residues in human epidermoid carcinoma KB cells. This protein displays significant homology with the EGF receptor and has the structural characteristic of a growth factor receptor. This suggests that receptor kinase activities may be modulated by receptor-receptor interactions in the cell.

1.1.17 Guanosine triphosphate-binding proteins in insulin signal transduction

Over the past eight years an increasing number of reports have appeared in the literature providing indirect evidence of the interaction between the insulin receptor and specific guanosine triphosphate-binding proteins (G-proteins) during the process of insulin signalling (Heyworth, 1986; Jo, 1992; Gawler, 1987; Baldini, 1991; Luttrell, 1990; Ciaraldi, 1989; Mortensen, 1992; Davis, 1990; Russ, 1992; Russ, 1994). These studies have indicated that various bacterial toxins (Baldini, 1991; Luttrell, 1990; Ciaraldi, 1989), guanidine nucleotides (Mortensen, 1992; Davis, 1990; Russ, 1992) and G-proteins (Jo, 1992; Gawler, 1987) have effects on insulin action. Baldini *et al.* (1991) have proposed the involvement of G-proteins in insulin-stimulated recruitment of Glut 4 to the cell surface in rat adipocytes. This association of G-proteins and Glut 4 translocation following insulin stimulation has also been observed in rat skeletal muscle (Etgen, 1993). Most recently, Mortensen *et al.* (1992) demonstrated that the mild reduction of rat adipocytes and turkey erythrocyte plasma membranes with dithiothreitol prior to insulin binding increased the proportion of insulin receptors in the high affinity state. In the presence of guanosine 5'-[γ-thio]triphosphate, the proportion of insulin receptors in the reduced plasma membranes decreased to the level seen in the absence of reduction, and this decrease was specific for guanine nucleotides. From these observations they have suggested that a regulatory G-protein is involved in the effect of guanine nucleotides on the affinity of the insulin receptor. Two G-proteins associated with rat adipocyte- and human placental- insulin receptors have recently been identified and partially characterised (Jo, 1992). A 41 kDa and a 67 kDa G-protein were identified in wheat germ-purified insulin receptor preparations from human placenta. The removal of these two G-proteins resulted in loss of insulin-stimulated tyrosine kinase activity of the insulin receptor, again inferring that the interaction between the insulin receptor and specific G-proteins may be important in insulin signal transduction.

1.1.18 The role of protein phosphatases in the regulation of the insulin receptor tyrosine kinase activity and post-receptor signalling

Regulation of the tyrosine kinase activity of the insulin receptor appears to be a balance between phosphorylation and dephosphorylation mechanisms (Goldstein, 1992). The tyrosine kinase activity of the insulin receptor is activated by *tris*-autophosphorylation reactions whilst this activity is modulated by the action of protein tyrosine phosphatases that catalyse the dephosphorylation of tyrosine residues (White, 1988; King, 1991).

Protein tyrosine phosphatases can act to regulate insulin action by two mechanisms. Firstly, the direct dephosphorylation of the insulin receptor can inactivate its intrinsic tyrosine kinase activity. Studies of insulin receptor autophosphorylation following insulin-stimulation shows that in intact cells the *bis*-phosphorylated form predominates, whilst in purified insulin receptor preparations the *tris*-phosphorylated form is evident and the receptor tyrosine kinase is fully activated (reviewed by Goldstein, 1992). This could indicate that the *tris*-phosphorylated form is the preferred substrate for protein tyrosine phosphatases in intact cells, thus acting to limit the extent of insulin receptor kinase activation. Studies have indicated that the phosphatase activity associated with the insulin receptor is extrinsic to the receptor, although it is closely associated *in situ* (reviewed by Goldstein, 1992). Recently, investigation of the protein tyrosine phosphatase activity associated with the insulin and IGF-1 receptors in tissues from diabetic and pregnant rats demonstrated that protein tyrosine phosphatase activity in insulin responsive tissues was altered in these two states of insulin resistance (Haguel de-Mouzon, 1993). This suggests a possible relationship between the defective activity of the receptor tyrosine kinase and protein tyrosine phosphatase activity in insulin responsive tissues. Secondly, protein tyrosine phosphatases also appear to be involved in the dephosphorylation of the substrates of the insulin receptor tyrosine kinase. Two protein tyrosine phosphatases that catalyse the dephosphorylation of the putative insulin signalling mediator, pp15, have been

characterised in 3T3-L1 adipocytes (Liao, 1991), although as yet protein tyrosine phosphatase activity against IRS-1 has not been documented.

1.2 INSULIN ACTION DURING FOETAL DEVELOPMENT, PREGNANCY AND LACTATION

1.2.1 Mammary gland morphology

Prior to puberty the female mammary glands are largely undeveloped. At the onset of puberty specific hormonal changes occur resulting in partial mammary gland growth and development (reviewed by DiFiore, 1981). Increased levels of oestrogen stimulates the growth and branching of the duct system within the gland which is accompanied by breast enlargement due to adipose deposition. Increased progesterone levels at this time also contribute to mammary gland growth. Cyclic changes in mammary gland morphology may occur with the menstrual cycle, however these changes regress at the end of the cycle. Alveolar development in the mammary gland during puberty is incomplete and full maturation of the tissue is not achieved until the onset of pregnancy and lactation. During pregnancy, mammary gland growth and maturation is primarily due to the action of oestrogen, progesterone, prolactin and, following the development of the feto-placental unit, placental lactogen. This results in physiological hyperplasia of the mammary gland by promoting lobulo-alveolar proliferation. A principle inhibitor of milk synthesis and secretion during pregnancy is the elevated level of progesterone. The loss of the placenta at parturition results in a rapid decrease in the concentration of this hormone, thereby stimulating lactogenesis. In addition, the lactating mammary gland becomes resistant to the action of oestrogen action (Shyamala, 1982). The mechanism of this apparent oestrogen resistance in not clear although it is thought that it may arise from changes in the sensitivity of mammary tissue to other hormones such as prolactin (Bolander, 1981). Measurement of the lactose levels in the blood of non-pregnant, pregnant and lactating women suggests that mammary tissue begins actively synthesising milk-constituents

by week 10-21 of gestation and reaches a maximum 3-5 days subsequent to parturition (Arthur, 1991).

1.2.2 Maternal metabolism during pregnancy and lactation

"Lactation is the final phase of the complete reproductive cycle of mammals. In almost all species the newborn are dependent on maternal milk during the neonatal period; in most the young are dependent for a considerable time. Adequate lactation is therefore essential for reproduction and the survival of the species and, biologically, failure to lactate can be just as much a cause of failure to reproduce as is failure to mate or to ovulate.

World Health Organisation Technical Report (1965)

Lactation is a fundamental stage in the perpetuation of most mammalian species. Whilst the timing of lactation is directly related to the reproductive cycle of the mother, the constituents of the milk and the magnitude of milk production and delivery is a function of both the nutritional status of the mother and the requirements of the neonate. Lactation is the corollary to specific physiological changes that occur during pregnancy, resulting in the total reprioritisation of maternal metabolism.

Following fertilisation, the maternal endocrine system regulates specific hormone concentrations which maintains the pregnant state, promotes foetal development, provides nutrients for constant foetal growth and prepares the maternal tissues for parturition and lactation (reviewed in Spence and Mason, 1983). These hormones are derived from both maternal and foetal tissues including the developing placenta which evolves to be the most important endocrine gland during pregnancy.

1.2.3 Insulin resistance during pregnancy

During pregnancy, the maternal metabolism adapts to satisfy the mothers energy requirements in addition to providing sufficient nutrients for the developing foetus. One of the most notable metabolic changes that occurs during pregnancy is the gradual development of insulin resistance in peripheral target tissues.

The development of insulin resistance is not evident during the first trimester of pregnancy however, during the second and third trimesters certain metabolic adaptations occur which induce an insulin resistant state in certain maternal tissues (Jirasek, 1980). Studies have identified that insulin action during the later stages of normal pregnancy is reduced some 50-70% when compared with the normal, non-pregnant female (reviewed by Buchanan, 1991). Despite the development of relatively severe insulin resistance in maternal tissues, glucose tolerance during normal pregnancy remains stable or diminishes only slightly due to increased pancreatic β–cell responsiveness (Buchanan, 1990). This enhanced β-cell responsiveness is, in part, due to the direct effects of placental hormones on β-cell function, but primarily reflects metabolic compensation for the developing insulin resistance (Buchanan, 1990; Howell, 1977; Nielsen, 1982; reviewed by Freinkel, 1980).

It has been suggested that the net metabolic effect of maternal insulin resistance during pregnancy is to preferentially remove carbohydrates, following feeding, to the foetus by delaying glucose uptake in maternal tissues (reviewed by Buchanan, 1991; Freinkel, 1974). Maternal insulin resistance during pregnancy may favour maternal fat anabolism during feeding due to the insulin resistance in muscle in conjunction with the compensatory hyperinsulinaemia (Caro, 1989; Phelps, 1981). This could result in the accumulation of adipose stores during early pregnancy and compensates for the rapid catabolism of adipose stores which occurs during fasting in the later stages of pregnancy.

1.2.4 Insulin action during lactation

Insulin plays a crucial role in regulating metabolism in the mammary gland during lactation. This is demonstrated by the effect of both diabetes (Walters, 1968) and *in vivo* treatment with anti-insulin antibodies (Martin, 1971) in reducing lipids, lactose and casein synthesis.

The action of insulin during lactation is intriguing since the anabolic effects of insulin are minimised in peripheral insulin-sensitive tissues but are concomitantly promoted in the mammary gland. In the first instance, the hypertrophy of mammary tissue during pregnancy (and in mice, rats and rabbits during the early stages of lactation) results in the mammary gland becoming a significant target organ for insulin action (Chatwin, 1969; Cowie, 1980). Insulin receptors are present in rat mammary cell plasma membranes (Posner, 1974) and in isolated mammary cells from mice and rats (O'Keefe, 1974), and their numbers rapidly increase as the mammary epithelial cells proliferate. Secondly, circulating insulin levels in the lactating animal are generally lower than in the virgin animal (Flint, 1979). This is reflected in the circulating insulin:glucagon ratio which is decreased 2-fold in the lactating animal (Robinson, 1978). As a result, the activity of the lipogenic pathway will be diminished in adipose tissue which is sensitive to the insulin:glucagon ratio, whilst lipogenic activity in the mammary gland will be relatively unaffected since the mammary gland is not sensitive to glucagon (Robson, 1984). Finally, brown and white adipose tissue becomes insulin resistant during lactation whilst hepatic and mammary tissues remain sensitive to the hormone (Burnol, 1987; Burnol, 1986). The exact mechanism for these changes in the insulin-sensitivity of these tissues during lactation is unclear, however it does not appear to be due to changes in receptor numbers in white or brown adipocytes (Burnol, 1990a; Flint, 1979; Kilgour, 1988), or hepatocytes (Flint, 1980). Furthermore, there is neither altered tyrosine kinase activity of the receptors (Burnol, 1990a; Burnol, 1990b) nor any apparent defect in the processing of insulin in the primary endosomes (Fernig, 1989), suggesting a post-

receptor signalling defect. This hypothesis is supported by the observation of Kilgour and Vernon (1988) who demonstrated that whilst the incubation of enriched white adipocyte plasma membranes with permeabilized mitochondrial membranes from virgin rats in the presence of insulin resulted in the activation of pyruvate dehydrogenase (PDH), when plasma and mitochondrial membranes from the adipocytes of lactating rats were used, no effect on PDH activity was evident. This observation further suggests that the post-receptor defect resulting in a failure to activate PDH in white adipose tissue from the lactating rat may be due to the absence of a plasma membrane-associated second messenger, or a failure to release this putative second messenger. All of these changes occur concomitantly in maternal tissues to create a metabolic state where nutrients are preferentially directed to the mammary gland and to promote the mobilisation of maternal fuel stores for milk synthesis.

Insulin also regulates the transcription of several genes whose products are essential for lactation (Bolander, 1981; Chomczynski, 1984; Yamashita, 1986; Stanley, 1988; Collet, 1992; Warner, 1993). In synergy with glucocorticoids and prolactin, insulin stimulates the expression of casein genes by increasing the rate of gene transcription (Chomczynski, 1984; Bolander, 1981). Insulin also stimulates the expression of the prolactin gene (Stanley, 1988), and inhibits the *de novo* synthesis of growth hormone mRNA (Yamashita, 1986).

1.2.5 Insulin action in the foetus

During intra-uterine life the foetus is in a continual state of anabolism. Due to the presence of maternally derived glucose and amino acids, a high insulin to glucagon ratio and limited responsiveness to catabolic hormones such as glucagon, the metabolic state of the foetus is primarily concerned with growth and fuel storage (Girard, 1973; Blazquez, 1976; Vinicor, 1976). In the rat foetus, pancreatic insulin concentrations increase at 14-18 days of gestation with maximal levels reached on day 18 (Pictet, 1972). Insulin is synthesised and secreted in the human foetus as early as

eight weeks of gestation with levels increasing rapidly between 11 to 20 weeks (Rastogi, 1970). The phase at which insulin is thought to exert its influence on embryogenesis is somewhat unclear. Studies by Sara *et al* (1983) on human tissues have suggested that the insulin-like growth factors are the principle modulators of early proliferative growth whilst insulin acts during the later stage of cell maturation and hypertrophic growth (Sara, 1983). This is supported by the observation of hypertrophy and hyperplasia in certain organs in hyperinsulinaemic infants from diabetic mothers (Naeye, 1965).

The appearance of a functional insulin receptor is crucial in the initiation of insulin action in the foetus. In foetal rat liver, significant insulin binding capacity is evident by day 16-18 of gestation, indicating the presence of membrane-associated insulin receptors (Neufeld, 1980). Foetal hepatic insulin receptor autophosphorylation increases 10-fold from day 17 to day 21 which parallels the significant increase in circulating insulin concentrations evident with advancing gestation (Gruppuso, 1992). Moreover, insulin receptor autophosphorylation was diminished in hepatic membranes from growth-retarded, hypoinsulinaemic foetuses (Gruppuso, 1992; Gruppuso, 1989). Benedict and Richman (1991) have identified that the degradative and retroendocytotic insulin processing pathways are present in foetal rat hepatocytes at day 17 of gestation. Furthermore, this ability for insulin-receptor processing is functionally related to the glycogenic action of insulin by day 19 of gestation (Benedict, 1991). These studies indicate that mature and functional insulin receptors are present during the early stages of foetal development.

Several studies have demonstrated that the insulin binding capacity in foetal tissues such as rat hepatocytes (Neufeld, 1980; Steven, 1979), rabbit lung (Devaskar, 1982) and human erythrocytes (Herzberg, 1980) increase as a function of gestation, reaching a maximum immediately prior to parturition. Subsequently, insulin binding capacities in rat hepatocytes decline until at around day 10 in the neonate when insulin binding levels are similar to those seen in the adult (Vinicor, 1982). Devaskar *et al.*

(1982) have observed similar changes in the insulin binding capacities of rabbit lung cells which were shown to be due to alterations in receptor numbers rather than the affinity of the hormone for the receptor. These changes in the insulin binding capacities parallel a peak in the level of endogenous glucocorticoids (Mulay, 1973; Martin, 1977). Since glucocorticoid have been shown to be important in the maturation of several enzyme systems in the foetus it is tempting to speculate that glucocorticoids may play a role in the regulation of insulin receptor numbers in certain foetal tissues. Recently, Kosaki *et al.* (1993) and Norgren *et al.* (1994a) demonstrated that dexamethasone alters the expression of the insulin receptor isoforms in cultured HepG2 cells.

1.3 THE PRESENT STUDY

It is apparent from this review of the literature that whilst the general structure of the insulin receptors in several species is known, the structural details of these proteins can differ from tissue to tissue. Furthermore, the bulk of the research in these areas focus on liver, muscle and adipose tissues. Whilst these tissues are principal insulin-sensitive tissues in mammals, mammary and placental tissues proliferate during the various stages of the reproductive cycle to become critical insulin-sensitive organs which sequester a significant proportion of the body's nutrients to maintain foetal and neonatal growth. Yet, perhaps suprisingly, relatively little is known about the structure of the insulin receptor or insulin receptor isoform distribution in mammary and foetal tissues. The major focus of this study was two-fold; firstly, this study characterised the structure of the mammary insulin receptor to further our understanding of the role insulin plays in the lactating mammary gland and secondly, this study characterised the distribution of insulin receptor isoforms in a range of rat tissues throughout the various stages of foetal and neonatal development and during lactation.

CHAPTER TWO

PURIFICATION OF A14-TYROSYL[^{125}I]IODOINSULIN USING C18 REVERSE PHASE CARTRIDGES

The development of a novel method of purifying A14-tyrosyl[^{125}I]iodoinsulin resulted from the need for an inexpensive source of the radiolabelled tracer. This chapter presents an expanded version of work which was published in the American peer-reviewed journal, Analytical Biochemistry. Deleo D.T., Helmerhorst E. 1992 Purification of A14-tyrosyl[^{125}I]iodoinsulin using C18 reverse phase cartridges. Analytical Biochemistry 206, 207-210 (Appendix A).

2.1 INTRODUCTION

Radioiodinated insulin is utilised in many applications including radioreceptor and radioimmunoassays. The validity of these applications often demand that the binding and biological properties of the radioiodinated insulin analogue be essentially indistinguishable from the native hormone. A14 tyrosyl monoiodinated insulin is an iodination product of insulin which best satisfy this criteria (Hamlin, 1974; Gliemann, 1979; Danho, 1980; Linde, 1981; Chu, 1992).

The iodination of insulin results in a heterogeneous population of radioiodinated insulin molecules variably modified on tyrosyl residues at positions 14 and 19 on the A chain, and 16 and 26 on the B chain of insulin (Linde, 1980; Sodoyez, 1975). The iodine distribution depends on the iodination reaction conditions and the state of aggregation of insulin (Linde, 1980; Regoeczi, 1984). Iodination conditions which favour modification of the A14 tyrosyl residue have been reported. However, it generally is desirable to remove unwanted by-products which minimally constitutes 20-30% of the radiotracer and native insulin which dilutes the specific activity of the product several fold.

Various purification techniques including high performance liquid chromatography (HPLC), ion exchange chromatography and polyacrylamide gel electrophoresis (PAGE) have been used to isolate of A14-tyrosyl[^{125}I]iodoinsulin from the other species of radiolabelled insulin and native insulin (Frank, 1983; Lioubin, 1984; Linde, 1981). However, all these methods have significant limitations either in terms of expense, convenience or safety of the procedure. The cost of purchasing A14-tyrosyl[^{125}I]iodoinsulin can be prohibitive for laboratories which routinely use this tracer.

The purpose of this work was to develop a relatively simple, rapid and most importantly, an inexpensive method for obtaining a highly purified preparation of A14-tyrosyl[^{125}I]iodoinsulin with binding potency and storage stability comparable to commercially available preparations of the product.

2.2 *EXPERIMENTAL PROCEDURES*

2.2.1 *Preparation of zinc-free insulin*

Zinc-free insulin was prepared according to the method outlined by Sodoyez *et al.* (1975). In brief, 100 mg of commercial porcine zinc insulin (Sigma Chemical Company, Castle Hill, NSW) was dissolved in 25 mL of 0.01 N HCl. Zinc ions were chelated by the addition of 1 mL of 50 mM ethylene diamine tetraacetic acid (EDTA). Zinc-free insulin was precipitated by the addition of 13 mL of 0.2 M sodium citrate, pH 5.6 at room temperature. The precipitate was recovered by centrifugation at 4,000g for 10 minutes and lyophilised. The recovery of insulin was determined by protein analysis according to the Lowry protein assay (1951). Zinc-free porcine insulin was used as the insulin source in all experiments unless otherwise stated and was stored at -20°C until required.

2.2.2 *Iodination of zinc-free insulin*

Zinc-free insulin (10 µg) in 10 µL of 0.4 M phosphate buffer, pH 7.4 was mixed with 37 MBq of sodium[^{125}iodide] (70 TBq/mmol purchased from Amersham Australia Pty. Ltd., North Ryde, NSW) or 80 ng of sodium[^{127}iodide] in 0.1 mM sodium hydroxide. Oxidation of iodine in the presence of insulin was catalysed by the addition of 10 µL of 15 mM N-chloro-p-toluene sulphonamide sodium salt (chloramine-T) in 0.4 M phosphate buffer, pH 7.4 with rapid mixing at room temperature. The reaction was stopped after 20 seconds by the addition of 50 µL of 15 mM sodium metabisulphite in 0.4 M phosphate buffer, pH 7.4. The iodination mixture was then immediately purified as described in the following section.

2.2.3 *Purification of A14-tyrosyl[^{125}I]iodoinsulin: a novel procedure*

The following is a description of the final procedure which I developed for the purification of A14-tyrosyl[^{125}I]-iodoinsulin. A 10 mL syringe was mounted on a SEP-PAK C18 cartridge (Waters Chromatography Division, Millipore Corporation, MA, USA) to facilitate safe washing, sample loading and elution steps (between washes the syringe was separated from the cartridge to enable the plunger to be withdrawn). The cartridge was washed prior to sample application using 10 mL of 50% (v/v) acetonitrile containing 50 mM triethylamine (TEA) which had been adjusted to pH 3 using orthophosphoric acid. The cartridge was washed with 10 mL of distilled, deionised water and the iodination mixture applied to the prewashed cartridge. The sample was washed through the cartridge with 5 mL of 0.4 M phosphate buffer, pH 7.4. This was followed by 10 mL of 29% (v/v) acetonitrile containing 50 mM TEA and 5 mL of 10% (v/v) acetonitrile containing 0.2 M ammonium acetate, pH 5.5. A14-tyrosyl[^{125}I]iodoinsulin was then eluted free from native insulin and unwanted iodinated products using 5 mL of 50% (v/v) acetonitrile containing 0.2 M ammonium acetate, pH 5.5. All steps were carried out in a fume hood, at room temperature, using the appropriate radioisotope handling procedures.

The A14-tyrosyl[^{125}I]iodoinsulin solution was aliquoted into 500 μL lots. The aliquots were placed at -70°C until frozen followed by lyophilisation and storage at -20°C until required. For certain experiments a storage buffer was added to the eluate to give a final concentration of 50 mM tris(hydroxymethyl)amino methane-HCl (Tris-HCl) pH 7.4 at 4°C containing 0.1% (w/v) bovine serum albumin, 5% (w/v) glycerol and 100 U/mL bacitracin. These samples were stored at -20°C until required.

2.2.4 High performance liquid chromatography analysis

HPLC of the various iodination reaction mixtures and purified A14-tyrosyl[^{125}I]iodoinsulin was carried out on a standard commercial system consisting of a LKB Bromma 2150 HPLC pump in conjunction with an LKB 2152 controller (Pharmacia-LKB Biotechnology, Uppsala, Sweden). A 0.46 x 25 cm Beckman ODS C18, 5 micron column (Beckman Instrument Inc.-Altex Division, CA, USA) was used for the separation of the various iodinated products.

2.2.5 N-terminal amino acid sequencing of purified A14-tyrosyl[^{125}I]iodoinsulin

A lyophilised aliquot of the iodoinsulin derivative was manually subjected to 26 successive Edman degradation cycles as outlined by Yarwood (1989) to establish the amino acid residue(s) at which iodine-125 was incorporated into the insulin molecule. The peptide (15 MBq of A14-tyrosyl[^{125}I]iodoinsulin plus 0.5 mg of carrier porcine insulin) was dissolved in 20 μL of 50% (v/v) pyridine and mixed with 100 μL of 5% (v/v) phenylisothiocyanate (PITC) in pyridine in an acid-washed tube. The solution was flushed with nitrogen, sealed and incubated for 1 h at 45°C. Following this incubation, the peptide was lyophilised in the presence of phosphorous pentoxide. The dried peptide was resuspended in 200 μL of anhydrous trifluoroacetic acid (TFA), the tube purged with nitrogen, sealed and incubated for a further 30 minutes at 45°C. Following lyophilisation, the peptide was resuspended in 150 μL of water and the derivatised amino acid extracted three times with 1.5 mL aliquots of n-

butyl acetate. The n-butyl acetate fraction (upper phase) containing the derivatised amino acid was separated from the water layer and the radioactivity in the fraction monitored in a Hewlett Packard Cobra™ II auto-gamma® counter. The remaining peptide in the water fraction was lyophilised before being subjected to the next degradation cycle.

2.2.6 Preparation of human placental microsomal membranes

Human placental tissue was obtained within two hours post partum from King Edward Memorial Hospital, Subiaco, WA. Placental microsomal membranes were prepared according to the procedure outlined by Fujita-Yamaguchi *et al.* (1983). In brief, the placental tissues were placed on ice and rinsed several times using 0.9% (w/v) sodium chloride containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) to remove excess blood. The amnion and chorion membranes were removed and the tissue cut into smaller pieces. The tissue was rinsed twice in 50 mM 4-(2-hydroxy ethyl)-1-piperazine ethane-sulphonic acid buffer (HEPES), pH 7.6 at 4°C containing 250 mM sucrose and 0.1 mM PMSF (HSP buffer). This tissue was homogenised in 1L of HSP buffer using a Polytron PCU-2 tissue homogeniser (Kinematica, Lucern, Switzerland) followed by centrifugation at 12,500g for 40 minutes at 4°C. Magnesium chloride and sodium chloride were added to the supernatant to a final concentration of 0.2 mM and 0.1 M respectively and the solution stirred on ice for 10 minutes. This solution was centrifuged at 22,000g for 2 h at 4°C, from which the supernatant was discarded and the pellets resuspended in 50 mM HEPES pH 7.6 at 4°C containing 0.1 mM PMSF and 0.1 M sodium chloride. This suspension was centrifuged at 112,000g for 20 minutes at 4°C. The resulting pellets were again resuspended in 50 mM HEPES pH 7.6 at 4°C containing 0.1 mM PMSF and centrifuged at 112,000g for 20 minutes at 4°C. The protein concentration in these placental membrane preparations were estimated using the Lowry protein assay (1951) and the pellets stored at -70°C until required.

2.2.7 Competitive displacement binding studies

A14-tyrosyl[^{127}I]iodoinsulin purified using a C18 cartridge or native porcine insulin (600 - 0.05 ng/50 µL) were incubated for 16 h at 4°C with 1 KBq (50 µL) of Amersham human A14-tyrosyl[^{125}I]iodoinsulin (70 TBq/mmol) and placental microsomal membranes (70 µg protein). The membranes were quantitatively recovered by the addition of 300 µL of an ice-cold bovine gamma globulin-polyethylene glycol solution. This solution of 0.07% (w/v) bovine gamma globulin and 16.7% (w/v) polyethylene glycol 6000 prepared in 0.05 M HEPES, pH 7.8 at 4°C containing 0.1 mM PMSF facilitated the quantitative recovery of the microsomal membranes. The membrane particles were recovered by centrifugation at 1,800g for 30 minutes at 6°C. The supernatant was aspirated and the radioactivity in the remaining pellet was monitored in a Hewlett Packard Cobra™ II auto-gamma® counter.

2.2.8 Analysis of binding data

Data reduction was performed using the method of Scatchard (1949) as incorporated in the Biosoft Ebda V 2.0 and Ligand programs (McPherson, 1985). The data was fitted with either a one-site or two-site model and the goodness of fit determined using the Runs test of Bennett and Franklin (1954). This test predicts whether the scatter of points about a fit is likely due to chance, and therefore, whether a given model provides a significant fit for the data. The statistical comparison of various sets of data for a given model was also performed using the Ligand program. All points within the analysis were given equal weighting and non-specific binding was handled as a computer fitted parameter. Mathematical comparisons of binding experiments were analysed using both the Runs test and the F-test which is based on the hypothesis of a second fit being a significant improvement on the first fit by determining the statistical significance of the difference between the mean square values for the two sets of data.

2.3 RESULTS

Insulin was iodinated using chloramine-T as the oxidant at a 3 : 1 molar ratio of insulin to iodine, and the products were separated by reverse phase chromatography. Five major peaks of radioactivity were resolved (figure 2.1). Based on published data, peaks 1 to 5 corresponded to free iodine, A19-tyrosyl[^{125}I]-iodoinsulin, B26-tyrosyl[^{125}I]iodoinsulin, B16-tyrosyl[^{125}I]iodoinsulin and A14-tyrosyl[^{125}I]iodoinsulin respectively (Frank, 1983; Lioubin, 1984). Integration of the areas under peaks 1 to 5 indicated that 42% of the radioactivity was located on the A14 tyrosyl residue, 11% on the B26 tyrosyl residue, 13% on the B16 tyrosyl residue and 21% on the A19 tyrosyl residue.

Since the A14-tyrosyl[^{125}I]insulin derivative adsorbed more strongly to C18 supports than the other monoiodinated forms (Frank, 1983; Lioubin, 1984), I evaluated small, disposable cartridges packed with a C18 matrix for selectively purifying the A14-tyrosyl[^{125}I]iodoinsulin derivative. An iodination mixture of insulin was applied to a C18 cartridge which was washed with 0.4 M phosphate buffer to remove any free Na[^{125}I]. Increasing acetonitrile concentrations were applied to the cartridge until the tracer was eluted. With 29% (v/v) acetonitrile in 50 mM TEA pH 3, 100% of the free iodine and more than 96% of the native insulin eluted off the cartridge (figure 2.2). A subsequent wash with 10% (v/v) acetonitrile containing 0.2 M ammonium acetate, pH 5.5 facilitated the removal of TEA from the system. This was necessary as TEA had an adverse effect on the stability of the purified insulin derivative during prolonged storage. The A14-tyrosyl[^{125}I]-iodoinsulin derivative was eluted quantitatively in 5 mL with 50% (v/v) acetonitrile in 0.2 M ammonium acetate pH 5.5 at room temperature.

The 50% (v/v) acetonitrile eluate pooled from peak D (figure 2.2) was rechromatographed using a 0.46 x 25 cm reverse phase column in line with a HPLC system. HPLC analysis of this preparation resolved as a single peak (figure 2.3). Furthermore, N-terminal amino acid sequencing data from the analysis of the C18

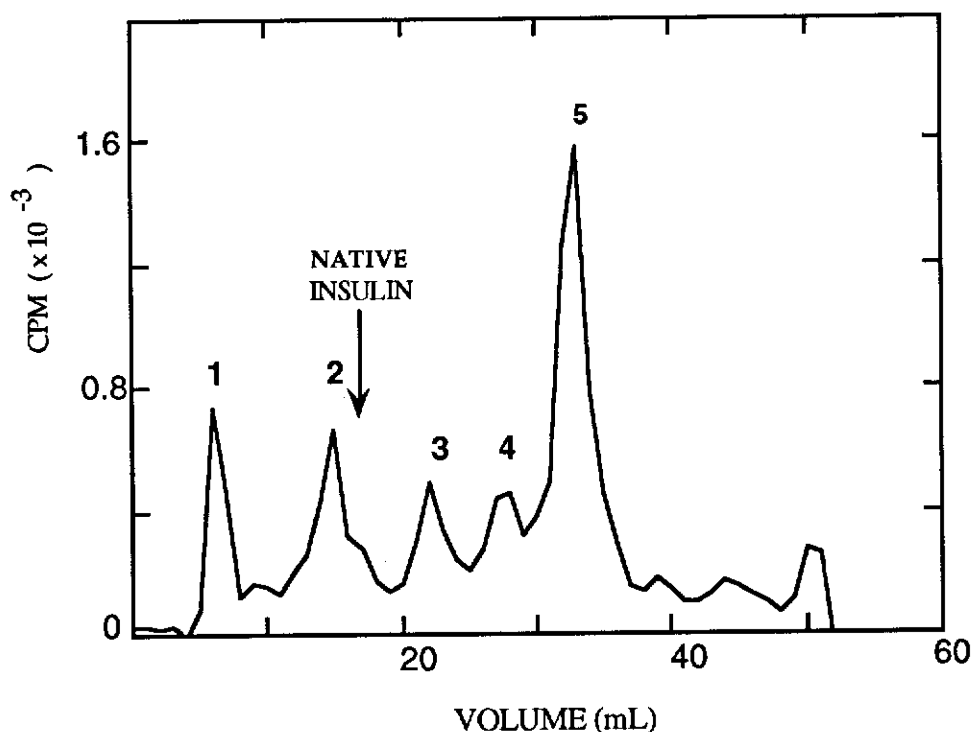

Figure 2.1 High-performance liquid chromatography of an insulin iodination mixture. Insulin was iodinated using the chloramine-T method as described in the methods section. The iodination mixture was chromatographed on a Beckman Ultrasphere ODS-C18 column (5 microns, 4.6 mm x 25 cm) using a mobile phase of 29 % (v/v) acetonitrile in 0.2 M ammonium acetate, pH 5.5 at a flow rate of 1 mL/min (—). The arrow indicates the position of the A_{280} absorbance peak corresponding to native insulin.

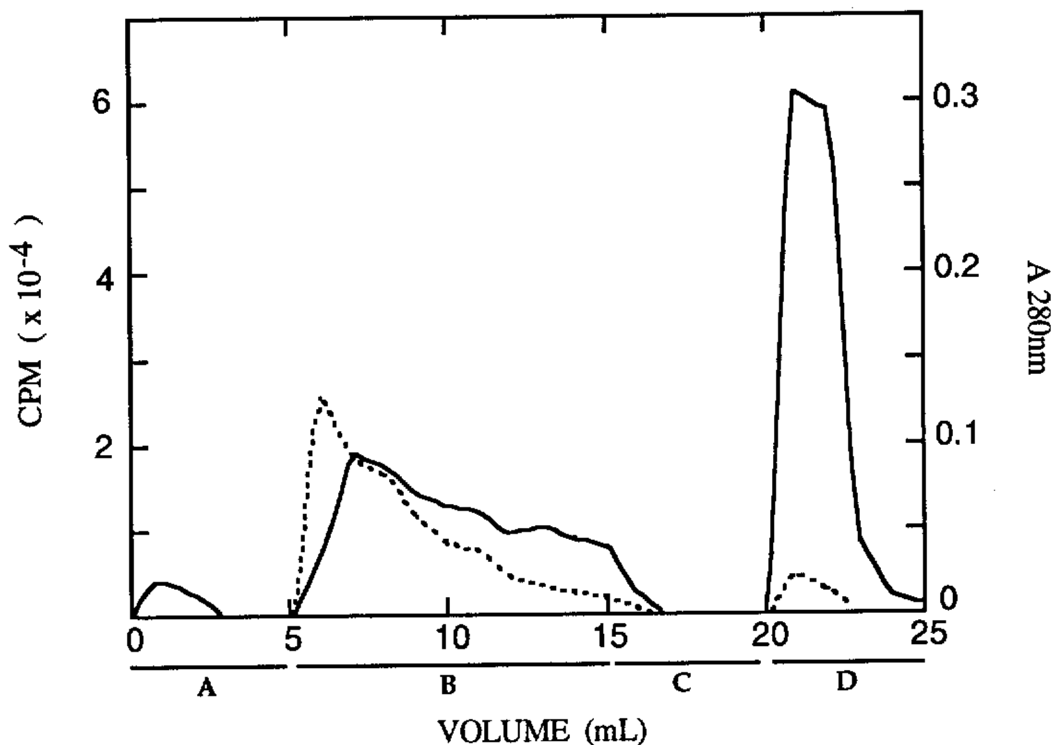

Figure 2.2 Step-wise elution of iodoinsulin derivatives on a C18 cartridge. Insulin was iodinated and resolved on a C18 cartridge. Radioactivity (—) was monitored in all wash and elution fractions. The wash fractions corresponded to 0.4 M phosphate buffer, pH 7.4 (**A**), 29 % (v/v) acetonitrile in 50 mM TEA, pH 3 (**B**) and 10 % (v/v) acetonitrile in 0.2 M ammonium acetate, pH 5.5 (**C**). Elution of the A14-tyrosyl [^{125}I]insulin was accomplished with 50 % (v/v) acetonitrile in 0.2 M ammonium acetate, pH 5.5 (**D**). The elution of native insulin was monitored by its absorbance at 280nm (----). The concentration of the iodinated insulin derivatives were sufficiently low not to be detected by A_{280} measurement.

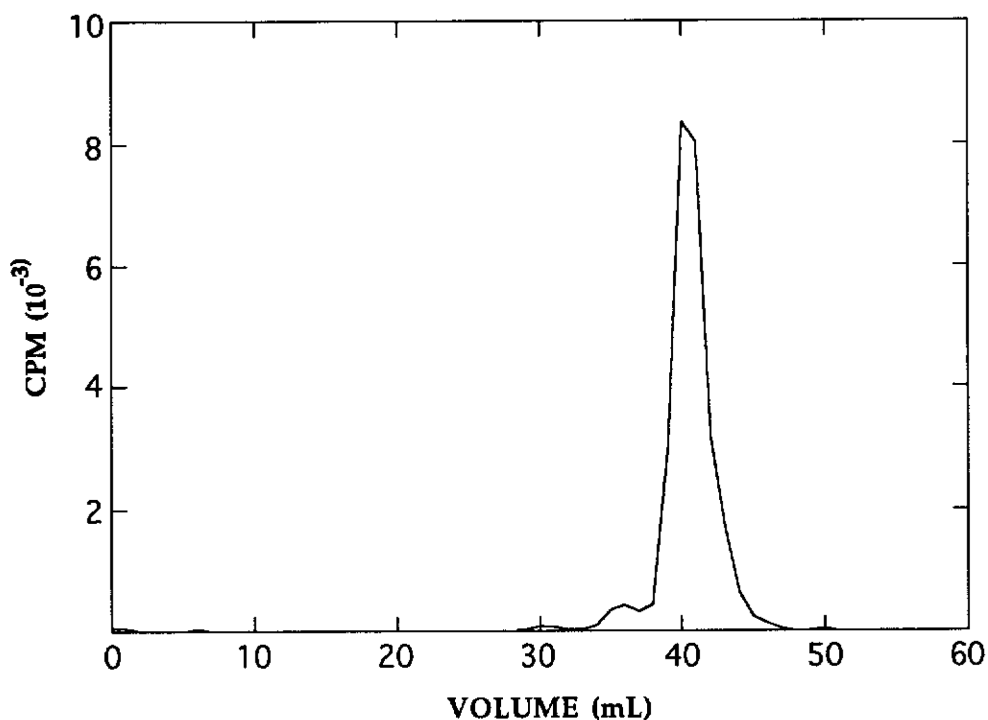

Figure 2.3 Rechromatography of the A14-tyrosyl[^{125}I]insulin purified using a C18 cartridge. A14-tyrosyl[^{125}I]insulin was purified using a C18 cartridge and the purified insulin analogue rechromatographed on a Beckman Ultrasphere ODS-C18 column (5 microns, 4.6 mm x 25 cm) using a mobile phase of 29 % (v/v) acetonitrile in 0.2 M ammonium acetate, pH 5.5 at a flow rate of 1 mL/min (——).

purified A14-tyrosyl[^{125}I]iodoinsulin indicated that the predominant release of radioactivity occurred in the fourteenth cycle (figure 2.4). Carryover of the radioactivity in the subsequent cycles was due to incomplete coupling associated with each cycle (Frank, 1983) and was an unavoidable limitation of carrying out this procedure manually. As no radioactivity would be expected in the 15th cycle, the carryover due to incomplete coupling was estimated to be approximately 50-75%. Based on this calculation the radioactivity appearing in the 16th cycle, which corresponds to the B16 tyrosyl residue, could be completely accounted for by carryover from previous cycles. Negligible levels of radioactivity were released in the 19th and 26th cycles. The profile obtained using a commercially purchased A14-tyrosyl[^{125}I]iodoinsulin was superimposable with the profile obtained for the A14-tyrosyl[^{125}I]iodoinsulin prepared using the C18 cartridge.

The binding of A14-tyrosyl[^{127}I]iodoinsulin purified using C18 cartridges and native porcine insulin to microsomal membranes from human placenta were compared. A14-tyrosyl[^{127}I]iodoinsulin was prepared according to the protocol outlined in section 2.2.3, and commercially available A14-tyrosyl[^{125}I]iodoinsulin was used as the tracer in these competitive displacement-binding studies. The resulting competitive displacement plots comparing A14-tyrosyl[^{127}I]iodoinsulin and native insulin were virtually superimposable (figure 2.5). No significant difference was observed between the two sets of data using the Students paired t-test (P=0.08).

The stability of the purified A14-tyrosyl[^{125}I]iodoinsulin tracer at -20°C was estimated by monitoring the binding affinity of the tracer for placental insulin receptors at days 1, 30 and 60 of storage. Identical preparations of the purified radiolabel was stored in the presence and absence of 50 mM Tris buffer, pH 7.4 at 4°C containing 0.1% (w/v) bovine serum albumin, 5% (w/v) glycerol and 100 U/mL bacitracin (storage buffer). At each interval, Scatchard analysis was performed using the stored radiolabel as the tracer, and native insulin and human placental microsomal membranes as the ligand and receptor source, respectively. Storage of the tracer in the absence of

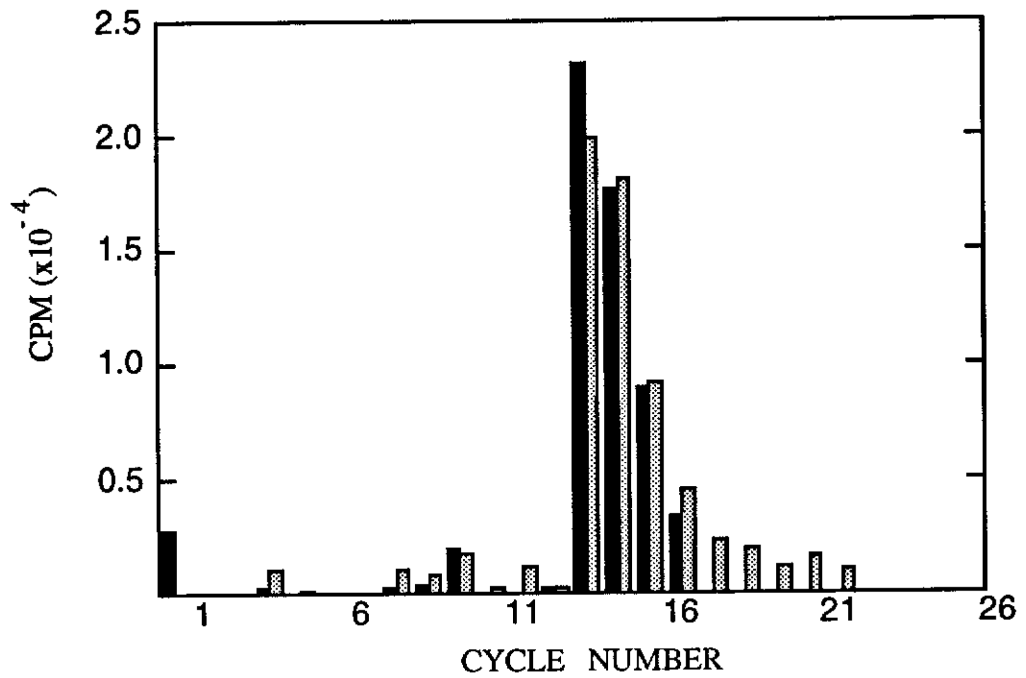

Figure 2.4 N-terminal amino acid sequencing of the purified iodoinsulin derivatives. A14-tyrosyl[^{125}I]iodoinsulin purified using a cartridge packed with a C18 support (■) and commercially purchased A14-tyrosyl[^{125}I]-iodoinsulin (▩) were sequenced as described in the methods section.

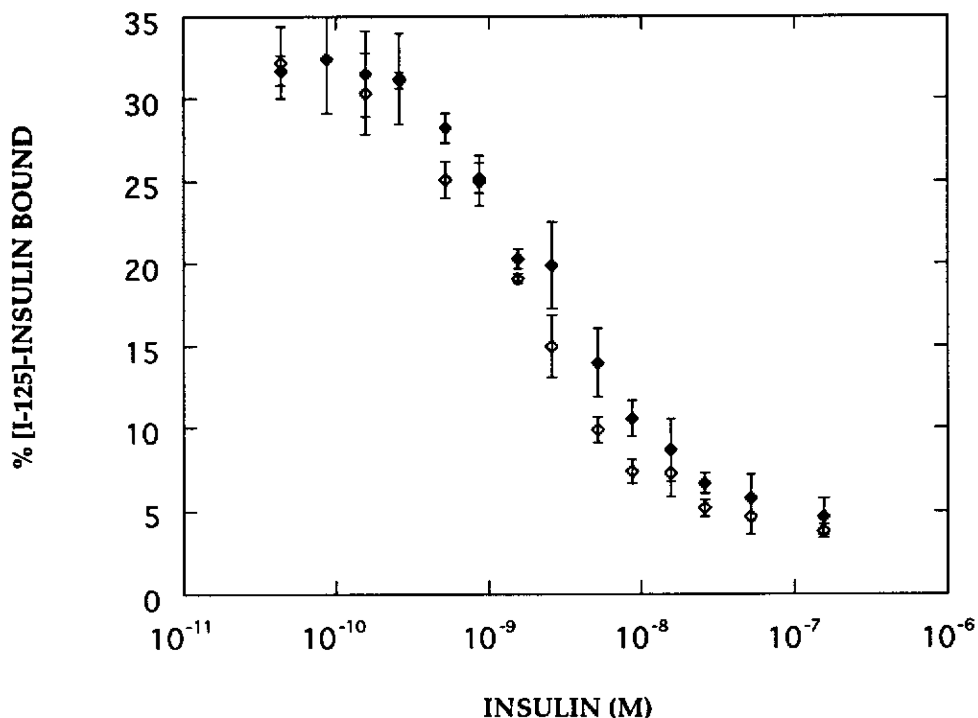

Figure 2.5 Comparison of C18 cartridge-purified A14-tyrosyl[^{127}I]-iodoinsulin and native insulin binding affinity. Competitive displacement plots of A14-tyrosyl[^{127}I]iodoinsulin purified using a cartridge packed with a C18 support (◇) and native insulin (◆) against commercially available A14-tyrosyl[^{125}I]-iodoinsulin. Human placental membranes were used as the insulin receptor source as outlined in the methods section. Each data point represent the mean from the experiment performed in triplicate, error bars represent one SD from the mean.

buffer was detrimental to the shelf-life of the radiotracer (figure 2.6A). Statistical analysis of the competitive displacement data obtained over a two month period indicated that their was a significant difference (P=0.01) between the day 1 and day 60 experimental results. Indeed, the competitive displacement plot of the A14-tyrosyl[^{125}I]iodoinsulin on day 60 indicates a complete loss of insulin-binding specificity. In contrast, A14-tyrosyl[^{125}I]iodoinsulin stored in the presence of storage buffer (figure 2.6B) extended the stability of the tracer to over 60 days (comparison of day 1 and day 60 data, P=0.25).

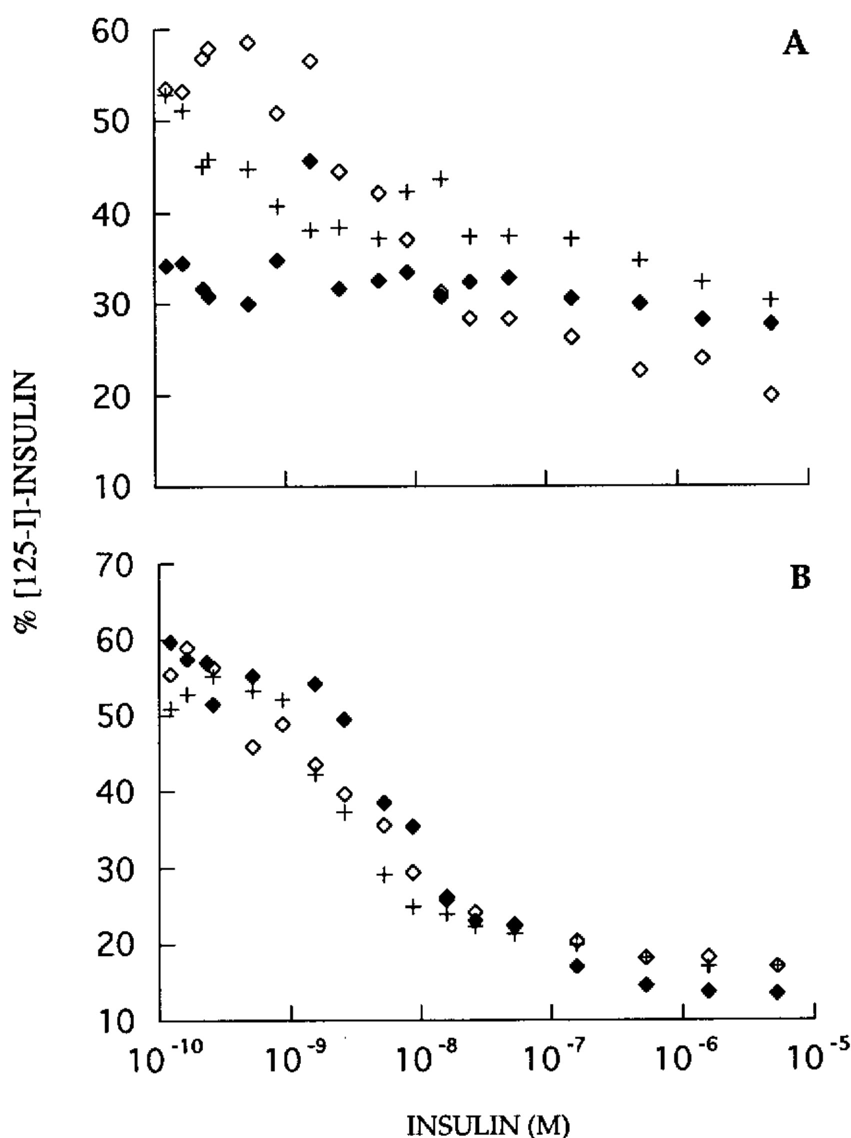

Figure 2.6 Stability of the C18 cartridge-purified A14-tyrosyl[^{125}I]-iodoinsulin over a 60 day period. Porcine insulin competitive displacement experiments were performed using A14-tyrosyl[^{125}I]iodoinsulin as the radiotracer and human placental membranes as the insulin receptor source as outlined in the methods section. Competitive displacement experiments were performed on days 1 (◆), 30 (+) and 60 (◇), using A14-tyrosyl[125I]iodoinsulin stored in the absence (graph A) and in the presence of 50 mM Tris buffer, pH 7.4 at 4°C containing 0.1 % (w/v) bovine serum albumin, 5 % (w/v) glycerol and 100 U/mL bacitracin (graph B). Each data point represent the mean from an experiment performed in triplicate.

2.4 DISCUSSION

A14-tyrosyl[^{125}I]iodoinsulin has been shown to be the radioactive tracer which exhibits activity most closely resembling that of native insulin (Hamlin, 1974; Gliemann, 1979; Danho, 1980; Linde, 1981; Chu, 1992). The secondary structure of the insulin A-chain consists of two distorted α-helices including residues A2-A8 and A13-A19 which run almost antiparallel and are connected by the peptide segment A9-A12 (Blundell, 1972). The A14 residue forms part of the second helical region and is located on the surface of the monomer which is fully exposed to the external environment. In most mammalian species, with the exception of the coypu and guinea pig, the A14 position is occupied by a tyrosine residue (Dayhoff, 1969). Chu *et al.* (1992) have demonstrated that the substitution of a hydrophilic acidic or basic group or of bulky hydrophobic groups at the A14 position results in only small changes in biological activity. However, significant reduction in biological activity does occur when a change at this position, for example the substitution of cyclohexalanine at the A14 position, disrupts the three dimensional configuration of the hormone. The monoiodination of insulin at the A14 position has no apparent effect on the biological activity of the hormone (Danho, 1980; Hamlin, 1974; Gliemann, 1979; Linde, 1981; Chu, 1992)

The A19 tyrosine residue located on the A chain also forms part of the A13-A19 helical region. However, in contrast to the A14 tyrosine, amino acids substitutions at A19 essentially abolishes biological activity (reviewed by Chu, 1992). Monoiodination of insulin at the A19 position reduces biological activity to ~50% that of the native hormone (reviewed by Chu, 1992).

The monoiodination of insulin within the B chain, at either the B16 or B26 position, also results in insulin analogues with binding affinities significantly different from the native hormone. Iodination at the B26 position increases the binding affinity of the analogue to rat adipocytes, IM-9 (human lymphocytes) and HT-29 (human adenocarcinoma cell line) cells to approximately 180-220% that of the A14-

tyrosyl[^{125}I]iodoinsulin (Sonne, 1983). Interestingly, the B16-tyrosyl monoiodinated insulin exhibits binding characteristics similar to those of the A14 analogue in rat adipocytes and hepatocytes, however, this analogue displays a significantly higher affinity in the IM-9 cell line suggesting that the insulin receptor in these tissues have significantly different insulin-binding properties (Sonne, 1983).

The product eluted from the C18 cartridge using the novel method developed in this study, was unambiguously identified by N-terminal sequencing as the A14-tyrosyl[^{125}I]iodoinsulin and was adjudged to be free of other contaminating monoiodinated insulin derivatives. HPLC rechromatography of the product confirmed this conclusion in addition to demonstrating that this product was not contaminated by free iodine (figure 2.1). I also estimated that less than 4% of unmodified insulin coeluted with the product (figure 2.2). Thus the specific activity of this product could be estimated from the known specific activity of the Na[^{125}I] assuming that one iodine molecule was incorporated per molecule of insulin and accounting for a 4% dilution of the specific activity by native insulin. On this basis, the A14-tyrosyl[^{125}I]iodoinsulin purified using the C18 cartridge was estimated to have a specific activity of approximately 67-74 TBq/mmol. This specific activity compares favourably with commercial preparations of this radiolabel, making it highly suited for competitive binding analysis.

The exclusion of TEA from the A14-tyrosyl[^{125}I]iodoinsulin elution buffer ensured that TEA contamination in the eluted product was minimal. This was advantageous since the presence of TEA during the storage of the tracer dramatically decreased storage stability (data not shown). Although the precise reason for this effect was not investigated, when the A14-tyrosyl[^{125}I]iodoinsulin product was lyophilised in the presence of TEA, a residue remained which had the characteristic odour of TEA. This suggested that the A14-tyrosyl[^{125}I]iodoinsulin tracer was in an environment of concentrated TEA which was detrimental to the storage-life of the

product. The exclusion of TEA from the buffers prior to the elution step significantly improved the storage stability of the radiotracer.

Although a chloramine-T iodination procedure was used in this study, it should be noted that the lactoperoxidase iodination method is better suited to the preparation of A14-tyrosyl[^{125}I]iodoinsulin. Chloramine-T is a relatively small molecule which enables easy access to most parts of the protein. In contrast, the lactoperoxidase molecule is significantly larger which limits its accessibility to the surface tyrosine residues only. The lactoperoxidase catalysed protein iodination employs milder conditions and a slower reaction rate than chloramine-T which allows the degree of iodination to be more readily controlled. The lactoperoxidase enzymatic reaction is also more specific when compared with the chloramine-T chemical based reaction. Finally, lactoperoxidase iodination of porcine insulin in an aqueous environment results in the about 85% of the iodine being introduced into the A-chain (Frank, 1983). Indeed, the lactoperoxidase catalysed iodination of insulin is the current method of choice in our laboratory. The chloramine-T iodination method utilised in this study yielded about 63% of the iodination product as A chain [^{125}I]-iodoinsulin. The chloramine-T iodination method was evaluated in this study to maximally test the efficiency of the protocol described for purifying A14-tyrosyl[^{125}I]iodoinsulin.

The stability of the purified A14-tyrosyl[^{125}I]iodoinsulin was shown to be greatly improved when stored in the presence of 50 mM Tris buffer, pH 7.4 at 4°C containing 0.1% (w/v) bovine serum albumin, 5% (w/v) glycerol and 100 U/mL bacitracin (figure 2.6). Subsequently, a laboratory finding by Helmerhorst and Bailey (personal communication) suggests that the stability of the monoiodinated insulin tracer is further improved when stored in the absence of glycerol. This observation would support the findings of Brange and Langkjaer (1992) who suggest that the use of glycerol as an excipient adversely effects the stability of insulin at neutral pH.

HPLC and PAGE methods for purifying the A14-tyrosyl[^{125}I]iodoinsulin derivative have significant limitations. Firstly, they often require extensive handling of the iodination mixtures outside the safe confines of a fumehood. Secondly, because of the levels of radioactivity involved in an iodination, expensive equipment must be dedicated to the task. Finally, HPLC and PAGE methods are relatively laborious. The method described in this chapter is extremely simple and can be entirely confined to a fumehood environment, making it particularly safe. No expensive equipment needs to be devoted to the procedure. Furthermore, the method developed in this study is inexpensive, costing less than AUD$10 per 0.4 MBq of product. In contrast, commercially available A14-tyrosyl[^{125}I]iodoinsulin preparations are prohibitively expensive to purchase on a routine basis (approximately AUD$500 per 0.4 MBq).

CHAPTER THREE

STRUCTURE OF THE INSULIN RECEPTOR IN MAMMARY AND LIVER TISSUES

Results from this work was presented, in part, as a poster at the 35th Annual Conference of the Australian Society for Biochemistry and Molecular Biology, Canberra 1991, and the abstract for which has been published in the Proceedings of the aforementioned conference (SP57) (Appendix B).

3.1 INTRODUCTION

Insulin acts in mammary and other target tissues by first binding to the extracellularly located α-subunit of the insulin receptor. Size variations of the α-subunit have been described in several tissues including brain, skeletal muscle, liver and monocytes (Yip, 1980; Heidenreich 1983; Burant, 1986a; McElduff, 1985; Heidenreich, 1986). In certain tissues, this size variation appears to be associated with the extent of post-translational receptor glycosylation (Heidenreich 1983; Burant, 1986a). However, in these instances neither the primary structure of the glycoprotein nor the effect of proteolysis have been evaluated.

More recently, Burnol *et al.* (1990b) and Helmerhorst *et al.* (unpublished) have observed a variation in the size of the insulin-binding subunit from mammary and liver insulin receptors from lactating rats. The purpose of this study was to confirm this observation and to establish if any difference in size of the α-subunit of the insulin receptor was due to changes in receptor glycosylation or proteolysis of the insulin receptor.

3.2 EXPERIMENTAL PROCEDURES

3.2.1 Animals

Wistar albino rats (*Rattus norvegicus*) with a gestation period of 22 days were obtained from the Animal Resource Centre (Perth, WA). The day after parturition was defined as day 1 of lactation. The animals were housed at a constant temperature (21-23°C) and under controlled lighting conditions. Unless otherwise stated, the animals were fed *ad libitum* on standard rat chowder. All experiment were performed in accordance with the guidlines stipulated by the Australian Animal Ethics Committee.

3.2.2 Specialised reagents

High and low molecular weight standards (200 kDa to 31 kDa) were purchased from Bio-Rad Laboratories (Richmond, USA). $N^{\varepsilon B29}$-mono(azidobenzoyl)insulin ($N^{\varepsilon B29}$-MABI) was a gift from Dr Cecil Yip of the Banting and Best Department of Medical Research, Toronto, Canada. Sodium[^{125}iodide] (70 TBq/mmol) was obtained from Amersham Australia Pty. Ltd. (Sydney, NSW). Neuraminidase from *Clostridium perfringens* type V (EC 3.2.1.18) and glycopeptidase F from *Flavobacterium meningosepticum* (EC 3.2.2.18) were obtained from Sigma (St. Louis, MO, USA).

3.2.3 Radioiodination of $N^{\varepsilon B29}$-mono(azidobenzoyl)insulin

The protocol for the radioiodination of $N^{\varepsilon B29}$-MABI was based on that described by Yip (1984). All procedures involving the photosensitive insulin analogue were performed in the dark. Sodium[^{125}iodide] (18 MBq) was added to 5 μL of 1 mg/mL $N^{\varepsilon B29}$-MABI and 10 μL of 0.4 M sodium phosphate buffer, pH 7.5. Iodination of the peptide was initiated by the addition of 5 μL of 15 mM chloramine-T and was allowed to continue for 20 seconds with continuous mixing. The reaction was halted by the addition of 25 μL of 15 mM sodium metabisulphite. Bovine serum albumin was added to give a final concentration of 1.6 % (w/v) and

the mixture was then applied to a Sephadex G-25M column prewashed with 25 mM Tris, pH 7.4. Nine 0.75 mL aliquots of 0.25 M Tris buffer, pH 7.4 were individually applied to and collected from the column. The radioactivity present in each 0.75 mL fraction was counted and the fractions containing the first peak of radioactivity, corresponding to the elution peak of insulin, were pooled and stored at -70°C until required.

3.2.4 Preparation of liver plasma membranes

Liver plasma membranes were obtained from rats on days 1, 5, 10, 15 and 20 of lactation according to the method of Ray (1970). Liver tissue (~15g) was placed in 1 mM sodium bicarbonate, pH 7.5 containing 0.5 mM calcium chloride (homogenising buffer). The liver was coarsely minced and then homogenised using a Dounce tissue grinder fitted with the loose pestle (Wheaton, NJ, USA). The homogenate was diluted 100-fold w/v and incubated on ice for 10 minutes. Large tissue pieces were removed by filtration through double layers of nylon gauze. The filtrate was centrifuged at 1,900g for 30 minutes at 4°C. The membrane pellet was resuspended in homogenising buffer and diluted to one half of its original volume was recentrifugation at 1,700g for 15 minutes at 4°C. The membrane pellet was resuspended again in one quarter of the original volume of homogenising buffer and the suspension was centrifuged at 1,700g for 15 minutes at 4°C. The remaining pellet was resuspended in sufficient 70% (w/v) sucrose solution such that the final sucrose concentration was 50% (w/v). This mixture was applied to a discontinuous sucrose density gradient comprising of 37%, 41% and 45% sucrose (measured using a sucrose refractometer) and centrifuged at 100,000g for 2 h at 4°C. The plasma membranes which migrated to the 37%:41% sucrose interface were removed and diluted 5-fold in 50 mM Tris, pH 7.5, containing 100 U/mL bacitracin. Following centrifugation at 25,000g for 20 minutes at 4°C the pellet was resuspended in 50 mM Tris, pH 7.5, containing 100 U/mL bacitracin and stored at -70°C until required.

3.2.5 Preparation of mammary membranes

Mammary membrane preparations were obtained from Wistar rats on days 1, 5, 10, 15 and 20 of lactation. Mammary membranes were partially purified according to the method of Dijane et al. (1977). Mammary tissue (~10g) was excised into 25 mM Tris, pH 7.5 containing 0.3 M sucrose, 1 mM PMSF, 2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 2 mg/mL bacitracin, 1,000 trypsin inhibitor U aprotinin/mL, 100 mM sodium fluoride and 0.2 mM sodium orthovanadate. The tissue was diluted 10-fold (w/v) in the Tris buffer and homogenised using a Polytron PCU-2 tissue homogeniser (Kinematica, Lucern, Switzerland). The homogenate was centrifuged at 3,000g for 15 minutes at 4°C. The supernatant was then recentrifuged at 100,000g for 2 h at 4°C. The microsomal pellet was resuspended in 50 mM HEPES, pH 7.6 containing 150 mM sodium chloride, 1 mM PMSF, 2 mg/mL bacitracin, 0.25 trypsin inhibitor U aprotinin/mL, 100 mM sodium fluoride and 0.2 mM sodium orthovanadate and stored at -70°C until required.

3.2.6 Estimation of protein concentration

Membrane protein concentration was estimated using the method of Lowry *et al.* (1951). In brief, an aliquot of the membrane suspension was centrifuged at 2,000g for 10 minutes. This pellet was resuspended in distilled water and the centrifugation-resuspension steps were repeated. A 200 μL aliquot of this suspension was incubated with 0.19 M sodium carbonate containing 0.1 M sodium hydroxide, 8 mM sodium tartrate, 35 mM sodium dodecyl sulphate (SDS) and 1.6 mM cupric sulphate pentahydrate for 10 minutes at room temperature. Sixty microlitres of a 50% (v/v) Folin-Ciocalteau phenol reagent was added and the solution incubated for a further 45 minutes at room temperature. The absorbance of the final solution was measured at 660 nm and compared with the absorbances obtained for bovine serum albumin standards over the concentration range of 0-100 μg/mL.

3.2.7 Measurement of 5'-nucleotidase activity

The relative purity of the membrane preparations was determined by measuring the 5'-nucleotidase activity in the tissue homogenates and the purified membranes. The 5'-nucleotidase activity of these membrane preparations was determined indirectly by measuring the amount of phosphate generated from the action of the enzyme on 5'adenosine monophosphate (5'AMP) (Ames, 1960). Briefly, the membrane was resuspended in distilled water to give a final concentration of approximately 50 μg of protein/135 μL of water. Twenty five μL of 0.1 M magnesium chloride, 25 μL of 1M glycine buffer, pH 8.5 and 5.8 mM 5'AMP was added to the membrane suspension and the solution incubated for 15 minutes at 37°C. Protein in the sample was precipitated by the addition of 65 μL of 30% (w/v) trichloroacetic acid (TCA) solution, followed by centrifugation at 2,000g for 15 minutes at room temperature. The phosphate concentration in a 300 μL aliquot of this supernatant was determined by the addition of 700 μL of 1 N sulphuric acid containing 80 mM ascorbic acid and 2 mM ammonium molybdenate tetrahydrate. This solution was incubated for 30 minutes at 37°C and the absorbance of the final solution measured at 660 nm. The absorbance from the test samples were compared against those of phosphate standards prepared from a 0.4 mM monobasic sodium phosphate monohydrate standard.

3.2.8 Photoaffinity labelling of mammary and liver membranes

Mammary and liver membranes (1.5 mg/mL protein) in 50 mM Tris, pH 7.5 containing 0.1% (w/v) bovine serum albumin and 1 mM bacitracin were incubated in the dark with 10-50 nM of [^{125}I]-N$^{\varepsilon B29}$-MABI for 16 h at 4°C. Non-specific binding of the N$^{\varepsilon B29}$-MABI to the membranes was determined by the addition of 20 μM bovine insulin to the reaction mixture. Samples were photolysed for 30 seconds on ice using the focused light source of a high intensity 100 W high-pressure mercury lamp. The membranes then were centrifuged at 25,000 g for 15 minutes at 4°C. The supernatant was discarded and the membranes were solubilised by incubating at 100°C for 5 minutes in 62.5 mM Tris, pH 6.8 containing 3% (w/v)

SDS, 10% (v/v) glycerol and 100 mM dithiothreitol (DTT). Membranes were also solubilised without reduction by incubation at 100 °C for 5 minutes in 62.5 mM Tris, pH 6.8 containing 3% (w/v) SDS, 10% (v/v) glycerol and 10 mM N-ethylmaleimide. The inclusion of the sulfhydryl alkylating reagent, N-ethylmaleimide, maintains the insulin receptor in its native form by preventing the reduction of disulfides by receptor-associated sulfhydryl groups (Helmerhorst, 1986). Solubilised proteins were resolved on a 7.5% polyacrylamide gel in conjunction with high and low molecular weight markers to establish the relative molecular masses of the protein bands. Following autoradiography, densitometric scanning was performed using the Tracktel Video Densitometer software package in association with an NEC SX Plus computer and an Adpro PRO-CAM 205 video camera (Vision Systems Limited, SA).

3.2.9 Neuraminidase and glycopeptidase F treatment of the eluted 125 kDa /130 kDa bands from mammary and liver tissues

The effect of desialation and removal of N-linked carbohydrate moieties on the size of the 125 kDa and 130 kDa photoaffinity labelled bands from mammary and liver tissues, respectively, was assessed. The radiolabelled 125 kDa protein band was excised from the polyacrylamide gel and macerated in 150 µl of 25 mM Tris, pH 8.3 containing 10% (w/v) methanol and 0.1% SDS (elution buffer). The gel/buffer suspension was incubated at 4°C for 2h with constant mixing followed by centrifugation at 2,000g for 5 minutes at 4°C. The supernatant was removed and an further 100 µl of elution buffer was added to the macerated gel. This was incubated at 4°C for 16h with constant mixing followed by centrifugation at 2,000g for 5 minutes at 4°C. The supernatants were pooled and concentrated using Centricon-30 microconcentrators (Amicon, MA, USA). The concentrated protein was incubated with 20 µL of 50 mM HEPES, pH 5.1 containing 0.4 U of neuraminidase for 1 h at 37 °C or, alternatively, with 20 µL of 0.25 mM sodium phosphate buffer, pH 7.4 containing 10 mM EDTA, 10 mM β-mercaptoethanol, 15 mM 1,10-phenanthroline, 0.1 mM PMSF, 25 mM benzamidine, 10 trypsin inhibitor U aprotinin and 1.2 U

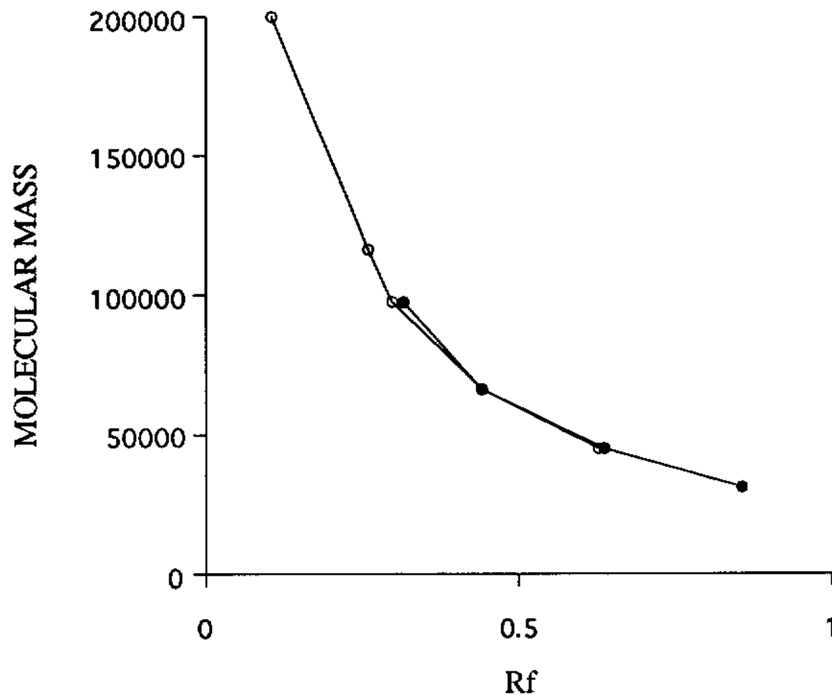

Figure 3.1 Typical protein molecular weight marker standard curve. This standard curve was obtained from the electrophoresis of Bio-Rad high and low molecular weight standards using a 7.5% polyacrylamide gel. High molecular weight standards include rabbit skeletal muscle myosin (200,000 Da), *E. Coli* β-galactosidase (116,250 Da), rabbit muscle phosphorylase b (97,400 Da), bovine serum albumin (66,200 Da), hen egg white ovalbumin (45,000 Da) (o). Low molecular weight standards include rabbit muscle phosphorylase b (97,400 Da), bovine serum albumin (66,200 Da), hen egg white ovalbumin (45,000 Da), bovine carbonic anhydrase (31,000 Da) (•).

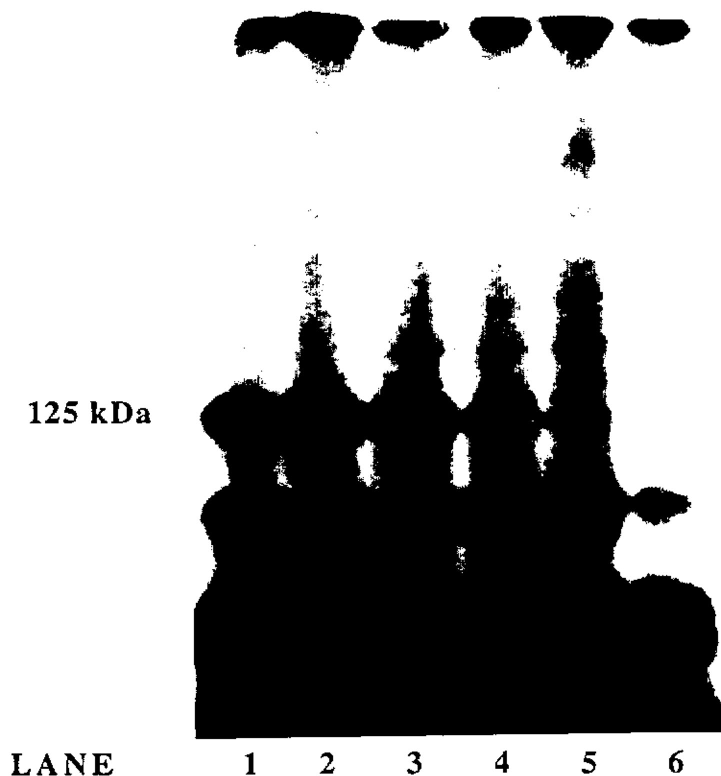

Figure 3.2 The effect of increasing native insulin concentrations of the specificity of [^{125}I]-N$^{\varepsilon B29}$-MABI labelling. Mammary membranes from day 14 lactating rats were incubated for 16 h at 4°C with approximately 10 nM [^{125}I]-N$^{\varepsilon B29}$-MABI in the presence of 50 mM Tris, pH 7.5 containing 0.1% (w/v) bovine serum albumin and 1 mM bacitracin and increasing concentrations of bovine insulin (lane 1, 0.9 nM bovine insulin; lane 2, 2.5 nM bovine insulin; lane 3, 9.5 nM bovine insulin; lane 4, 28 nM bovine insulin; lane 5, 170 nM bovine insulin; lane 6, 1.7 μM bovine insulin). Following photolysis, the membranes were solubilised and resolved by electrophoresis in a 7.5% polyacrylamide gel. The gel was subsequently dried and autoradiographed.

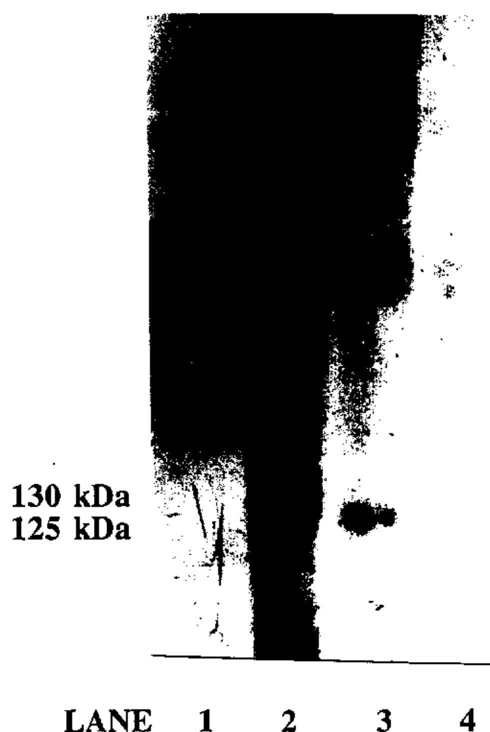

Figure 3.3 Photoaffinity labelled α-subunits of the rat mammary and liver insulin receptors. Liver (lanes 1 and 2) and mammary (lanes 3 and 4) membranes from day 14 lactating rats were incubated with 10-50 nM $[^{125}I]$-$N^{\varepsilon B29}$-MABI in the presence (lanes 1 and 4) or absence (lanes 2 and 3) of 20 μM bovine insulin. Following photolysis, the photoaffinity labelled membranes were solubilised and resolved by electrophoresis in a 7.5% polyacrylamide gel. The gel was dried and autoradiographed.

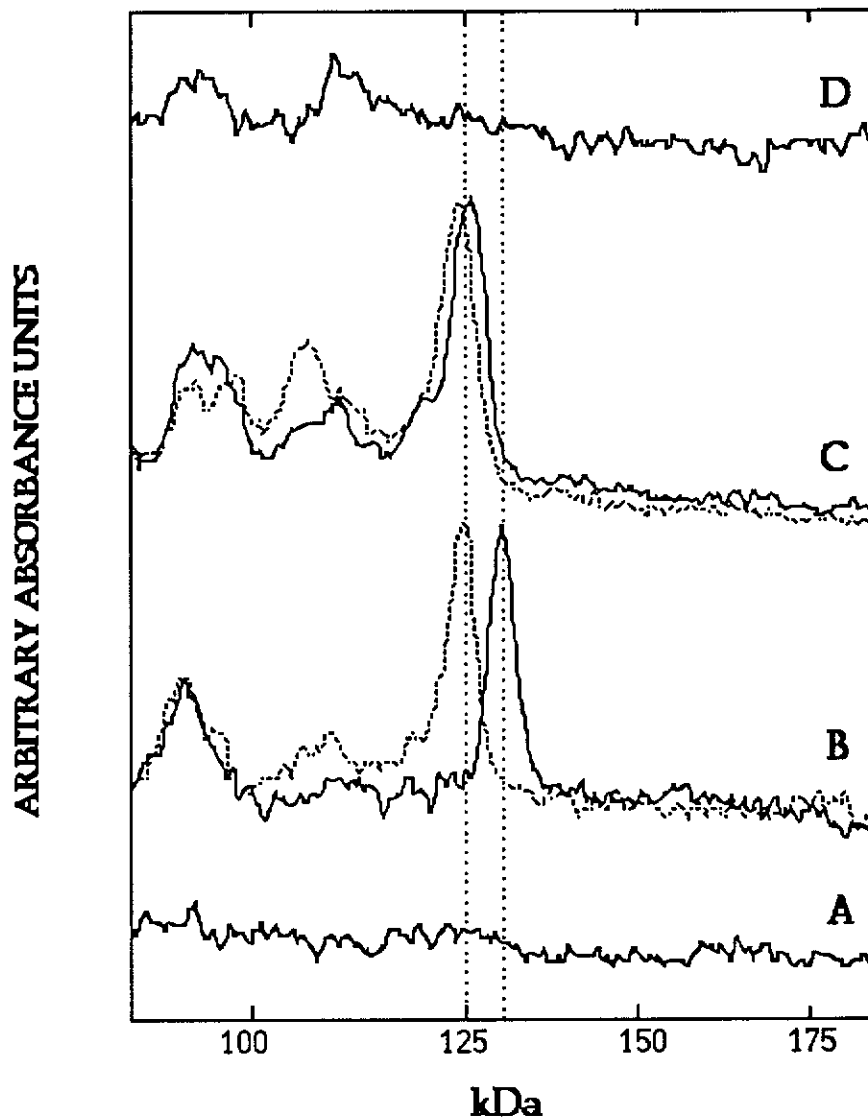

Figure 3.4 The effect of neuraminidase on the mobility of the photoaffinity labelled mammary and liver insulin receptors. Membranes from the liver tissue (A and B) and mammary tissue (C and D) from a 10 day lactating rat were incubated in the dark with 10-50 nM [^{125}I]-N$^{\varepsilon B29}$-MABI with 20 μM bovine insulin (A and D, solid line) or without 20 μM bovine insulin (B and C, solid line) for 16 h at 4°C, followed by electrophoresis in a 7.5 % polyacrylamide gel. The 125 kDa/130 kDa photoaffinity labelled bands were excised from the gel and eluted as described in the Methods section. Liver (B, dashed line) and mammary (C, dashed line) photoaffinity labelled membranes were incubated with neuraminidase type V for 1 h at 37°C. Solubilised proteins were resolved on a 7.5% polyacrylamide gel, autoradiographed and densitometrically scanned. The horizontal dotted lines are included to facilitate the comparison of the mobility of the various protein bands.

the 130 kDa and 125 kDa bands in liver and mammary membranes respectively was observed on days 1, 5, 10, 15 and 20 of lactation.

The specifically labelled 130 kDa protein of photoaffinity labelled liver membranes (figure 3.4B; solid line) migrated on a polyacrylamide gel with substantially increased mobility following treatment with neuraminidase which removes sialic acid residues from the oligosaccharide side-chains of the protein (figure 3.4B; dotted line). In contrast, the mobility of the specifically labelled 125 kDa protein of mammary membranes (figure 3.4C; solid line) migrated only fractionally faster following treatment with neuraminidase (figure 3.4C; dotted line).

Treatment of the mammary membrane preparation with glycopeptidase F, which cleaves the N,N'-diacetylchitobiose-asparagine bond resulting in the cleavage of the oligosaccharide chain from the protein anchor, resulted in the reduction of the 125 kDa protein band to a 100 kDa band (figure 3.5).

Photoaffinity labelled liver membranes were solubilised in 1% (w/v) Triton-X100 and incubated overnight in the absence (figure 3.6B) or presence (figure 3.6C) of unlabelled, solubilised mammary membranes. This experiment was carried out to establish if the 125 kDa radiolabelled mammary protein was derived from a 130 kDa form by proteases absent in liver membranes. The mobility of the 130 kDa protein specifically labelled in the liver membranes on a polyacrylamide gel was unaltered by prolonged exposure to the solubilised mammary membrane proteins. This suggests that the mammary tissue preparations did not contain proteases that were absent from the liver membrane extracts.

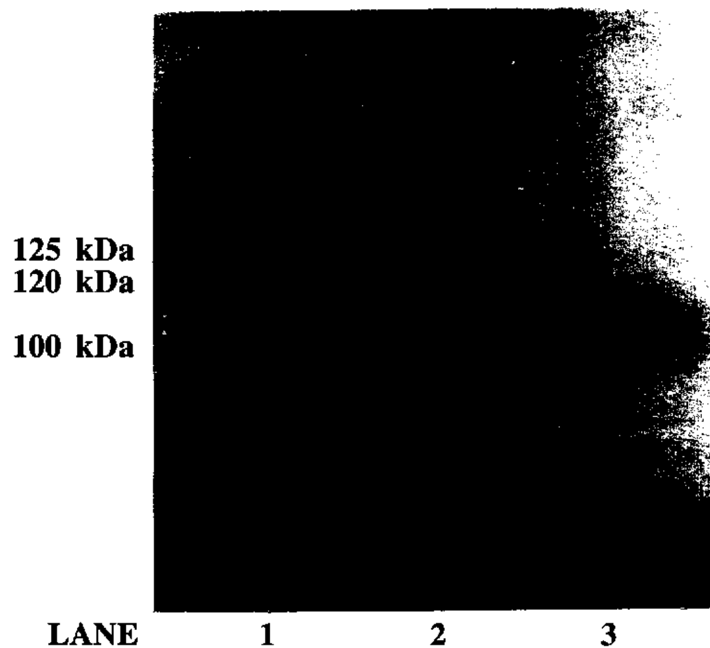

Figure 3.5 Effect of glycopeptidase F on 125 kDa photoaffinity labelled protein band from rat mammary plasma membranes. Mammary membranes from a day 10 lactating rat were incubated with 10-50 nM [^{125}I]-N$^{\varepsilon B29}$-MABI for 16 h at 4°C followed by electrophoresis in a 7.5 % polyacrylamide gel. The 125 kDa photoaffinity labelled band was excised from the gel and eluted as described in the Methods section. The eluted products were either untreated (lane 1), incubated with neuraminidase for 1 h at 37°C (lane 2), or glycopeptidase F for 6 h at 25°C (lane 3). Solubilised proteins were resolved on a 7.5% polyacrylamide gel and autoradiographed.

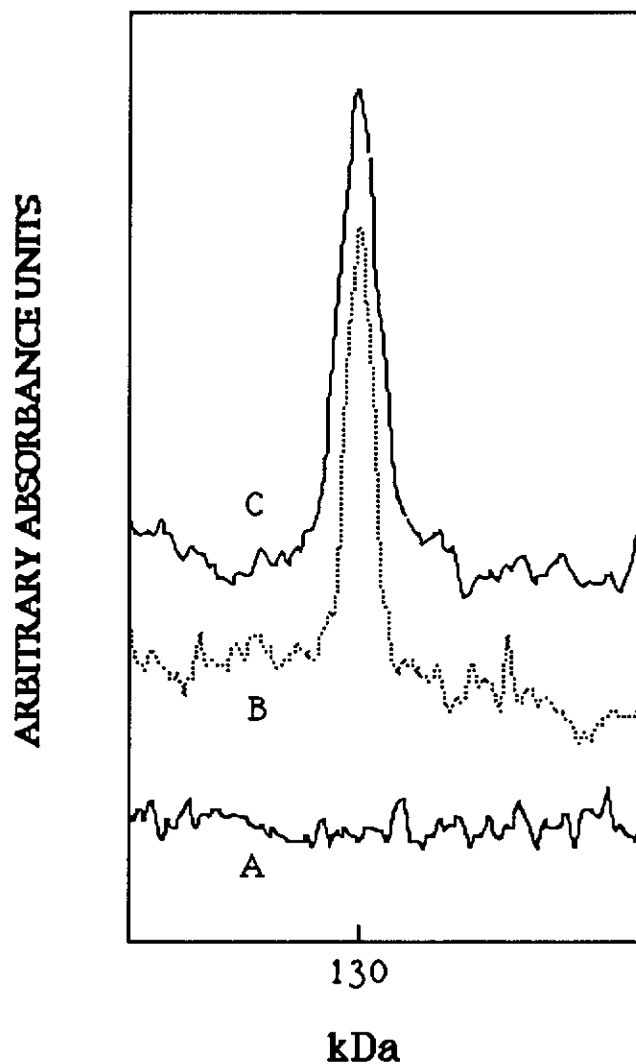

Figure 3.6 Effect of endogenous mammary tissue proteases on liver plasma membranes. Densitometric scan of polyacrylamide gel electrophoresis of photoaffinity labelled liver plasma membranes incubated with unlabelled, solubilised mammary membranes. Liver plasma membranes from a 10 day lactating rat were photoaffinity labelled followed by incubation with (C) or without (B) solubilised mammary membranes for 12 h at 4°C. Non-specific binding was determined by incubation of photoaffinity labelled liver plasma membrane with 20 μM bovine insulin (A). Solubilised proteins were resolved on a 5-10% gradient polyacrylamide gel, autoradiographed and densitometrically scanned.

3.4 DISCUSSION

I have confirmed that the α-subunit of the mammary insulin receptor is smaller compared with the α-subunit of the liver insulin receptor (125 kDa versus 130 kDa) following photoaffinity labelling and PAGE (figure 3.3). The 125 kDa α-subunit of the mammary insulin receptor was not derived as a consequence of proteolysis of a 130 kDa form since the mobility of the α-subunit of the photoaffinity labelled insulin receptor of rat liver was unaltered after prolonged incubation with unlabelled, solubilised mammary tissue extracts (figure 3.6). Furthermore, this was confirmed by the observation that the presence of a wide range of protease inhibitors still resulted in the appearance of the 125 kDa α-subunit of the mammary gland insulin receptor. Finally, the 125 kDa α-subunit of the mammary insulin receptor was evident from day 1 through to day 20 of lactation.

Desialation of the glycan side chains of both the mammary and liver insulin receptors resulted in a reduction in the size of the glycoprotein with both α-subunits having a Mr of approximately 120 kDa. This suggests that the differences in size evident between the tissues may be accounted for by the degree of sialation of the carbohydrate side-chains of the α-subunits. This observation is not entirely surprising as differences in the extent of receptor sialation have been identified in a number of other insulin sensitive tissues including the brain, adipocytes (Yip, 1980; Heidenreich, 1983; Heidenreich, 1986; Burant, 1986a) and circulating monocytes and erythrocytes (McElduff, 1985). In all instances neuraminidase treatment diminished the observed differences between insulin receptors from various tissues sources.

Glycopeptidase F treatment of the mammary α-subunit demonstrated that following deglycosylation, the remaining aglycoreceptor has a Mr of 100 kDa (figure 3.5). This is consistent with the observations by Heidenreich and Brandenberg (1986) and Herzberg *et al.* (1985) that the deglycosylated insulin-binding subunits of the rat adipocyte and brain and IM-9 lymphocytes have apparent molecular weights of

100 kDa. Furthermore, this is close to the expected size of the aglycosylated insulin-binding subunit deduced from both the human and rodent insulin receptor cDNA clone (Ebina, 1985; Ullrich, 1985; Goldstein, 1990).

Glycosylation differences of the same receptor in various tissues has been demonstrated for the cell surface receptors for IGF-I, IGF-II and the insulin receptor (McElduff, 1987). Variations in the size of the insulin receptor from several insulin-sensitive tissues including liver, skeletal muscle, brain and blood cells, have been identified (Yip, 1980; Heidenreich, 1983; Heidenreich, 1986; Burant, 1986a; McElduff, 1985). The nature of these differences in skeletal muscle and brain tissues can be explained by the variation in protein glycosylation, although this only accounts for part of the differences evident in monocytes.

The significance of these subtle tissue-specific differences in the extent of insulin receptor glycosylation still remains to be elucidated. However, the oligosaccharide moieties which form part of the receptor glycoprotein, are involved in the formation of the tertiary and quaternary structural changes necessary for the acquisition of insulin binding activity of the insulin receptor (Olson, 1987). Studies using inhibitors of the intracellular glycosylation apparatus have indicated that glycosylation of the proreceptor prior to insertion into the plasma membrane may be necessary for the conformational changes required for the production of an active receptor or to provide a recognition sites for associated proteins necessary for receptor activation (Rosen, 1979; Ronnett, 1981; Ronnett, 1984; Arakaki, 1987). The use of lectins which interact with specific carbohydrate groups have been shown to significantly reduce insulin binding to insulin receptors from rat adipocytes (Olden, 1982; Cherqui, 1982; Cherqui, 1981). Studies by Cherqui *et al.* (1981; 1982) have implicated the penultimate D-galactose, internal N-acetyl-glucosamine and D-mannose residues in insulin binding. Furthermore, the treatment of adipocytes and liver membranes with specific glycosidases (β-galactosidase and neuraminidase/β-galactosidase) also results in a reduction in insulin binding (Caron, 1978; Cherqui,

1981). Finally, defective protein glycosylation mechanisms in CHO cells have been shown to affect insulin binding (Podskalny, 1984; Podskalny, 1986; Rouiller, 1986). Here, Podskalny *et al.* (1984; 1986) have characterised the binding affinity in two mutant CHO cell lines with well-characterised defects in their glycosylation mechanisms; B4-2-1 cells, which lack mannose containing glycoproteins, and Lec1.3c cells, which lack glycoproteins containing complex carbohydrates. In these cell lines the insulin binding affinities were significantly higher and lower, respectively (Podskalny, 1984; Podskalny, 1986). Together these studies provide strong evidence that carbohydrate moieties of the insulin receptor can affect the insulin-receptor binding interaction. With work presented in this chapter, these studies give credence to a possible scenario where the tissue-specific differences in insulin receptor glycosylation between the mammary and liver tissues, provide a mechanism by which the insulin-affinity of these tissues could be finely modulated.

CHAPTER FOUR

ALTERNATIVE SPLICING OF THE INSULIN RECEPTOR MESSENGER RNA

4.1 INTRODUCTION

Insulin receptor-A and IR-B isoforms are expressed in a tissue-specific manner and the distribution of these isoforms has been characterised in several human and rat tissues from both healthy and NIDDM individuals (Moller, 1989; Seino, 1989b; Goldstein, 1990; Mosthaf, 1991; Benecke, 1992; Kellerer, 1992a; Kellerer, 1992b; Mosthaf, 1992; Anderson, 1993; Mosthaf, 1993; Kellerer, 1993). Several studies suggest that altered expression of the insulin receptor isoforms in the skeletal muscle of individuals with NIDDM could possibly be used as a marker of the disease (Mosthaf, 1991; Mosthaf, 1993; Mosthaf, 1992; Norgren, 1993; Kellerer, 1992a; Kellerer, 1993). Although independent studies by Benecke *et al.* (1992) and Anderson *et al.* (1993) have not identified any differences between the healthy and NIDDM individuals. Studies using polyclonal and monoclonal insulin receptor isoform-specific antibodies have shown that tissue expression of IR-A and IR-B approximately parallels observations at the mRNA level (Kellerer, 1992a; Benecke, 1992). Subtle differences in the functional properties of these receptor have been demonstrated (reviewed in Chapter 1).

In this study we have established a RT-PCR for the quantitation of insulin receptor isoform mRNA distribution. This method was subsequently used to characterise the distribution of the IR-A and IR-B insulin receptor isoforms in mammary and liver tissues throughout pregnancy and lactation to identify if changes in the insulin sensitivity which occur during this time reflect changes in insulin receptor isoform distribution. A corollary of this study was the assessment of IR-A

and IR-B distribution in various tissues during foetal/neonatal ontogeny to identify if changes in isoform distribution were developmentally regulated. Finally, this study presents preliminary data as to the effect of streptozotocin-induced insulin deficiency on the expression of the insulin receptor isoforms in rats. The development of the RT-PCR protocol and characterisation of the isoform distribution in foetal and neonatal rats were performed in collaboration with Ms. Alison Smith, an Honours student within our laboratory in 1992.

4.2 EXPERIMENTAL PROCEDURES

4.2.1 Animals

Lactating Wistar albino rats were obtained and housed as described in section 3.2.1. Pregnant Wistar albino rats were purchased from the Animal Laboratory No. 3 (University of Western Australia, Nedlands, WA). The onset of pregnancy was determined by the appearance of a vaginal plug with the normal gestation period for these animals being 22 days. Three female Wistar rats weighing 200-250 g were injected by tail vein with 50 mg/kg body weight of streptozotocin (Sigma Chemical Company, MO, USA) dissolved in 10 mM sodium citrate buffer, pH 4.0. This dose of streptozotocin was selected as it had been shown to reproducibly result in insulin deficiency in the absence of weight loss, ketosis or extreme hypertriglyceridaemia (Redgrave, 1990). The control group consisted of two female adult rats of a comparable size to the test group which were injected with citrate buffer only. Control and insulin deficient rats were fed *ad libitum* on standard rat chowder and maintained for three weeks prior to sacrifice.

4.2.2 RNA extraction

Pregnant, neonatal and adult Wistar rats were sacrificed by cervical dislocation and foetuses delivered by caesarean section. Identity of foetal tissues was verified by histological examination. Mammary, liver, brain, skeletal muscle (biceps femoris), heart and kidney were obtained from lactating animals. Liver, brain, skeletal muscle, heart, kidney, and lung were collected from foetuses, neonates and adults animals. Mammary and liver only were collected from the pregnant animals. Tissues were excised into ice-cold 25 mM sodium citrate, pH 7.0 containing 4 M guanidinium thiocyanate, 0.5 % (w/v) N-lauryl sarcosine and 0.1 mM DTT.

RNA was isolated according to the method of Chomczynski and Sacchi (1987). The RNA pellets were either stored in 70 % (v/v) ethanol and kept at -20°C or alternatively, the pellets were dried under vacuum, resuspended in diethyl pyrocarbonate (DEPC)-treated water and stored at -70°C. The quality of the isolated RNA was determined on a 1.2 % denaturing formaldehyde agarose gel.

Estimation of RNA concentration was evaluated by comparing the fluorescent emission intensity of extracted RNA stained with 0.04 mg/mL ethidium bromide with ethidium bromide stained salmon sperm DNA standards using a Perkin-Elmer LS-5 Luminescence Spectrophotometer at an excitation wavelength of 366 nm and emission wavelength of 590 nm.

4.2.3 Oligonucleotide primers

Oligonucleotide primers 5' AAG AAG CTT AGG CCA GAG ATG ACA AGT 3' (antisense) and 5' GAA GAA TTC ATT CAG GAA GAC CTT CGA 3' (sense) (Goldstein, 1990) were synthesised using an Oligonucleotide Synthesiser Model #381A (Applied Biosystems, Australia). These primers flank the region containing exon 11 corresponding to nucleotides 2181 to 2438 of the rat insulin receptor. Estimation of the yield of oligonucleotide primer was determined by

measuring the absorbance of the solution at 260 nm. Oligonucleotide primers were stored as lyophilised products at -70°C. The oligonucleotides were resuspended in sterile water to a concentration of 100 µg/mL for use.

Oligonucleotide primer integrity was visually assessed by observing the electrophoresis pattern of the [γ–^{32}P]-dATP (110 TBq/mmol, Amersham, North Ryde, Australia) end-labelled oligonucleotide. End-labelling of the oligonucleotide primer was performed according to the protocol outlined in the Promega fmol DNA Sequencing System Technical Manual (Promega, Madison, USA). End-labelled primers were resolved by electrophoresis in a 19% denaturing polyacrylamide gel.

4.2.4 Complimentary DNA synthesis

For synthesis of first strand cDNA, the RNA sample (5 µg) was heated for 5 minutes at 70°C and added to a final 20 µL reaction mix containing 4 µM oligo dT primer (Promega, Madison, USA) in 10 mM Tris-HCl, pH 7.5 containing 50 mM KCl, 4.5 mM $MgCl_2$, 0.01% (w/v) EIA pure gelatin, 1 mM DTT, 5 U AMV reverse transcriptase (Promega, Madison, USA), 8 U RNAsin (Promega, Madison, USA) and 0.5 mM of each dNTP. This mixture was incubated for 2 h at 42°C.

4.2.5 Quantitation of the cDNA yield

Estimation of the efficiency of cDNA synthesis was determined by the incorporation of [α-^{32}P]dCTP into the cDNA synthesis reaction followed by TCA precipitation of the cDNA. Radiophosphorylated dCTP (260 kBq) was included in the cDNA reaction. A 3 µL aliquot of the cDNA reaction mixture, one prior to the addition of the AMV reverse transcriptase enzyme and the second following the completion of the cDNA reaction, was added to each of two glass tubes containing 300 µL of a 4 mg/mL solution of salmon sperm DNA. A 5 mL aliquot of ice-cold 5% (w/v) TCA was added to each tube and the precipitates concentrated on filter paper prewashed with the TCA solution. Each tube was rinsed twice with the TCA solution to ensure the recovery of all of the precipitate. Following a final wash of the

filter paper with 95% (v/v) ethanol the filter paper was dried under a vacuum and the radioactivity associated with each sample measured using a Hewlett Packard Cobra™ II auto-gamma® counter for 10 minutes each with energy window settings of 15-1000 keV. The efficiency of cDNA synthesis was determine by the comparison of the "enzyme plus" and the "enzyme minus" tubes.

4.2.6 PCR amplification of insulin receptor cDNA

The parameters for the PCR amplification of the rat insulin receptor cDNA were originally based on those described by Goldstein and Dudley (1990). Within our laboratory we found it necessary to reassess several parameters to ensure optimal efficiency and yield of PCR products. The final conditions used in this study were as follows: a 12 μL aliquot of 10 mM Tris-HCl, pH 8.3 containing 50 mM KCl, 6 mM $MgCl_2$, 0.01% (w/v) EIA pure gelatin, 167 μM dATP, dTTP, dGTP and 0.083 μM dCTP, 260 KBq [α-^{32}P]dCTP (Amersham, North Ryde, NSW), 5 μM antisense primer, 5 μM sense primer and 1 U AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, USA) were mixed and this solution was overlaid with approximately 50 μL paraffin oil. The cDNA mix was heated to 95°C for 5 minutes and a 3 μL aliquot of this cDNA was then added to the reaction through the paraffin oil. The DNA was amplified for 30 cycles of 95°C for 30 seconds, 57°C for 30 seconds and 72°C for 90 seconds (Corbett FTS Thermocycler). The oligonucleotide primers chosen resulted in the amplification of DNA flanking exon 11 of the rat insulin receptor gene which was either 273 bp (including exon 11) or 237 bp (excluding exon 11) in size. The PCR products were resolved by electrophoresis in a 5% polyacrylamide gel before visualising the radioactivity by autoradiography. pGEM DNA markers (Promega, Madison, USA) were used for the estimation of the size of the PCR products. The gel slices were counted in a Hewlett Packard Cobra™ II auto-gamma® counter for 10 minutes each with energy window settings of 15-1000 keV enabling the relative distribution of the 273 bp and 237 bp PCR products to be determined. The measurement of β-emitters under these conditions resulted in

an approximate 17% efficiency of counting. This was deemed suitable as the % incorporation of ^{32}P into the PCR product was sufficient to be accurately measured. The use of a gamma counter for the measurement of the relative distribution of the PCR products was convenient and preferable as the liquid scintillation counter which was available was both unreliable and inefficient. Furthermore, the use of toxic scintillant fluids was avoided. Statistical analysis of the data employed the unpaired, two-tailed Students t-test which evaluates the statistical significance of the difference between the mean value of two sets of data.

4.2.7 PCR positive control

Oligonucleotide primers to the rat β-actin gene (Clontech, Palo Alto, USA) were used as a positive control for the PCR reaction. Positive control PCR reactions included 10 mM Tris-HCl, pH 8.3 containing 50 mM KCl, 2 mM $MgCl_2$, 0.01 % (w/v) EIA pure gelatin, 500 μM dATP, dTTP, dGTP, 250 μM dCTP, 260 KBq [α-^{32}P]dCTP, 1.25 μM antisense primer, 1.25 μM sense primer and 1 U AmpliTaq polymerase to which 2 μL of control DNA or cDNA sample was added to a final reaction volume of 20 μL. Paraffin oil (0.1 mL) was overlaid on the PCR reaction mixture and cDNA amplification carried out over 40 cycles of 95°C for 60 seconds, 60°C for 120 seconds and 72°C for 180 seconds. The PCR products were resolved on a 5 % polyacrylamide gel and visualised by autoradiography.

4.2.8 Structural analysis of the exon 11 encoded peptide of the rat insulin receptor

Secondary structure predictions and surface/antigenic profiles of the exon 11 encoded peptide were performed using the MacVector 4.0 Sequence Analysis Software. Secondary structure predictions utilise both the Chou-Fasman and the Robson-Garnier methods with each method having a 60% probability of being correct (Chou, 1978; Garnier, 1978). The peptide hydrophilicity profile is based on the Kyte-Doolittle scale of amino acid hydropathy. These values are estimated using

the water-vapour transfer free energies for amino acid side chains and the preference for the amino acids side chains from an internal or external environment (Kyte, 1982). The antigenic index locates possible exposed surface peaks of a protein by combining information from the surface probability, hydrophilicity, flexibility and secondary structure predictions (Jameson, 1988).

4.3 RESULTS

4.3.1 Evaluation of RNA integrity

A typical autoradiograph obtained from the electrophoresis of RNA samples prepared according to the method of Chomczynski and Sacchi (1987) is shown in figure 4.1. Resolution of total RNA samples in a 1.2% denaturing formaldehyde-agarose gel results in the observation of three bands corresponding to the 5S, 18S and 28S subunits of ribosomal RNA and are indicative of intact RNA. The fourth upper band evident in the autoradiograph probably represents genomic DNA.

4.3.2 Estimation of RNA concentrations

In early experiments, the concentration of RNA was estimated by measuring the absorbance of the RNA in solution at 260 nm. In certain instances, estimation of the RNA concentrations were confounded by protein contamination of the preparations in addition to low yields of RNA from foetal sources. Specificity and sensitivity was improved by the measurement of the fluorescent intensity emitted when the RNA is associated with ethidium bromide. Using salmon sperm DNA standards, a standard curve with an analytical range from 5 to 150 μg was obtained (figure 4.2) enabling adequate quantitation of unknown RNA concentrations. Data obtained using this technique in conjunction with the estimation of cDNA synthesis efficiency indicated that approximately 5 μg of RNA resulted in optimal cDNA synthesis (data not shown).

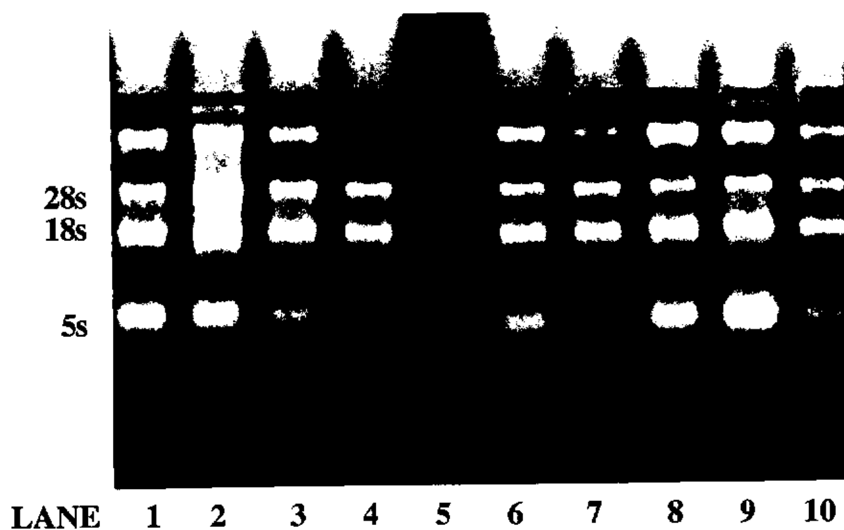

Figure 4.1 RNA isolated from mammary and liver tissues from day 7 lactating Wistar rats. RNA was isolated according to the protocol outlined in Materials and Methods and resolved by denaturing formaldehyde gel electrophoresis followed by visualisation using ethidium bromide. Mammary RNA is shown in lanes 1-4 and liver tissues in lanes 6-10.

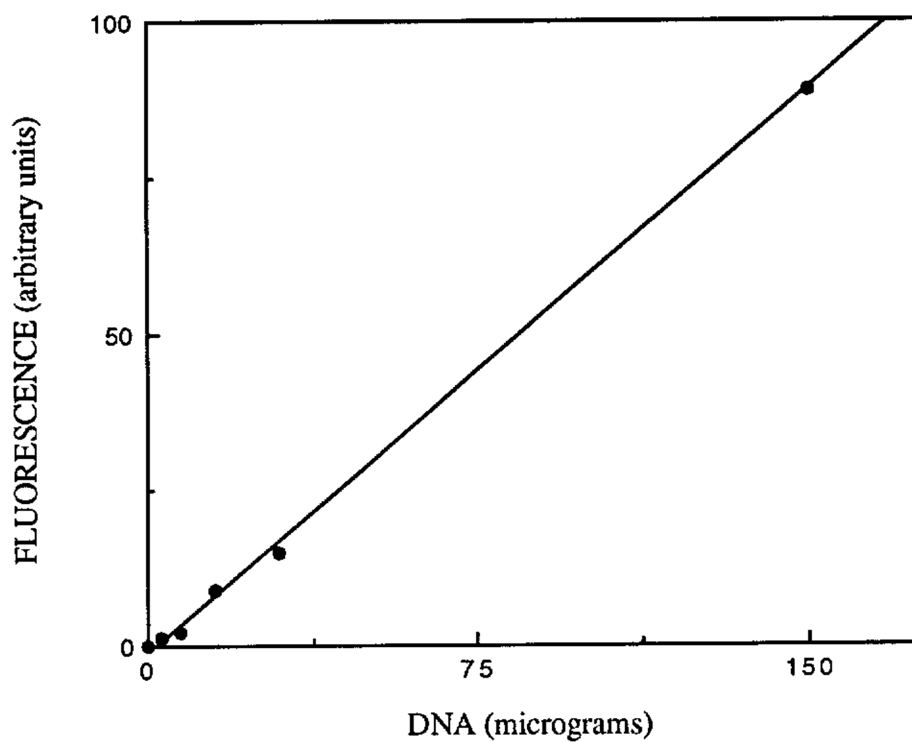

Figure 4.2 A typical standard curve from the fluorimetric measurement of RNA concentration. A typical standard curve of salmon sperm DNA measured as a function of fluorescent intensity at an excitation wavelength of 366 nm and emission wavelength of 590 nm.

4.3.3 Assessment of primer integrity

Oligonucleotide primers were synthesised and [γ–^{32}P]dATP end-labelled to assess primer integrity. The oligonucleotide primers were resolved by electrophoresis and visualised by autoradiography (figure 4.3). Primers that were deemed suitable for use in the cDNA and PCR reactions include the sense primers in lanes 1, 3 and 7 and the antisense primers in lanes 2 and 6 which migrated as a single band. Unsuitable primers were those which appeared as a smear within the lane or for which no band was present at the concentration assessed (such as lanes 4 and 5 in figure 4.3).

4.3.4 Optimisation of the PCR protocol for the amplification of the rat insulin receptor cDNA

Several modifications of the PCR methodology described by Goldstein and Dudley (1990) were necessary to reproducibly obtain suitable PCR products. The PCR protocol was optimised for both mammary and liver RNA isolated from day 14 lactating rats which exhibit predominantly the mRNA encoding the IR-A (77%) and IR-B (96 %) insulin receptor isoforms, respectively.

4.3.4.1 PCR cycle number

Evaluation of the effect of cycle number on the amplification of the PCR product demonstrated that optimal amplification was achieved by 30 cycles (lanes 2 and 6, figure 4.4). Further amplification of the DNA did not significantly increase the product yield but did result in the appearance of non-specific product bands on the autoradiograph.

4.3.4.2 PCR primer concentration

The effect of sense and antisense oligonucleotide primer concentration was assessed over the concentration range of 0.6 to 5 µM (data not shown). Low

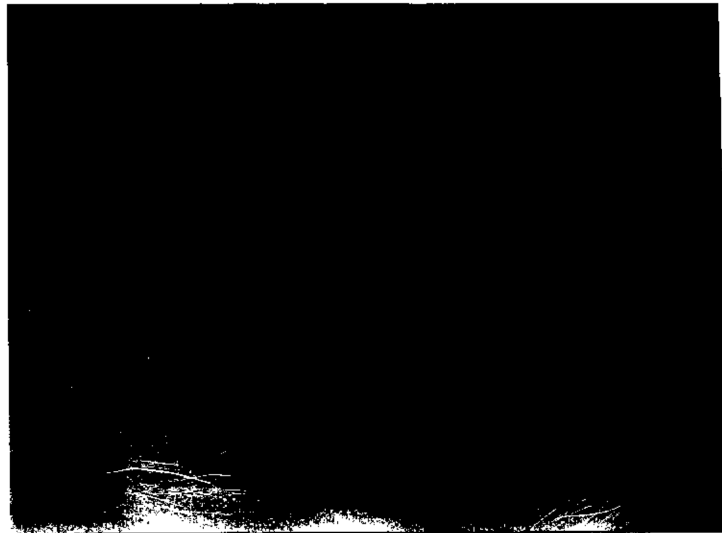

Figure 4.3 Assessment of primer quality. Primers were end-labelled and the products resolved on a 19 % denaturing acrylamide gel. Resolved products were visualised by autoradiography of the PAGE gel. Lanes 1, 3, 5 and 7 represent the 5' sense primer (27 bp) and lanes 2, 4 and 6 represent the 3' antisense primer (27 bp).

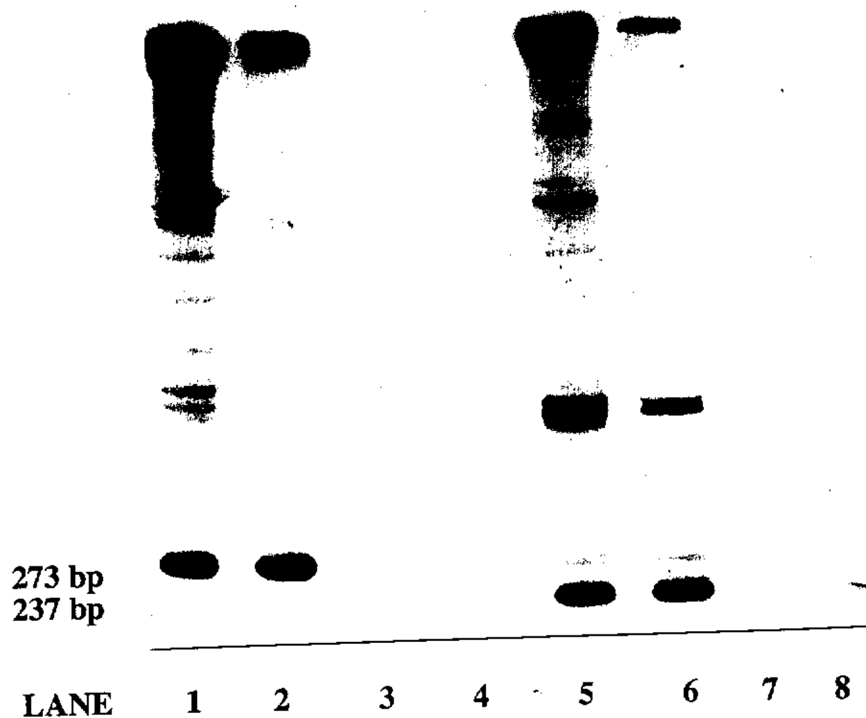

Figure 4.4 The effect of PCR cycle number on the amplification of IR-A and IR-B cDNA. This was determined by assessing the yield and specificity of PCR products obtained from PCR terminated at 40 cycles (lane 1, mammary cDNA; lane 5, liver cDNA), 30 cycles (lane 2, mammary cDNA; lane 6, liver cDNA), 20 cycles (lane 3, mammary cDNA; lane 7, liver cDNA), 10 cycles (lane 4, mammary cDNA; lane 8, liver cDNA). PCR products were resolved using a 5 % polyacrylamide gel and visualised by autoradiography.

concentrations of primers resulted in a general reduction in [α-32P]-dCTP incorporation into the PCR product. In contrast, excessively high levels of primers have been shown to promote the formation of primer dimers and non-specific PCR products. A 5 μM concentration of sense and antisense primers was deemed optimal for this PCR protocol.

4.3.4.3 Characterisation of annealing temperature

Amplification of the rat insulin receptor gene was performed at annealing temperatures over the range of 45°C to 62°C (figure 4.5). The specificity of product amplification was determined by calculating the radioactivity in the IR-A mRNA and IR-B mRNA bands as a proportion of the total radioactivity in each gel lane. At an annealing temperature of 45°C the specificity of amplification was poor, as indicated by the presence of several PCR products of various sizes. An increase in the annealing temperature to 55°C and 57°C resulted in an increase in product specificity increased whilst maintaining a high product yield Further increases in annealing temperature to 60°C and above resulted in high specificity of the PCR product obtained but with a concomitant decrease in yield. From these experiments, 57°C was chosen as the appropriate annealing temperature to provide both optimal PCR product specificity and yield.

4.3.4.4 Incorporation of [α-^{32}P]dCTP into the PCR product

The concentration of dCTP in the PCR was varied, relative to the other three deoxynucleotide, to achieve optimal incorporation of [α-^{32}P]dCTP into the final product. This was achieved using a final dCTP concentration of 83 nM (data not shown).

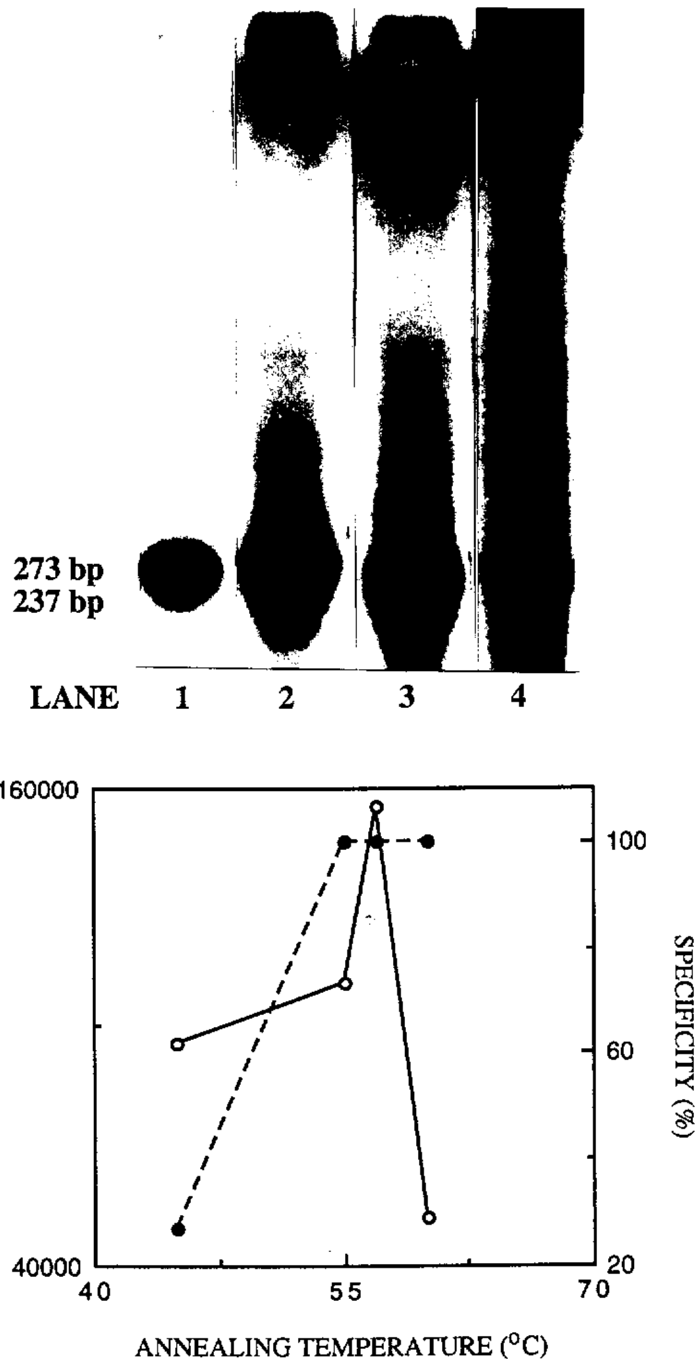

Figure 4.5 The effect of annealing temperature on the amplification of IR-A and IR-B cDNA. The PCR was performed on rat liver cDNA at annealing temperatures of 45°C (lane 1), 55°C (lane 2), 57°C (lane 3) and 60°C (lane 4). PCR products were resolved on a 5 % polyacrylamide gel and visualised by autoradiography. The amount of radioactivity associated with the IR-B PCR product (o) and the specificity of dCTP tracer incorporation into the insulin receptor PCR products (•) was determined as outlined in the Methods section.

4.3.4.5 Evaluation of the relative rates of amplification of the PCR products which encode the IR-A and the IR-B insulin receptor isoforms

When comparing the distribution of the insulin receptor isoform mRNA levels using the PCR technique it is necessary to ensure that the cDNA encoding both the IR-A and IR-B isoforms amplify at the same rate. An estimate of the rate of PCR product amplification was made by combining various ratios of liver and mammary cDNA from day 14 lactating animals which predominantly express IR-B mRNA (96%) and IR-A mRNA (77%) respectively. Excellent correlation (r = 0.947) was seen between the observed and expected proportion of IR-B seen in these tissues (figure 4.6).

4.3.5 Distribution of IR-A and IR-B in mammary and liver tissues from adult, pregnant and lactating animals

The distribution of the IR-A and IR-B insulin receptor isoforms was characterised in mammary and liver tissues as a function of pregnancy and lactation. Mammary and liver RNA was extracted from tissues from three animals on day 14 and day 20 of gestation, on the day of parturition, and days 1, 7, 14 and 20 of lactation. Each experiment was performed in duplicate. The mean intra-animal coefficients of variation were less than 4% and the mean inter-animal coefficients of variation were less than 7%.

Complimentary DNA derived from these RNA samples were amplified using the PCR technique. An autoradiograph from a typical experiment showing the PCR products from mammary and liver tissues from rats at various stages of pregnancy and lactation are shown in figure 4.7. Estimates of the relative proportions of the mRNA encoding the IR-A and IR-B insulin receptor isoforms in these tissues (figure 4.8a and 4.8b) indicate that whilst their is no significant variation in the distribution of mRNA encoding IR-A and IR-B in liver tissues of the pregnant and lactating animals ($P>0.1$ comparing data from the day of parturition with day 7 of lactation),

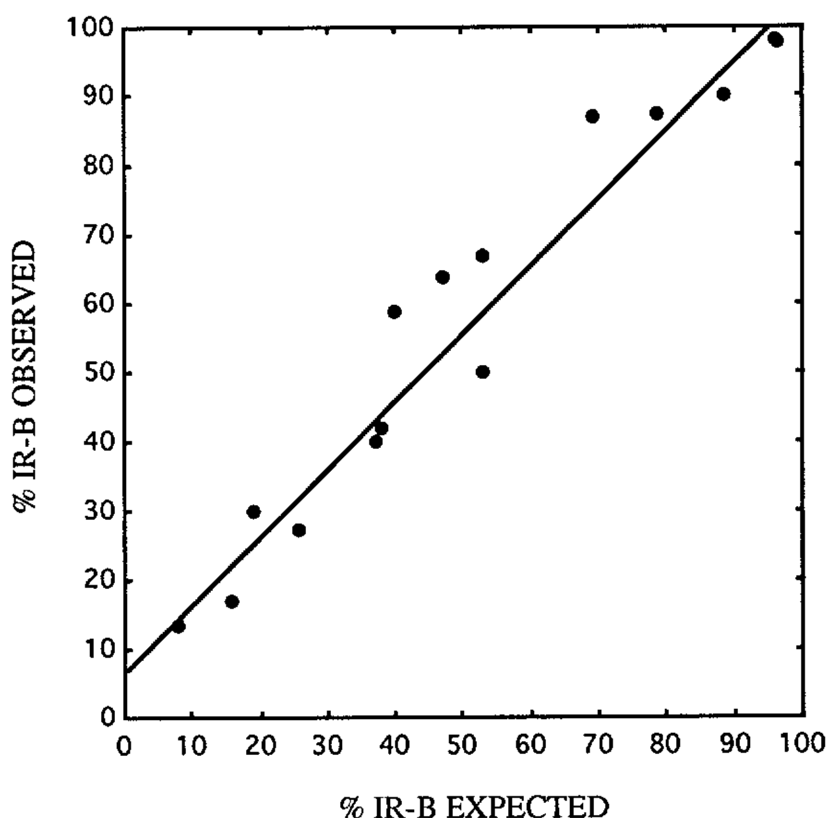

Figure 4.6 Assessment of the relative rates of IR-A and IR-B cDNA amplification. The rates of insulin receptor isoform cDNA amplification were assessed by comparing the relative proportion of exon 11-minus and exon 11-plus products expected (x axis) with the amounts measured (y axis) from mixing varying amounts of rat mammary and liver cDNA. Graph shows data from the experiment performed on two different occasions. Calculation of the regression line equation for the data was performed using the Statview statistical software on a Macintosh system. The calculated correlation coefficient for the data was 0.947.

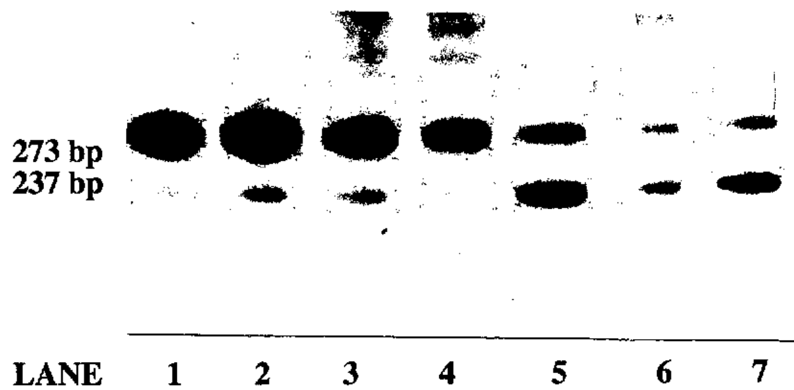

Figure 4.7 Distribution of IR-A and IR-B PCR products in mammary and liver tissues during pregnancy and lactation. Assessment of the relative distribution of the IR-A and IR-B PCR products generated from rat liver cDNA from the day of parturition (lane 1), day 1 of lactation (lane 2), day 7 of lactation (lane 3) and day 14 of lactation (lane 4) and in rat mammary tissue cDNA from day 1 of lactation (lane 5), day 7 of lactation (lane 6) and day 14 of lactation (lane 7).

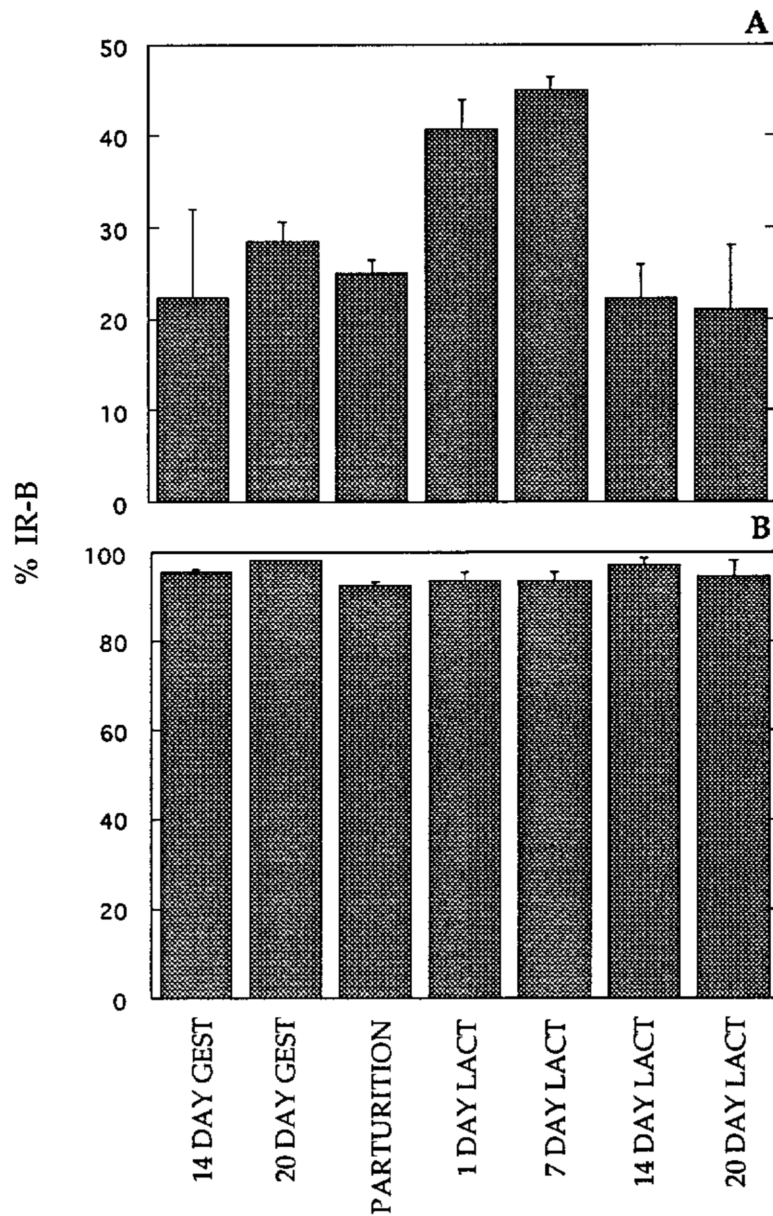

Figure 4.8 The relative expression of IR-B mRNA in mammary and liver tissue during pregnancy and lactation. The expression of the IR-B mRNA in rat mammary (A) and liver (B) tissues from day 14 and day 20 of gestation, the day of parturition and days 1, 7, 14, and 21 of lactation were compared. Error bars indicate one SD from the mean % IR-B mRNA.

changes are evident in mammary tissue. In mammary tissue the relative amount of IR-A changes from approximately 75% in the pregnant and peripartum period to 57% on day 1 of lactation (P=0.035 comparing data from the day of parturition with day 1 of lactation) and remained decreased up to day 7 of lactation (55% IR-A encoding mRNA). The level of mRNA encoding the IR-A isoform is restored to peripartum levels by day 14 of lactation (P>0.7 comparing data from the day of parturition with day 14 of lactation).

4.3.6 Distribution of IR-A and IR-B during development in rat tissues

The distribution of IR-A and IR-B mRNA was measured in several tissues from 14 day and 21 day foetal, 1 day and 7 day neonatal and 12 week old adult rats. In skeletal muscle (figure 4.9a) tissue specimens at various stages of development were limited and thus results were inconclusive. In brain (P>0.1, comparing the day 14 foetus and the adult animal) and lung (P>0.1, comparing day 21 foetus and the day 7 neonate), IR-A mRNA predominated and the distribution of IR-A and IR–B mRNA appeared to be independent of the stage of development (figures 4.9a, 4.9b, 4.9d). The proportion of IR-A mRNA generally decreased with age in the foetal and neonatal cardiac muscle (figure 4.9c), kidney (figure 4.9e) and liver (figure 4.9f). In cardiac muscle the decline was gradual to 7 days post-partum (P=0.01, comparing 21 day foetus and 7 day neonate) however a significant increase in IR-A mRNA expression was evident in the adult animal (P=0.04, comparing the day 7 neonate with the adult animal). A decline in the expression of the IR-A isoform was observed in kidney tissue between 7 days post-partum and the adult animal (P=0.001). A pronounced variation in IR-A and IR-B expression in the developing rat is seen in the liver. Peripartum the level of expression of IR-A changed from 53% in a 21 day old foetus to approximately 13% in the 1 day old neonate (P=0.02). By 7 days post-partum the level of IR-A mRNA expressed was indistinguishable from the adult animal (P=0.3).

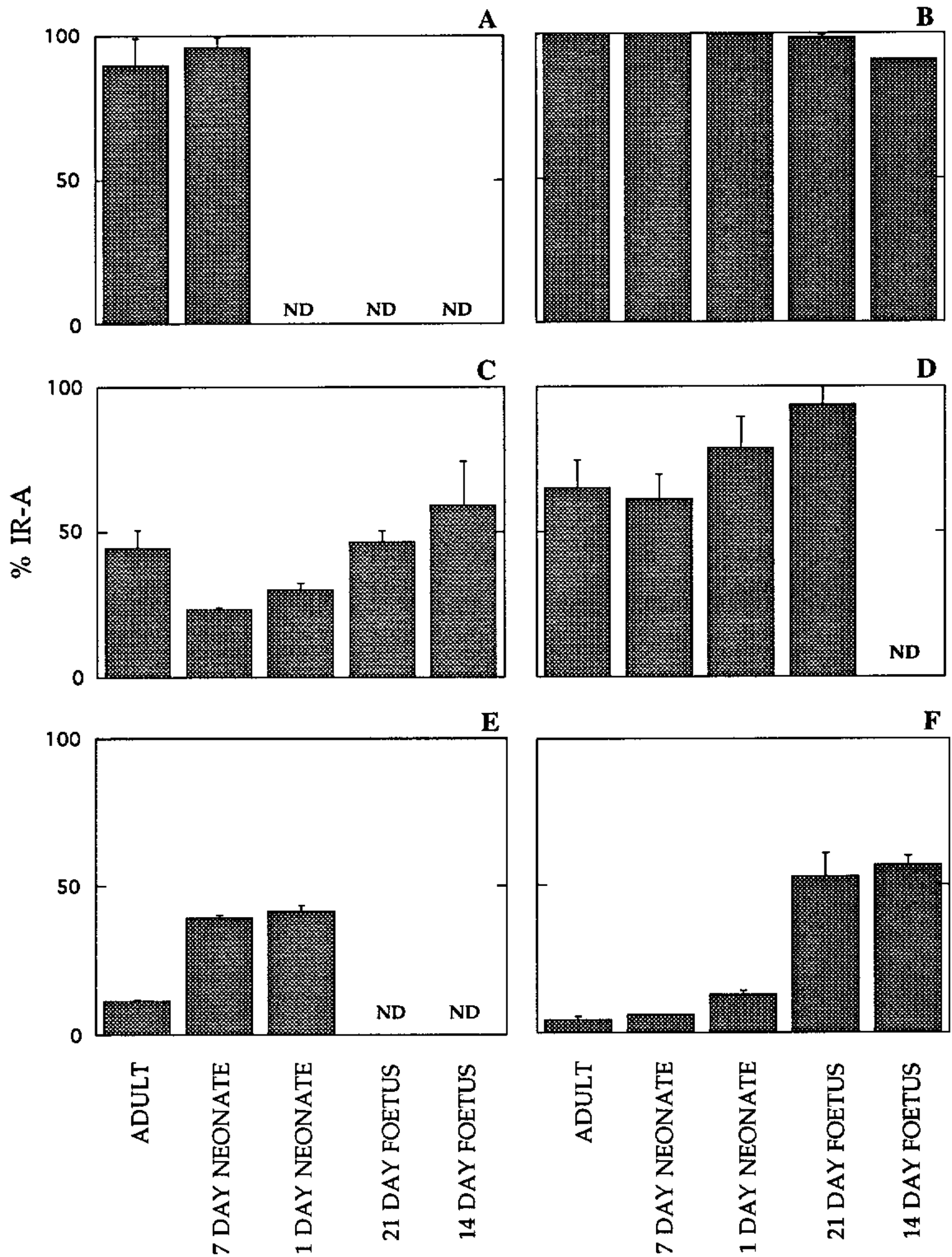

Figure 4.9 The relative expression of IR-A mRNA in foetal and neonatal rat tissues. Biceps femoris (A), brain (B), cardiac muscle (C), lung (D), kidney (E), liver tissues (F) were obtained from day 14, 21 foetuses and day 1, 7 neonatal and adult rats (**ND,** not done). Error bars indicate one SD from the mean % IR-A. See text for details on t-test probabilities.

4.3.7 Effect of streptozotocin-induced diabetes on the IR-A and IR-B isoform distribution in adult female rats

Preliminary studies were performed to assess the effect of hypoinsulinaemia on the expression of IR-A and IR-B mRNA. The relative abundance of the mRNA encoding the IR-A and IR-B insulin receptor isoforms was assessed in the liver, skeletal muscle, heart, kidney, brain, spleen, lung and adipose tissue from adult female rats with streptozotocin-induced diabetes and control animals (figure 4.10). Although the number of animals in both the test and control groups does not allow the statistical significance to be calculated, this preliminary data suggests that there is no difference in IR-A and IR-B distribution between the animals with streptozotocin-induced diabetes and the control population. The relative proportions of the insulin receptor isoforms mRNA were consistent with those previously reported in normal adult rat liver, kidney, brain and spleen (results from other tissue have not been reported in the literature to date).

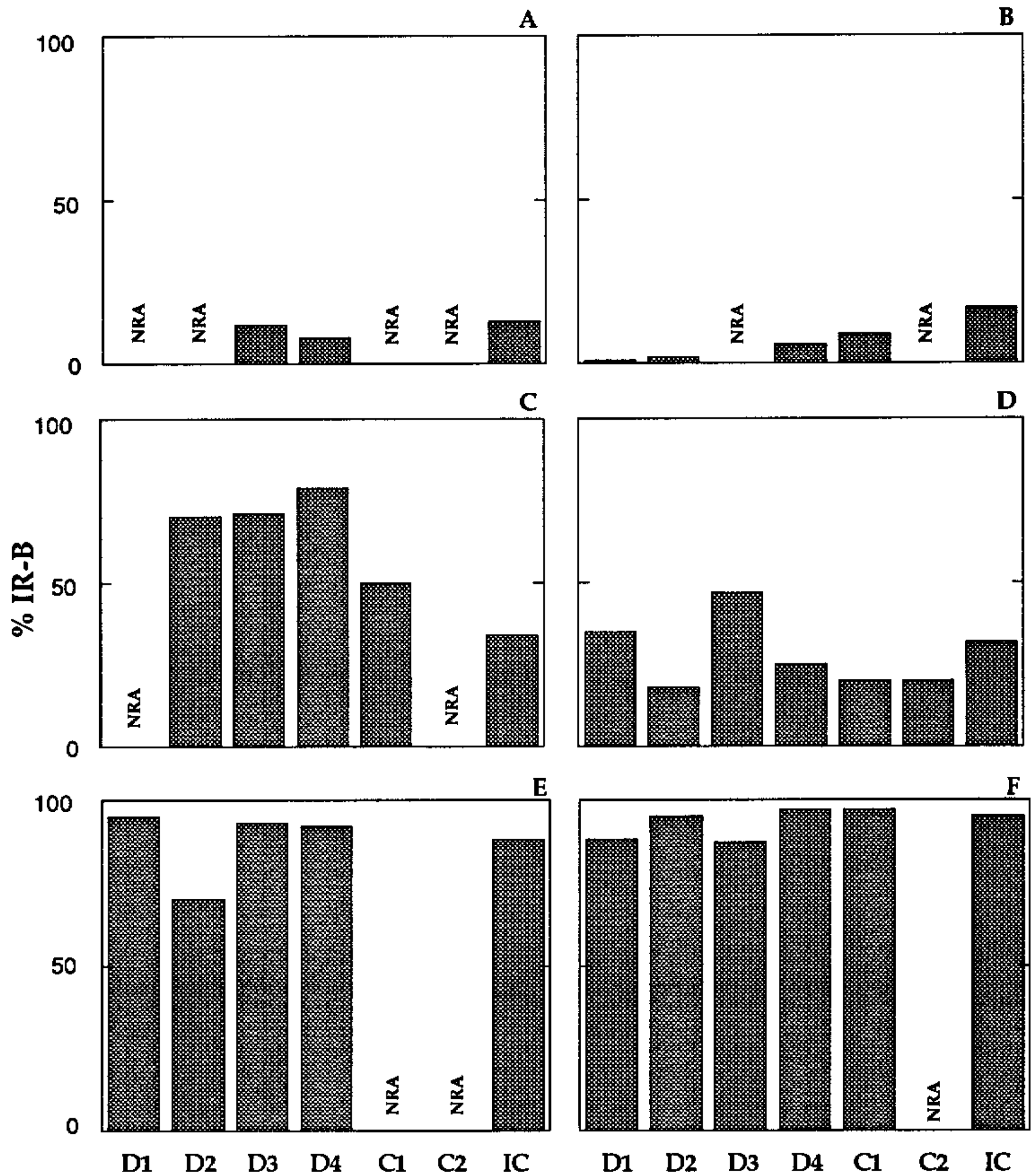

Figure 4.10 The relative expression of IR-B mRNA in tissues from streptozotocin-treated rats. The relative expression of the exon 11-plus insulin receptor mRNA isoform in biceps femoris skeletal muscle (A), brain (B), cardiac muscle (C), lung (D), kidney (E) and liver (F) from adult female Wistar rats displaying characteristics of streptozotocin-induced diabetes. Each column represents the IR-B mRNA expression in an individual animal. Prefixes indicate, **D** = diabetic (treated with streptozotocin in citrate buffer), **C** = control (animals were injected with citrate buffer only), and **IC** = internal control (untreated). **NRA** = no result available.

4.4 DISCUSSION

4.4.1 Development of reverse transcriptase-polymerase chain reaction

The RT-PCR method used in this study was adapted from that of Goldstein and Dudley (1990). Modification of the protocol was necessary to ensure optimal conditions for reproducible product amplification were met. The RNA extraction protocol from Chomczynski and Sacchi (1987) enabled the isolation of intact RNA relatively free from genomic DNA in high yields (figure 4.1). The synthesis of cDNA from the RNA template used an oligo-dT primer which specifically binds the poly-A tail of mRNA molecules. The use of this primer provided a consistently high yield of cDNA for use in the PCR reaction. Alternatively, gene-specific primers which generate cDNA from the region of interest could have been used. The principle disadvantage of this method is that cDNA synthesis conditions such as annealing temperature and primer concentration would need to be optimised for each specific primer (Sambrook, 1989).

When the PCR technique is employed to determine the *in vivo* distribution of specific DNA or RNA sequences it is crucial to ensure that the amplification of the PCR products are not limited by decreased enzyme activity, the accumulation of PCR products nor the exhaustion of reaction components (Saiki, 1988). Therefore the number of amplification cycles must be sufficient to yield enough PCR product for subsequent use but must be few enough to ensure that the relative amounts of PCR products formed are solely dependent on the initial amount of cDNA in the system. For this study a PCR cycle number of 30 was shown to yield adequate PCR products whilst amplification remained independent of factors other than the amount template available (figure 4.4). Further increases in the cycle number to 40 increased the non-specificity of cDNA amplification.

Reduced primer levels have been shown to increase the PCR specificity by limiting the amplification of non-specific sequences (Sambrook, 1989). Assessment

of the effect of primer concentration revealed that decreasing the concentration of PCR primers resulted in a reduction in the yield of product with no apparent change in the insulin receptor PCR specificity.

Evaluation of the annealing temperature for the insulin receptor PCR identified an optimal primer annealing temperature of 57°C. The choice of annealing temperature represents a compromise between the specificity of the PCR products generated and the yield of the product of interest. At this temperature the generation of non-specific fragments is minimised.

The PCR reaction is based on the exponential amplification of a specific area of cDNA which is determined by the nature of the primers used. As a consequence, small variations in the rate of amplification will greatly affect amplification efficiency (Saiki, 1988). By combining different ratios of mammary cDNA (predominantly exon 11 minus insulin receptor mRNA) and liver cDNA (predominantly exon 11 plus insulin receptor mRNA) this study has demonstrated that over the range of mammary cDNA:liver cDNA ratios assessed, the rate of amplification does not significantly change ($r=0.947$) (figure 4.6). This observation is fundamental to the validity of the results presented in this study.

Radiolabelled [α-^{32}P]dCTP competes with the unlabelled dCTP for incorporation into the PCR product. Hence theoretically, the lower the concentration of dCTP the higher the radioactivity of the PCR product and the greater the signal on the autoradiograph. However, sufficient dCTP must be present for *de novo* synthesis from the cDNA template. Titration of the dCTP concentration demonstrated that a dCTP concentration approximately 2000-fold less than the other three deoxynucleotides resulted in adequate [α-^{32}P]dCTP incorporation whilst maintaining sufficient product yield.

In certain experiments, two slow migrating PCR products were evident in the autoradiographs, characteristic of the formation of heteroduplexes The formation of

these DNA hybrids have been shown to be a significant source of inaccuracy in the assessment of the distribution of insulin receptor isoforms (Norgren, 1993). Heteroduplexes are formed when the concentration of the PCR products are sufficiently high to compete with the primers during the annealing cycle resulting in a greater proportional loss of the less abundant PCR fragment. However in this study, heteroduplex formation is evident only in experiments which relate to the optimisation of the PCR technique. The final PCR protocol used did not result in the generation of heteroduplexes.

4.4.2 Distribution of IR-A and IR-B in rat tissues

The discussion which follows is based on the premise that the ratio of IR-A and IR-B mRNA in a particular cell type parallels the expression of the insulin receptor isoform proteins. I consider this to be a reasonable assumption based on reports by Kellerer *et al.* (1993) and Benecke *et al.* (1992) who have employed a novel immuno-precipitation method for the estimation of the IR-A and IR-B isoforms and have established that protein levels of the insulin receptor isoforms display high correlation with mRNA levels in several human tissues. This is an important observation as it indicates that the rate of protein translation and the half-life of the two proteins are relatively similar. Nevertheless, this assumption is made with a certain degree of caution and further studies characterising the ratio of the IR-A and IR-B proteins in various tissues and in different species also need to be completed to ensure its validity.

This study has determined the insulin receptor isoform mRNA present in rat mammary tissue and identified that the relative distribution of IR-A and IR-B mRNA in this tissue is dependent on the stage of lactation. Insulin receptor-A mRNA predominated throughout all stages of pregnancy and lactation studied (figure 4.8). If as suggested by several groups, the IR-A isoform has a higher affinity for insulin, and does not follow classical down-regulation pathways following insulin binding (Mosthaf, 1990; Vogt, 1991; Yamaguchi, 1991), tissues which predominantly

express this isoform would exhibit enhanced insulin-sensitivity when compared with tissues that expressed a greater proportion of IR-B. In contrast, liver expresses almost exclusively IR-B mRNA (figure 4.8) which displays lower insulin affinity and follows classical down-regulation pathways when expressed in transfected cell lines. This contrariety in insulin receptor isoform distribution in these tissues would be expected to promote nutrient partitioning which evolves during late pregnancy and lactation. In theory, the predominance of the high affinity IR-A isoform in mammary tissue would result in more insulin receptors being occupied for a given insulin concentration in addition to the diminished capacity for normal, insulin-stimulated receptor down-regulation. This would result in a greater insulin-stimulated signal into the mammary cells.

Investigation of IR-A and IR-B mRNA distribution in mammary tissue during late pregnancy and lactation also showed that their were changes in the relative proportions of the receptor isoform as lactation progressed. As shown in figure 4.8, a significant decrease in the IR-A:IR-B mRNA ratio is evident during the first week of lactation. The physiological trigger of the altered insulin receptor isoform distribution during the first week of lactation is unclear although this does suggest that the insulin receptor isoform expression could be subject to acute hormonal/metabolic control (see below) and may change as a function of the development of the tissue.

The predominance of IR-B mRNA in both human and rat liver has been confirmed in previous studies (Mosthaf, 1990; Goldstein, 1990) (table 1.2). Mosthaf *et al.* (1990) suggest that the physiological significance of the predominance of the IR-B isoform (with the reported lower affinity for insulin) in the liver is due to this tissue being constantly exposed to portal insulin concentration which are some 2-3 fold greater than peripheral insulin levels. Hence, they conclude that this would enable the liver to remain sensitive to normal variations in the portal insulin

concentrations, whereas the IR-A isoform would be relatively saturated at these elevated hormone levels.

The insulin receptor isoform mRNA expression in several insulin-sensitive tissues from foetal, neonatal and adult rats were measured to identify if isoform variations were evident throughout ontogeny. Specific expression of IR-A and IR-B mRNA was evident in 14 day old foetal rats suggesting that functional splicing machinery is present within the foetal tissue by at least day 14 of gestation. In three of the tissues studied (cardiac muscle, kidney and liver), the relative proportions of IR-A and IR-B mRNA varied within a single tissue type as the foetus developed, through parturition and in the maturing neonate (note: in kidney tissue, IR-A/IR-B distribution was only measured in neonates and adult rats). This demonstrates that insulin receptor isoform mRNA expression in these tissues does not only occur in a tissue-specific manner, but is also developmentally regulated. The most significant change in insulin receptor isoform distribution occurs peripartum. In cardiac muscle and most markedly in the liver, the proportion of IR-A significantly decreases over the 48 hours from 21 day foetus to 1 day neonate. This change in the expression of the insulin receptor isoforms coincides with a significant decrease in the concentration of both total (figure 4.11a) and free corticosteroids in foetal plasma (Martin, 1977). Interestingly, the change in insulin receptor isoform expression which occurs in the maternal mammary tissue immediately subsequent to parturition is also associated with a significant decrease in total corticosteroid concentration (figure 4.11b). Recently, Kosaki and Webster (1993) have shown that dexamethasone treatment of HepG2 cells in vitro induces a switch in the expression of the A to the B isoform of the insulin receptor that is associated with changes in the insulin-stimulated glucose incorporation into glycogen and 2-deoxyglucose uptake. Furthermore, they provide preliminary evidence that in 3T3-L1 adipocytes, the expression of the insulin receptor isoforms are developmentally regulated since differentiation of the cells occurs concomitantly with a change in expression from the IR-A to the IR-B isoform (Kosaki, 1993). This observation has been confirmed by

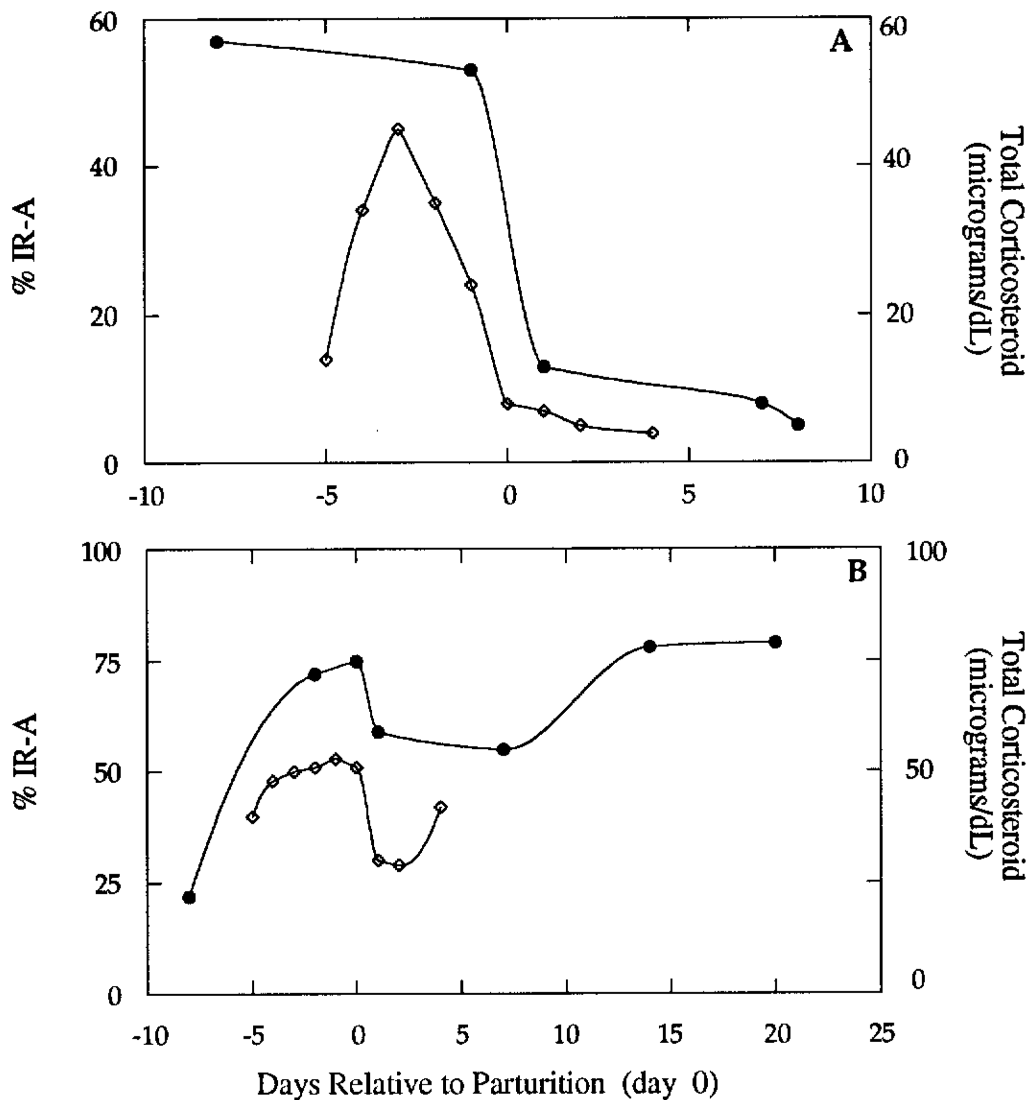

Figure 4.11 Insulin receptor isoform expression and circulating corticosteroid concentrations during foetal ontogeny and lactation. The proportion of IR-A mRNA in foetal liver (A) and maternal mammary (B) rat tissues prior and subsequent to parturition were determined using the RT-PCR. Total plasma corticosteroid concentrations were obtained from Martin *et al.* (1977). (•) represents the relative proportion of the mRNA encoding the IR-A insulin receptor isoform, (◇) represents the total plasma corticosteroid concentration.

Norgren *et al.* (1994a) who also demonstrated that dexamethasone increases the proportion of IR-B mRNA in HepG2 cells in a dose-dependent manner. In addition, they have shown that low glucose concentrations act to decrease the proportion of IR-B mRNA whilst insulin and IGF-1 appears to have no effect on IR-A/IR-B distribution. In another study by Norgren *et al.* (1994b) the ratio of IR-A mRNA in skeletal muscle from a NIDDM individual changed from 74% to 48% subsequent to the initiation of insulin treatment. This observation further implicates the level of glucose as a possible regulator of insulin receptor isoform expression.

In cardiac muscle, kidney and liver during organogenesis, the expression of the insulin receptor isoform changed from predominantly IR-A to IR-B. This may suggest that the functional role of the alternatively spliced insulin receptor isoforms is associated with the stage of organogenesis in these insulin-sensitive tissues. A number of studies have identified insulin gene expression and insulin synthesis by tissues other than the pancreas during ontogeny (Giddings, 1989; De Pablo, 1990; Giddings, 1990; Muglia, 1984). Furthermore, insulin gene expression is evident in the foetal rat liver and yolk sac prior to insulin gene expression in the pancreas, suggesting that insulin plays a significant physiological role during foetal growth and development (Kakita, 1983; Giddings, 1989; Giddings, 1990). Recently, Gruppuso *et al.* (1994) have demonstrated that insulin has a direct hepatotrophic effect on rat foetal liver cells which is independent from the actions transforming growth factor-α and hepatocyte growth factor. The importance of the growth promoting effects of insulin in the foetus is also supported by the observation that the abundance of liver insulin receptor mRNA in mid-gestational foetal rats is significantly greater than the IGF-1 mRNA (which is important in foetal growth) (Giddings, 1992). This observation is interesting since significant metabolic activity in the liver does not commence until later in the foetus (Giddings, 1992) suggesting that insulin receptors are present in the foetus during mid-gestation to transmit the insulin signal which promotes hepatocyte growth. Together, these observations suggest that insulin plays an important role in the regulation of foetal growth and development. From the

observations made in this study characterising the changes in insulin receptor isoform distribution which occur in the foetal and neonatal rat, I propose that the insulin receptor isoforms may have differential roles which influence the growth-promoting effect of insulin on insulin-sensitive tissues.

Finally, studies were initiated which investigated the effect of streptozotocin-induced diabetes on the expression of IR-A and IR-B mRNA. Streptozotocin-induced diabetes in rodents results in hypoinsulinaemia associated with decreased insulin action in adipose (Kasuga, 1978; Kobayashi, 1979; Karnieli, 1981) and liver tissue (Haft, 1968) despite increased insulin-insulin receptor binding. Increased insulin binding is attributed to both compensatory up-regulation of cell-surface insulin receptors in response to the decreased circulating insulin levels and the modulation of the steady-state insulin receptor mRNA levels (Okabayashi, 1989; Secchi, 1992). The net result of streptozotocin-induced diabetes is a state of post-receptor insulin resistance (Kasuga, 1978; Kobayashi, 1979; Davidson, 1976). Although only preliminary in nature, this experiment identified that in all of the tissues studied, streptozotocin-induced diabetes did not appear to alter the relative expression of insulin receptor isoform mRNA compared with the control group. This suggests that the hypoinsulinaemia which results from streptozotocin-induced diabetes does not appear to alter the expression of the insulin receptor isoforms. However, I reiterate that this is a preliminary study and a greater sample size is required to confirm this observation.

Some information as to the roles of the two insulin receptor isoforms may be gleaned from analysis of the nature of the peptide encoded by exon 11 (figure 4.12). Hydrophilicity profiles suggest that the entire 12 amino acid peptide is exposed on the outside of the protein. The antigenic profile of the exon 11 encoded peptide, which identifies possible exposed surface peaks which may be antigenic sites, indicates that residues lysine-720 to alanine 727 display a high antigenic index. Secondary structure predictions based on both the Chou-Fasman and the Robson-

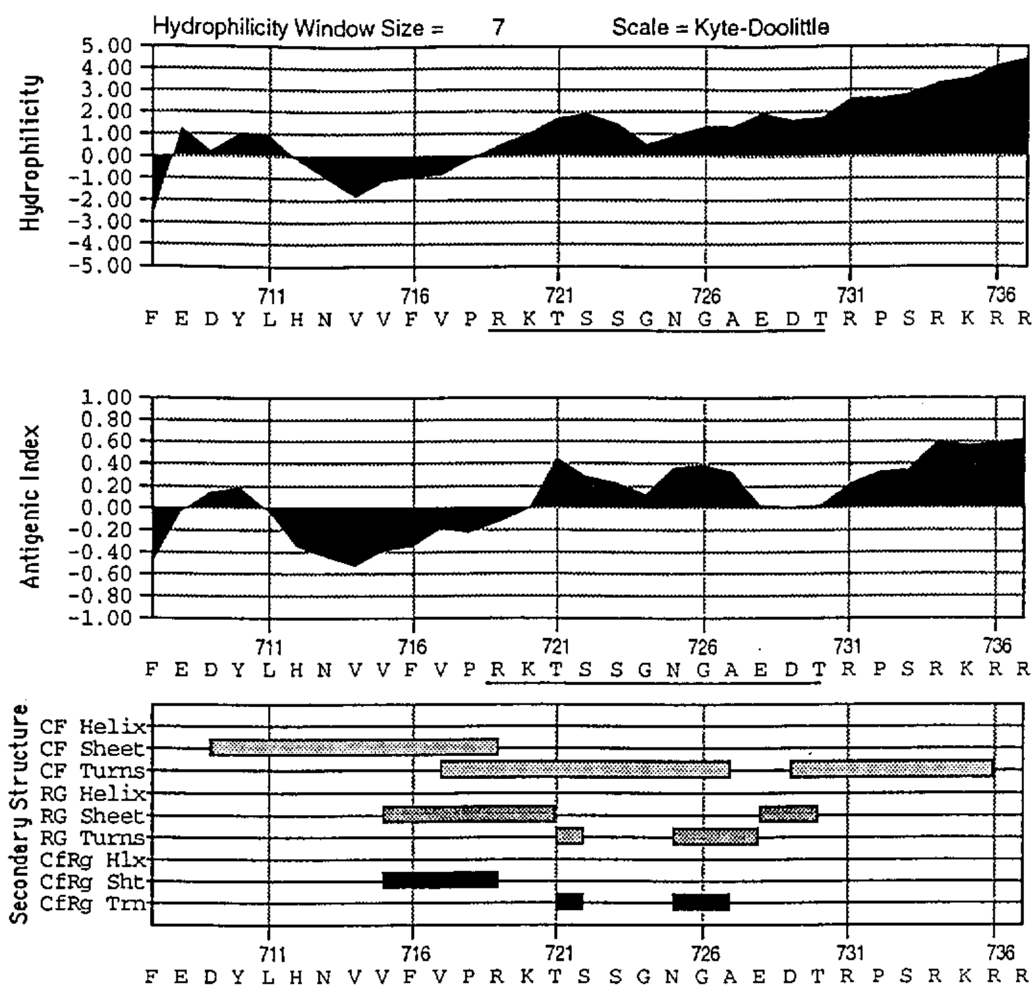

Figure 4.12 Predictions of hydrophilicity, antigenic probability and secondary structure within the twelve amino acid sequence encoded by exon 11 of the rat insulin receptor gene. The rat exon 11 sequence from Goldstein (1990) was translated into to the exon 11 encoded peptide (residues 719-730, underlined) and secondary structures predicted using the MacVector 4.0 Sequence Analysis Software. Hydrophilicity estimates were based on the Kyte-Doolittle scale of amino acid hydropathy and secondary structure predictions were made using both the Robson-Garnier (RG) and Chou-Fasman (CF).

Garnier models indicate the high probability of a reverse turn within the exon 11 encoded peptide at asparagine-725 to alanine-727. These combined data indicate that there is a high probability that the exon 11 encoded region is exposed to the extracellular environment and is a good candidate as an antigenic epitope. Examination of available data bases of both the DNA sequence and the translated protein identified no significant homology between exon 11 or the exon 11 encoded peptide and other gene or peptide sequences. Moreover, the syntenic regions in the human and rat insulin receptor genes are highly conserved suggesting that the region encoded by exon 11 is specific to the insulin receptor. Interestingly, Yamaguchi *et al.* (1993) observed that the ability of unlabelled IGF-1 to displace with ^{125}I-insulin binding was markedly different between two CHO cell lines expressing either the IR-A or IR-B isoforms. In these experiments the IC_{50} for IGF-1 inhibition of insulin binding was increased some 10-fold for the IR-B insulin receptor isoform in both intact cells and solubilised receptors. This observation implies that the presence or absence of the exon 11 encoded peptide (residues 719-730), located near the C-terminal end of the insulin receptor α-subunit, exerts an effect on the conformational state of the ligand-binding region of the receptor which has been localised to the cysteine-rich region of the α-subunit (residues 155-312).

This study has characterised the insulin receptor isoform distribution in lactating rat mammary and liver tissues, and in several foetal and neonatal tissues. I have established that the relative proportions of IR-A and IR-B mRNA varies within a single tissue type as a function of development which suggests that insulin receptor isoform expression may be regulated by hormonal and/or metabolic factors. This has been demonstrated during both pregnancy and lactation in mammary tissues, and in a number of foetal tissues during ontogeny. Interestingly, in both instances, changes in IR-A/IR-B mRNA expression occur concomitantly with significant decreases in the concentration of circulating corticosteroids. This observation in conjunction with a recent reports by Kosaki and Webster (1993) and Norgren *et al.* (1994a) suggests that changes in glucocorticoid levels may affect the mechanism which regulates

insulin receptor pre-mRNA splicing *in vivo*. In addition, glucose concentrations have also been shown to affect IR-A/IR-B mRNA expression (Norgren, 1994a). Finally, based on the observation of changes in the ratio of IR-A to IR-B in within certain tissues during foetal ontogeny, I propose that the predominance of the IR-A isoform in certain tissues during foetal ontogeny reflects the importance of the growth promoting effects of insulin in these tissues.

Post script

Results from this chapter form the basis of a manuscript which has been submitted for consideration for publication in the American, peer-reviewed journal Diabetes titled "Expression of insulin receptor isoforms in the developing rat" by Domenica Deleo, Alison Smith and Erik Helmerhorst.

CHAPTER FIVE

INSULIN-INSULIN RECEPTOR BINDING IN MAMMARY AND LIVER TISSUES

5.1 INTRODUCTION

Nutrient partitioning during lactation in maternal tissues follows several coordinated changes in the insulin/glucagon sensitivity of target tissues (reviewed in Chapter 1). In the mammary tissue of lactating rats, insulin induces an eight-fold increase in glucose uptake (Burnol, 1987). The magnitude of this insulin-stimulated action is greatest in lactating mammary tissue compared with other insulin-sensitive tissues (Burnol, 1987), illustrating the extreme insulin-sensitivity and responsiveness of the lactating mammary gland.

Following my observations of specific structural differences in the mammary insulin receptor relative to other typical insulin receptors, I postulated that these structural variations may lead to functional differences that promote the flow of nutrients into the mammary gland for milk synthesis. This postulate was based on conclusions from chapter 3, wherein I clearly demonstrated that the difference between the mammary and liver insulin receptors can, in part, be attributed to the altered glycosylation of the mammary insulin receptor, and that their is strong evidence suggesting that the carbohydrate moieties of the insulin receptor affects insulin binding (Olden, 1982; Cherqui, 1982; Caron, 1978; Cherqui, 1981; Podskalny, 1984; Podskalny, 1986; Rouiller, 1986; reviewed in Section 3.4). Secondly, in contrast to adult liver tissue, the mammary insulin receptor mRNA is spliced to predominantly yield the IR-A isoform which is purported to have a two-fold greater affinity for insulin compared with IR-B when expressed in CHO cells or Rat-1 fibroblasts (Mosthaf, 1990; Yamaguchi, 1991). As the first step to test my postulate I compared

insulin binding in mammary and liver tissues from lactating rats. The results from this study is presented in this chapter.

5.2 EXPERIMENTAL PROCEDURES

5.2.1 Animals

Lactating and nulliparous Wistar albino rats were obtained and housed as described in section 3.2.1.

5.2.2 Preparation of membranes from rat mammary and liver tissues

Liver plasma membrane preparations were obtained from eight week old, adult Wistar rats. Liver plasma membranes were partially purified using a modified method based on that of Ray (1970) as outlined in Section 3.2.4. Mammary and liver microsomal membrane preparations were obtained from lactating Wistar rats on the seventh day of lactation according to the method of Dijane *et al.* (1977), as outlined in Section 3.2.5. Particular care was taken to ensure all liver tissue and both ventral mammary glands were excised from the animal. Membrane protein concentration and the estimation of membrane purity was determined using the procedures outlined in sections 3.2.6 and 3.2.7.

5.2.3 Insulin binding to mammary and liver membranes

Membranes (12.5-800 μg/mL protein) were resuspended in 0.1 M HEPES, pH 7.8 at 4°C containing 0.1 % (w/v) bovine serum albumin-fraction V, 0.1 % (v/v) Triton-X and 100 U/mL bacitracin. A14-tyrosyl[^{125}I]iodoinsulin (0.16 kBq of 70 MBq/nmol) was added to the resuspended membrane in the presence of varying concentrations of porcine insulin (0-5.2 μM) in a final volume of 200 μL. Membranes were incubated at either 4°C for 16 h or 37°C for 1 h. Following the incubation, membrane pellets were recovered according to the protocol outline in

Section 2.2.7 and the radioactivity associated with each pellet measured using a Hewlett Packard Cobra™ II auto-gamma® counter.

5.2.4 Derivation of ligand affinity constants and receptor numbers using Scatchard analysis

The simplest model for the hormone-receptor interaction and the subsequent hormone-stimulated cellular responses is based on four assumptions;

(i) reversibility of the hormone-receptor interaction,

(ii) biomolecularity of the binding reaction,

(iii) non-cooperativity of receptors,

(iv) proportionality between receptor occupancy and cellular action.

These assumptions can be described by;

$$H + R \underset{kd}{\overset{ka}{\Leftrightarrow}} HR \overset{ke}{\rightarrow} \text{Effect} \qquad \text{equation 1}$$

where R = concentration of unbound receptor

H = concentration of unbound hormone

k_a = rate constant of association

k_d = rate constant of dissociation

k_e = proportionality constant between receptor occupancy and the biological response

The biological effect is directly related to the concentration of hormone-receptor complexes by the proportionality constant k_e;

$$\text{Effect} = k_e[RH] \qquad \text{equation 2}$$

Hormone-receptor binding affinities and receptor concentrations can be easily interpreted from the graphical representation, first introduced by Scatchard (1949).

Assuming that F is the amount of unbound (free) hormone, B is the proportion of bound hormone and Ro is the total receptor concentration;

$$F + (Ro - B) \Leftrightarrow B \qquad \text{equation 3}$$

$$Ka = \frac{B}{F(Ro - B)} \qquad \text{equation 4}$$

$$Ka\,(Ro - B) = \frac{B}{F} \qquad \text{equation 5}$$

$$KaRo - KaB = \frac{B}{F} \qquad \text{equation 6}$$

The Scatchard plot compares the ratio of free:bound hormone (B/F) with the amount of bound hormone (B) providing a linear representation of equation 6, where the negative gradient is equivalent to the association constant of the interaction, and the x-intercept is equivalent to the total receptor number.

5.2.5 Derivation of the concentration of hormone-receptor complexes

Knowing the number of receptors (Ro) and the affinity constant for the interaction (Ka) as estimated from Scatchard analysis the number of hormone receptor complexes at any given concentration of the hormone (Ho) can be derived.

Since F = Ho - B, where Ho = total concentration of hormone and B = the number of hormone-receptor complexes assuming a 1:1 binding stoichiometry, then equation 4 may be rewritten as;

$$Ka(Ro - B)(Ho - B) = B \qquad \text{equation 7}$$

$$KaRoHo - KaRoB - KaHoB + KaB^2 = B \qquad \text{equation 8}$$

$$KaRoHo - KaRoB - KaHoB + KaB^2 - B = 0 \qquad \text{equation 9}$$

$$KaB^2 - B(KaRo + KaHo + 1) + KaRoHo = 0 \qquad \text{equation 10}$$

Solving the quadratic equation;

$$aB^2 + bB + c = 0 \qquad \text{where } a = K_a$$
$$b = -(K_aR_o + K_aH_o + 1)$$
$$c = K_aR_oH_o$$

From this, the concentration of hormone-receptor complexes per unit of protein, and per organ can be estimated.

5.2.6 Analysis of binding data

Data analysis was carried out using the method of Scatchard (1949) as employed by the Biosoft Ebda V 2.0 and Ligand programs (McPherson, 1985). The nature of this analysis is outlined in section 2.2.8.

5.2.7 A14-tyrosyl[^{125}I]iodoinsulin degradation

The degree of ligand degradation was assessed by performing TCA precipitations on the supernatant containing the unbound A14-tyrosyl[^{125}I]iodoinsulin. Ice-cold TCA was added to the binding assay supernatant containing the unbound fraction of the ligand to a final concentration of 10% (v/v) TCA. The solution was centrifuged at 10,000g for 30 minutes at 4°C, and the radioactivity associated with the pellet measured using a Hewlett Packard Cobra™ II auto-gamma® counter.

5.2.8 Measurement of serum insulin concentrations

Insulin concentrations in serum obtained from day seven lactating rats and eight week old, nulliparous rats were estimated using the Phadeseph Insulin RIA kit (Pharmacia Diagnostics AB, Uppsala, Sweden). This assay uses guinea-pig anti-insulin antibody and test values are compared to those obtained from human insulin standards. For the estimation of hormone-receptor complex concentrations in liver tissues, portal insulin concentrations in both the nulliparous and day seven lactating

animals was assumed to be approximately 2.5-fold greater than the peripheral blood insulin concentrations determined for each set of animals (Mosthaf, 1990).

5.3 RESULTS

5.3.1 Enrichment of plasma membranes

Typical data from the purification of liver plasma membranes from eight week old nulliparous rats, and the enrichment of the microsomal membranes from mammary and liver tissues from day seven lactating rats are shown in table 5.1. Using the method of Ray (1970), plasma membranes from the liver of eight week old nulliparous rats were purified 12-fold as estimated from the increased 5'-nucleotidase activity per milligram of protein. Mammary and liver microsomal membranes recovered using this method (Dijane, 1977) resulted in an approximate 7-fold increase in the 5'-nucleotidase activity compared with the tissue homogenate. Both methods of cell membrane isolation resulted in a significant increase in the amount of insulin bound per unit of protein.

5.3.2 Validation of hormone-receptor binding experiments

5.3.2.1 Time course of insulin binding

Insulin binding to liver and mammary membranes was assessed over a 24 hour period at 4°C, and over a 4 hour period at 37°C. A typical time course for insulin binding to liver microsomal membranes is shown in figure 5.1. The equilibria were stable at all temperatures over the range from 4°C to 37°C, and in the presence of bacitracin which inhibited insulin degradation. Furthermore, the time taken to reach equilibrium was independent of hormone concentrations and similar time course profiles were obtained over a wide range of receptor concentrations (data not shown).

	Protein per Organ (mg)	Yield (%) 5'-nucleotidase activity	Specific Activity	Purification Factor
Method of Ray (1970);				
Liver Homogenate	4095	-	611	-
Liver Plasma Membrane	43.2	43.0%	7268	11.9
Method of Dijane (1977);				
Mammary Homogenate	157.5	-	18.4	-
Mammary Membrane	13.6	67.3%	143.4	7.8
Liver Homogenate	477.0	-	36.7	-
Liver Membrane	14.0	19.7%	246.4	6.7

Table 5.1 Mammary and liver membrane isolation. Data from a typical purification of liver plasma membranes from an 8 week old Wistar rat (Ray, 1970) and mammary and liver membranes from a day seven lactating Wistar rat. Membranes purification (Dijane, 1977), protein estimation and measurement of the 5' nucleotidase activity were assessed according to the procedures outlined in the methods section. Specific activity is expressed as the amount of phosphate produced (nmol) per hour per mg of protein.

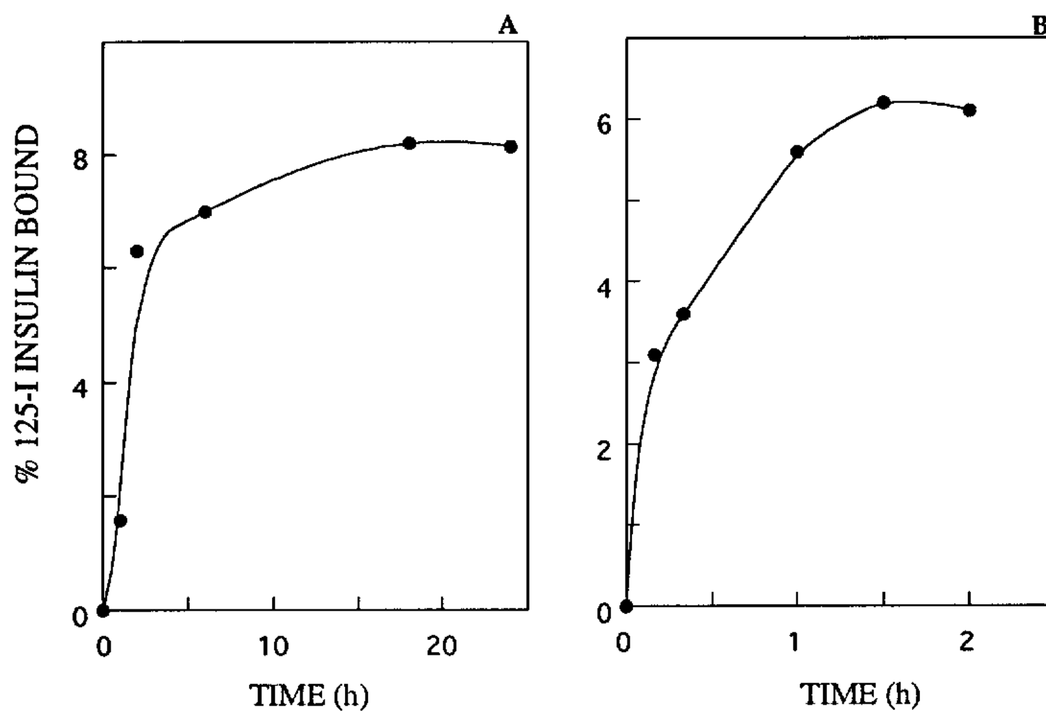

Figure 5.1 Binding of A14-tyrosyl[^{125}I]iodoinsulin to liver membrane as a function of time. Comparison of % A14-tyrosyl[^{125}I]iodoinsulin bound to liver microsomal membranes at 4°C (A) and 37°C (B).

5.3.2.2 Assessment of insulin degradation

Insulin degradation was assessed by measuring the TCA precipitability of the ligand following a 45 minute incubation at 37°C. Receptor-bound insulin was greater than 90% precipitable over the insulin range of 10 mM to 10 pM, whilst the unbound ligand was less than 60% precipitable at insulin concentrations below 10 nM. Both bacitracin and N-ethylmaleimide were assessed as potential inhibitors of ligand degradation with bacitracin being the most effective. The inclusion of 100 units of bacitracin per mL of buffer resulted in 100% of bound insulin and greater than 95% of unbound insulin being precipitable at all insulin concentrations. There was no apparent difference in the Hill coefficient or the association constant from binding experiments performed at 4°C or 37°C in the presence or absence of ligand degradation inhibitors when data derived in the absence of bacitracin or N-ethylmaleimide was corrected for ligand degradation.

5.3.3 Insulin binding in purified mammary and liver membranes

The binding affinity constants from mammary and liver insulin receptors from day seven lactating rats were determined by Scatchard analysis performed at 4°C. These experiments were performed in triplicate. In each experiment, data derived from both tissues was best fitted assuming a one site model ($P>0.05$). Scatchard plots of typical experiments (figure 5.2, table 5.2) indicate a Ka of 1.8×10^9 for mammary tissue and 1.2×10^9 for liver. Individual comparisons of mammary and liver insulin binding data from all three experiments were made. In each instance, data obtained for the mammary tissue was mathematically modelled on the liver data affinity constant. This was determined to be a significant fit using the Run's test of Bennett and Franklin (1954) which indicates that these two sets of data were not significantly different.

The interaction between insulin and receptors present in mammary and liver plasma membranes obtained from day seven lactating animals was also analysed at 37°C. This experiment was performed in duplicate. A two-site model was required to

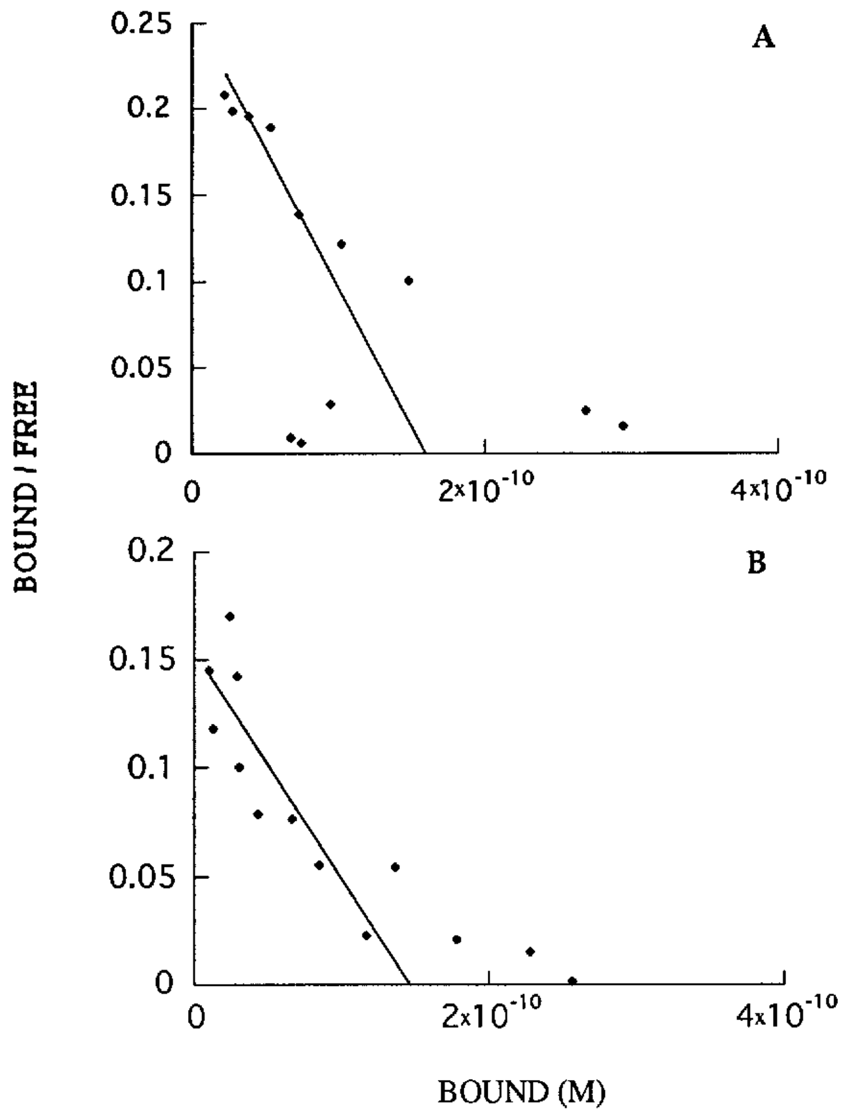

Figure 5.2 Scatchard plots from mammary and liver membranes from lactating Wistar rats (4°C). The interaction of native porcine insulin with mammary (A) and liver (B) microsomal membranes obtained from day seven lactating rats was assessed at 4°C.

	Mammary	Liver
Insulin (M)	5.4×10^{-11}	1.35×10^{-10}
Insulin receptor (M)	1.46×10^{-10}	1.34×10^{-10}
K_a	1.78×10^{9}	1.16×10^{9}
% occupancy	7.16	12.1
Insulin receptors (moles/organ)	1.30×10^{-11}	1.94×10^{-11}
I-IR complexes (moles/organ)	9.32×10^{-13}	2.35×10^{-12}

Table 5.2 Data from the analysis of insulin binding at 4°C in mammary and liver membranes from day seven lactating Wistar rats. Scatchard analysis of insulin-insulin receptor binding was carried out at 4°C as described in the Methods section and data reduction performed using the Biosoft Ebda V 2.0 and Ligand programs (McPherson, 1985) (I-IR = insulin-insulin receptor complexes).

provide a significant fit to both the mammary and liver data derived at 37°C (P>0.05). Data from a typical experiment is shown in figure 5.3 and table 5.3. Curvilinear Scatchard plots were obtained from both mammary and liver insulin receptors from equilibrium binding experiments performed at 37°C. Estimates of the percentage of receptor sites occupied at physiological concentrations of the hormone indicate that the low affinity site in both tissues is only minimally occupied compared with the high affinity site (< 0.5% of total occupied sites).

5.3.4 Estimation of the insulin binding affinity of liver plasma membranes from nulliparous rats

Insulin binding to the liver plasma membranes purified according to the method of Ray (1970) and obtained from 8 week old, nulliparous rats was assessed at 4°C. This experiment was performed twice with tissues from two animals. Data from these experiments were best fitted assuming a model of a single class of insulin receptor binding sites (P>0.05) (figure 5.4, table 5.4).

A comparison of the binding affinity of liver insulin receptors from the eight week old, nulliparous animals with that of the day seven lactating animals indicated that their was no significant difference between the estimated binding affinities for the two tissues (forced fit of K_a obtained from the liver from the nulliparous animal to the binding data from the liver of the day seven lactating animal was a significant fit, P>0.05).

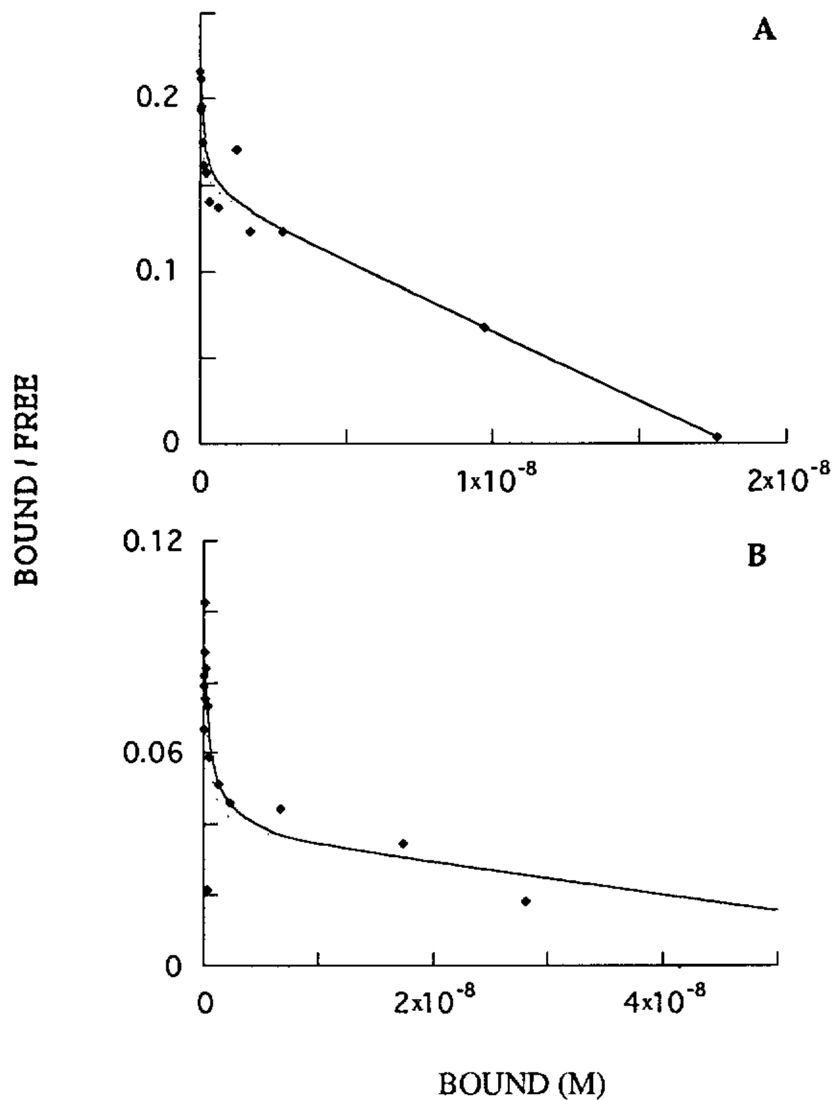

Figure 5.3 Scatchard plots from mammary and liver membranes from lactating Wistar rats (37°C). The interaction of native porcine insulin with mammary (A) and liver (B) microsomal membranes obtained from day seven lactating rats was assessed at 37°C.

	Mammary Site 1	Mammary Site 2	Liver Site 1	Liver Site 2
Insulin (M)	5.4×10^{-11}		1.35×10^{-10}	
Insulin receptor (M)	1.7×10^{-11}	1.6×10^{-8}	2.3×10^{-10}	4.8×10^{-8}
K_a	4.5×10^{9}	1.1×10^{7}	2.1×10^{8}	7.1×10^{5}
% occupancy	18.6	0.04	2.64	0.01
Insulin receptors (moles/organ)	1.5×10^{-12}	1.4×10^{-9}	3.3×10^{-11}	6.7×10^{-9}
I-IR complexes (moles/organ)	2.8×10^{-13}	6.1×10^{-13}	9.0×10^{-13}	6.2×10^{-13}

Table 5.3 Data from the analysis of insulin binding at 37°C in mammary and liver membranes from day seven lactating Wistar rats. Scatchard analysis of insulin-insulin receptor binding was carried out at 37°C as described in the Methods section and data reduction performed using the Biosoft Ebda V 2.0 and Ligand programs (McPherson, 1985) (I-IR = insulin-insulin receptor complexes).

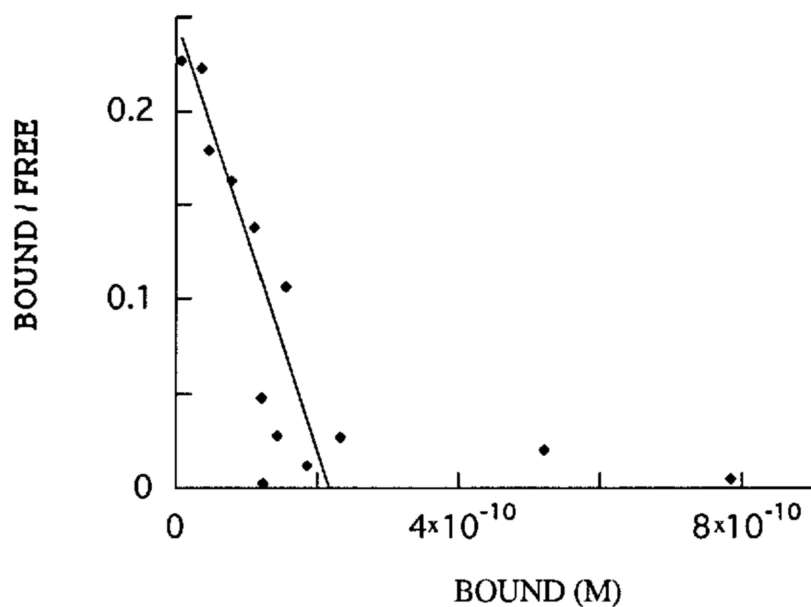

Figure 5.4 Scatchard plots from liver plasma membranes from 8 week old, nulliparous Wistar rats (4°C). The interaction of native porcine insulin with liver plasma membranes obtained from 8 week old, nulliparous Wistar rats was assessed at 4°C.

	Liver
Insulin (M)	1.3E-10
Insulin receptor (M)	$2.0x10^{-10}$
K_a	$1.2x10^9$
% occupancy	11.4
Insulin receptors (moles/organ)	$1.3x10^{-11}$
I-IR complexes (moles/organ)	$1.6x10^{-12}$

Table 5.4 Data from the analysis of insulin binding at 4°C to liver plasma membranes from 8 week old, nulliparous Wistar rats. Insulin binding experiments were performed as described in the Methods section. (I-IR = insulin-insulin receptor complexes).

5.4 DISCUSSION

The mammary insulin receptor was first identified by O'Keefe and Cuatrecasas (1974) who demonstrated the specific and saturable binding of insulin to murine mammary cells. Moreover, they identified that cells from lactating mice bound 3-4 fold more insulin than mammary cells from pregnant or nulliparous animals. This observation was also confirmed using rat mammary cells (Flint, 1982). Preliminary studies of the interaction between the insulin and the mammary insulin receptor suggest that the increased binding capacity of the cells was predominantly due to an increase in the insulin receptor number and not due to a change in insulin affinity (Inagaki, 1982; Flint, 1982). This observation is somewhat suprising since the structural differences I have characterised between the mammary and liver insulin receptors, both in terms of glycosylational differences and differences in the distribution of IR-A and IR-B mRNA, support the suggestion of a difference in the insulin binding affinity of these two tissues.

Several technical limitations were inherent in a number of these previous studies (O'Keefe, 1974; Flint, 1982; Inagaki, 1982) that may account for their inability to differentiate between the affinity of insulin binding in these tissues. Firstly, heterogeneous [^{125}I]insulin preparations were used which have been shown to display binding properties significantly different from the native hormone (Gammeltoft, 1984; see chapter 2). In the studies by Flint (1982) and Inagaki and Kohmoto (1982), collagenase-dissociated mammary cells were the receptor source of choice. It has been the experience of this author and others (personal communication Associate Professor Erik Helmerhorst and Professor Cecil Yip) that the isolation of mammary epithelial cells and other cells by collagenase digestion affects the integrity of the insulin receptor, probably through the actions of contaminating proteases present in many commercial collagenase preparations. Moreover, in two of these previous studies (O'Keefe, 1974; Inagaki, 1982) neither ligand nor receptor degradation were evaluated

at any point during the binding experiments, even though the experiments were carried out in the absence of any inhibitors of protein degradation.

I first characterised the binding assay methodology described in this chapter to verify that implicit assumptions in my equilibrium binding analyses do, in fact, hold true. Time course experiments demonstrated that a stable, steady-state equilibrium was achieved by the incubation of the ligand and receptor at 4°C for 16 hours, or 37°C for 1 hour (figure 5.1). The inclusion of bacitracin in the system ensured that the equilibrium was not disturbed by insulin degradation. Furthermore, the receptor remained structurally and functionally intact over the time and temperature of incubation, and the polyethylene glycol precipitation of bound ligand was shown not to affect equilibrium (Helmerhorst, 1993). Finally, an assumption inherent in the estimation of binding affinity between two molecules is that the tracer used in the experiment exhibits binding properties identical to the native ligand. For these binding studies, A14-tyrosyl[^{125}I]iodoinsulin was the tracer used which has binding characteristics indistinguishable from native insulin (Deleo, 1992; see Chapter 2).

The source of the insulin receptor to be used in these experiments was carefully considered prior to the commencement of this study. As previously alluded to, initial attempts to isolate collagenase-digested mammary epithelial cells were unsuccessful as these cells retained only a minimal capacity to specifically bind insulin. Partially purified membranes were the receptor source of choice in this study as sufficient insulin binding could be demonstrated in these preparations, and unlike purified insulin receptors, adequate amounts of membranes could be recovered from the tissue from a single animal to perform the necessary binding experiments. Further purification of insulin receptor using conventional methods, such as wheat-germ agglutinin and insulin-agarose chromatography, markedly increased the purity of the receptor preparation but insufficient receptor numbers were recovered for subsequent Scatchard analysis (data not shown). Attempts were made to purify plasma membranes from mammary tissue using the method of Ray (1970), which is frequently cited as the

method of choice for the isolation of liver plasma membranes. This method incorporates a gentle homogenisation step which, whilst disrupting the cell membrane, allows relatively large sheets of plasma membranes to remain intact which facilitates their subsequent isolation by ultracentrifugation in a discontinuous sucrose density gradient. In contrast to liver tissue, mammary tissue contains significant amounts of interlobular connective tissue and as a result, harsh mechanical homogenisation is required. Subsequent attempts to isolate plasma membrane from this mammary homogenate using the method of Ray (1970) failed to provide sufficient recovery of plasma membrane for using in binding analyses. The method first described by Dijane *et al.* (1977) was used for the enrichment of membranes from both mammary and liver tissues to allow direct comparison of insulin binding data to be made. Both mammary and liver membranes were enriched approximately 7-fold based on the 5'-nucleotidase activity of these preparations (table 5.1), with sufficient recovery of protein for subsequent Scatchard analysis. Comparisons of two methods for membrane isolation were made using liver tissue which was purified using both the methods of Ray (1970) and Dijane *et al.* (1977). These experiments demonstrated that the protocol described by Dijane *et al.* (1977) was comparable to the more commonly used method of Ray (1970) as no significant differences were evident between the insulin binding affinities of the two membrane preparations. A comparison of the insulin binding affinity in mammary tissue from nulliparous rats with mammary tissue from the lactating animal was not able to be made since proliferation of the mammary epithelium does not occur until the onset of pregnancy/lactation.

The affinity of insulin for the insulin receptor in mammary and liver membranes from day seven lactating rats were not significantly different under the conditions of this study (table 5.2). The exposure of the liver tissue to the elevated (although still non-saturating) portal concentrations of insulin is reflected in the greater proportion of hormone-occupied receptor sites, in addition to a higher number of hormone-receptor complexes per organ. These results suggest that the structural differences evident between the mammary and liver insulin receptors during lactation do not appear to alter

the affinities with which these receptors bind insulin, thus supporting the results from previous studies. This was somewhat suprising since, as alluded to earlier, a difference in insulin receptor affinity might be expected based on the observation of the two-fold difference in affinity between the IR-A and IR-B isoforms when expressed in either Rat 1 or CHO heterologous cell lines (Mosthaf, 1990; Yamaguchi, 1991), or from the perceived role of insulin receptor carbohydrate moieties on insulin binding (Olden, 1982; Cherqui, 1982; Caron, 1978; Cherqui, 1981; Podskalny, 1984; Podskalny, 1986; Rouiller, 1986).

During lactation mammary tissue expresses predominantly the IR-A isoform, although the relative ratio of IR-A to IR-B in this tissue changes as lactation progresses (see Chapter 4). On day seven of lactation (binding studies were performed using tissues from day seven lactating rats) the average proportion of the IR-A mRNA in mammary tissue was 54.5% (SD = 0.7), which was the lowest proportion of the IR-A isoform expressed at any stage during pregnancy or lactation. This may, in fact, have masked any difference in affinity which was due to variations in the proportions of the two alternatively spliced insulin receptor isoforms and it would be interesting to compare the insulin binding affinity in mammary tissues from a day 14 lactating animal.

I believe the two-fold difference in insulin affinity observed in IR-A/IR-B transfected cell lines must be considered with a certain degree of caution. Firstly, heterologous cell lines were used in these studies as tools to express either the IR-A or IR-B isoforms and to assess their function. Therefore, since these proteins are expressed in a foreign environment, parallels to the expected *in vivo* responses of these protein must be made with reserve. In the case of glycoprotein receptors such as the insulin receptor, any differences in the post-translational glycosylation mechanisms between the native cell and the transfected cell, differences in the targeting of the receptor to the membrane, or in the signalling pathways following ligand-stimulation of the receptor could influence the evaluation of protein function. Furthermore, the

expressed insulin receptors may form hybrids with the native insulin receptors which could influence the estimated insulin binding affinity if the equilibrium binding constants of these two receptors are not the same. Recently, Kato *et al.* (1993) compared the effect of insulin receptor and IGF-1 receptor over-expression in CHO cells and NIH-3T3 fibroblasts for their use in experiments which aim to measure the biological responses of their respective ligands. From this study, they have concluded that changes in the biological response of a particular cell line will be largely dependent on the level of the native receptors rather than the level of the over-expressed receptor. Hence clonal variations, even within a particular cell line, can affect the biological response of expressed receptors (Kato, 1993). Accordingly they advise that caution be taken when interpreting results obtained using CHO cells. The second reservation to be made when interpreting the significance of the difference in affinity between the IR-A and IR-B isoforms in transfected cell lines, or indeed in my study and those of others (O'Keefe, 1974, Flint, 1982; Inagaki, 1982), is that a two-fold difference in binding affinity is a relatively small difference and would be a difficult parameter to estimate accurately. The normal errors associated with Scatchard analyses allows a certain flexibility in the best fit for the data. Indeed, a small change in the binding affinity constant may be compensated with a reciprocal change in receptor number providing a equally good fit. For this reason, it may be more appropriate not to dissociate Scatchard data into separate Ka, Ro and Ho parameters, but instead to discuss the results based on the number of hormone-receptor complexes since this estimate would depend on the concentration of hormone and receptor, and the affinity of their interaction. In this study, the number of hormone-receptor complexes was determined based on the Ka and Ro derived from Scatchard analysis at physiological concentrations of the insulin, however as the parameter of interest in this study was the affinity of the insulin-insulin receptor interaction, it was necessary to make conclusions from comparisons of the estimated Ka values.

The interaction of insulin with the liver insulin receptor in isolated membranes from the nulliparous (table 5.4) and day seven lactating (table 5.2) animal were also

compared. No differences in the insulin binding affinity of these two tissues were observed. This confirms a previous report by Flint (1980) who demonstrated that their is no significant difference between the insulin binding affinity or insulin receptor numbers in liver from virgin and lactating rats.

Equilibrium binding analyses performed at 4°C and 37°C using identical sources of ligand and receptor displayed significantly different binding characteristics. At the lower temperature binding data was best fitted assuming a one site model (figure 5.2), whilst at 37°C the insulin binding data appeared more complex (figure 5.3). The observation of curvilinearity of the insulin-receptor interaction at 37°C is consistent with a previous report by Helmerhorst and Yip (1993) who describe the temperature-dependence of insulin binding in rat liver tissues. Furthermore, they propose that the complexity of insulin binding and the temperature-dependence of the interaction may be explained by the presence of a plasma membrane-associated glycoprotein that modulates insulin binding. The association of this putative insulin affinity modulator appears to be temperature-dependent with the inhibition of insulin binding affinity apparent above, but not below, 17°C. This theory is supported by a number of other studies (Kohanski, 1985; Baldini, 1991; Ciaraldi, 1989; Davis, 1990). The temperature-dependence of insulin binding described in this chapter, which was evident in both mammary and liver tissues from the lactating rat, supports the theory that a temperature-dependent regulator of insulin binding affinity may exist. The characterisation of this putative modulator of insulin binding in these tissues awaits elucidation and may have pivotal implications on the interpretation of binding studies, such as those described herein.

This study has demonstrated that subtle structural differences in insulin receptor glycosylation, and insulin receptor isoform distribution between mammary and liver tissues, does not appear to alter the affinity with which these receptors bind insulin. Furthermore, I have demonstrated that the temperature-dependent affinity of the insulin receptor that has been reported previously in liver and adipose tissue, and thought to be

due to the interaction of a glycoprotein modulator of insulin binding (Helmerhorst, 1993; Kohanski, 1985; Baldini, 1991; Ciaraldi, 1989; Davis, 1990), may also exists in mammary tissue from the lactating rat.

CHAPTER SIX

CONCLUDING COMMENTS

At the commencement of this study no reports had appeared in the literature characterising the structure of the mammary insulin receptor. However, a number of studies performed between 1974 and 1982 described the specific and saturable binding of insulin to mammary epithelial cells and that insulin binding in mammary tissues was significantly increased during lactation (O'Keefe, 1974; Flint, 1982; Inagaki, 1982). In this study, I have identified the mammary insulin receptor using photoaffinity labelling techniques and I have shown that significant structural differences exist between the rat mammary insulin receptor and the more frequently studied liver insulin receptor. The mammary and liver insulin receptors differ in both the extent of receptor glycosylation, and the distribution of the insulin receptor isoforms which are generated from the alternative splicing of insulin receptor mRNA.

The literature suggests that receptor glycosylation and the alternative splicing of the insulin receptor affects the affinity of insulin for its receptor (Olden, 1982; Cherqui, 1982; Caron, 1978; Cherqui, 1981; Podskalny, 1984; Podskalny, 1986; Rouiller, 1986; Mosthaf, 1990; Yamaguchi, 1991). Therefore, as part of this study, I compared the affinity of insulin for the mammary and liver insulin receptors. I concluded from my studies that the binding affinities were not significantly different, suggesting that the structural differences between these two tissues did not alter the insulin binding affinity.

It is apparent from the literature that during lactation there are changes in the insulin sensitivity and responsiveness in mammary and other target tissues (reviewed in chapter 1). Clearly, these changes cannot be accounted for by the structural differences that I have observed in mammary tissues since, as concluded above, these

differences do not appear to affect insulin binding. Recent studies have shown that despite no changes in the binding affinity or the insulin receptor tyrosine kinase activity, the ability of insulin to activate pyruvate dehydrogenase (PDH) is significantly diminished in adipose tissue during lactation (Kilgour, 1988), and in the lactating mammary gland 24 hours after the removal of the litter (Burnol, 1990b). Thus, it would appear that an event distal to the insulin-binding and activation of the insulin receptor tyrosine kinase regulates the insulin responsiveness of these tissues during lactation. This may be due to the presence of a novel, tissue-specific regulator of insulin action in mammary tissue or due to the absence or inactivation of a ubiquitous regulator of insulin action.

The eventual identification of the second messengers of insulin action will greatly improve the current understanding of how insulin promotes nutrient partitioning during lactation. To this end, the activity of the substrates of the insulin receptor tyrosine kinase which have already been identified (see section 1.1.16) should be examined for their role during lactation. Interestingly, insulin-stimulated activation of PDH has been shown to be independent of the insulin receptor tyrosine kinase activity (Gottschalk, 1991). Putative mediators of insulin action could be isolated from the cellular milieu using the intracellular region of the insulin receptor β-subunit cross-linked to an affinity support. This approach would be highly specific for the immediate second messengers of insulin action, however, the recovery of mediators which are present in minute concentrations would be difficult. Nonetheless, this would provide an excellent starting point for the isolation of novel second messengers of insulin action.

This study also focussed on the expression of the alternatively spliced insulin receptor isoforms (IR-A and IR-B) during periods of organogenesis in several rat tissues. I demonstrated that in cardiac muscle and most markedly in liver tissue, the expression of IR-A and IR-B mRNA changes during ontogeny. This observation of tissue-specific expression and developmental regulation of the insulin receptor

isoforms implies that the IR-A and IR-B isoforms have distinct functional roles *in vivo*. To date, studies examining the functional distinctions between the IR-A and IR-B isoforms have been carried out *in vitro* using cell lines transfected with either IR-A or IR-B cDNA. The perceived advantages and disadvantages of using cell lines for examining specific protein function have been discussed in chapter 5. An alternative approach to examining the effect of insulin receptor isoform expression *in vivo* could be the development of a transgenic animal which expresses only one of the insulin receptor isoforms. If this approach was viable it would provide an animal model for the study of the insulin receptor isoforms *in vivo*.

Identification of the hormonal and/or metabolic factors regulating insulin receptor splicing would facilitate the characterisation of the functional distinctions between the IR-A and IR-B isoforms. In HepG2 cells, both dexamethasone and glucose have been shown to affect insulin receptor isoform expression (Kosaki, 1993; Norgren, 1994a) whilst insulin and IGF-1 have been shown to have no effect (Norgren, 1994a). *In vivo*, I have demonstrated that changes in the expression of the IR-A and IR-B isoforms parallel changes in the concentration of circulating glucocorticoids which occur peripartum in the foetus/neonate, and during lactation in maternal tissues (figure 4.11). Cultured foetal rat hepatocytes would provide a particularly sensitive vehicle in which to identify the trigger(s) that initiates the marked change in IR-A/IR-B expression in the liver around parturition. This *in vitro* system could also be used to examine the effects of single and multiple stimuli on insulin receptor isoform expression. Furthermore, it would be necessary to examine the effect of these putative regulators of insulin receptor mRNA splicing in a number of other insulin-sensitive tissues such as cardiac muscle and kidney which also exhibit developmentally regulated expression of IR-A and IR-B. Preliminary data obtained from streptozotocin-diabetic rats presented in chapter 4 was inconclusive due to the limited population size, however I believe this would be a useful experiment to repeat to assess the effect of hypoinsulinaemia and glycemic control on IR-A and IR-B expression. A precedence for the *in vivo* changes in insulin receptor isoforms

expression in the skeletal muscle of a NIDDM patient following insulin treatment has been reported by Norgren *et al.* (1994b). This observation presents the possibility that the discrepancies evident between a number of studies which have determined the distribution of the IR-A and IR-B isoforms in tissues from NIDDM patients (Benecke, 1992; Anderson, 1993; Norgren, 1993; Mosthaf, 1993; Mosthaf, 1991; Moller, 1989) may reflect the extent of glycemic control of these patients.

My evaluation of insulin binding in mammary tissues identified complex, temperature-dependent insulin-binding characteristics similar to those of the liver insulin receptor (Helmerhorst, 1993). This observation is consistent with the model of Helmerhorst and Yip (1993) who propose that the two states of the insulin receptor result from the temperature-dependent binding of the receptor with an regulator of insulin-binding affinity. Several others have also suggested that an insulin receptor-associated protein may regulate the affinity state of the receptor (Kohanski, 1985; Baldini, 1991; Ciaraldi, 1989; Davis, 1990). Although entirely speculative, it is possible that the 12 amino acid peptide encode by exon of 11 of the insulin receptor gene may act as an epitope for the binding of the putative affinity regulator. Computer-aided secondary structure predictions have identified the exon 11 encoded region of the insulin receptor as a good candidate as an antigenic site. Further studies should address the role of the exon 11 encoded region on the conformational state of the insulin receptor α-subunit. A useful tool for these studies would be the production of an anti-exon 11 peptide antibody to ascertain the effect that blocking the peptide has on receptor function. Attempts to isolate and characterise the putative affinity-regulator of the insulin receptor will be an important step in furthering our understanding of its role in insulin action.

The work described in this thesis has significantly contributed to the characterisation of the mammary insulin receptor. This has been achieved through; 1) the comparison of size and extent of glycosylation of the mammary insulin receptor, 2) characterisation of the distribution of the IR-A and IR-B isoforms in mammary

tissue during pregnancy and lactation and, 3) the evaluation of the insulin binding affinity of the mammary insulin receptor relative to other tissues. Furthermore, this study has established that expression of the IR-A and IR-B insulin receptor isoforms occurs not only in a tissue-specific manner, but can also change within a particular tissue during development in response to other stimuli, as evident in foetal tissues during ontogeny and in mammary tissue during lactation. Finally, study has led to the development of highly useful and novel method for the convenient and inexpensive preparation of A14-tyrosyl[^{125}I]insulin which is an essential tool for the study of insulin action in laboratories worldwide.

REFERENCES

Ames, B.N., Dubin, D. T. (1960) The role of polyamines in the neutralisation of bacteriophage deoxyribonucleic acid. *Journal of Biological Chemistry*, 235: 769-775.

Anderson, C., Henry, R., Knudson, P., Olefsky, J., Webster, N. (1993) Relative expression of insulin receptor isoforms does not differ in lean, obese, and noninsulin-dependent diabetes mellitus subjects. *Journal of Clinical Endocrinology and Metabolism*, 76(5): 1380-1382.

Arakaki, R.F., Hedo, J.A., Collier, E., Gorden, P. (1987) Effects of castanospermine and 1-deoxynojirimycin on insulin receptor biogenesis. *Journal of Biological Chemistry*, 262: 11886-11892.

Araki, E., Shimada, F., Uzawa, H., Mori, M., Ebina, Y. (1987) Characterisation of the promoter region of the human insulin receptor gene. *Journal of Biological Chemistry*, 262(33): 16186-16191.

Arthur, P.G., Kent, J.C., Potter, J.M., Hartmann, P.E. (1991) Lactose in blood in nonpregnant, pregnant and lactating women. *Journal of Paediatric Gastroenterology and Nutrition*, 13(3): 254-259.

Ashcroft, S. J. H., Bunce, J., Lowry, M., Hanson, S.E., Hedeskov, C.J. (1978) The effect of sugars on (pro)insulin biosynthesis. *Biochemical Journal*, 174: 517-526.

Avruch, J., Nemenoff, R.A., Blackshear, P.J., Pierce, M.W., Osathanondh, R. (1982) Insulin-stimulated tyrosine phosphorylation of the insulin receptor in detergent extracts of human placental membranes. *Journal of Biological Chemistry*, 257(24): 15162-15166.

Avruch, J., Tornqvist, H.E., Gunsalus, J.R., Yurkow, E.J., Kyriakis, J.M., Price, D.J. (1990) Handbook in experimental pharmacology. Berlin: Springer-Verlag.

Baldini, G., Hohman, R., Charron, M.J., Lodish, H.F. (1991) Insulin and nonhydrolyzable GTP analogues induce translocation of Glut 4 to the plasma membrane in a-toxin-permeabilised rat adipose cells. *Journal of Biological Chemistry*, 266: 4037-4040.

Baron, V., Gautier, N., Kaliman, P., Dolais-Kitabgi, J., Van Obberghen, E. (1991) The carboxyl-terminal domain of the insulin receptor: Its potential in growth-promoting effects. *Biochemistry*, 30: 9365-9370.

Bell, G.I., Kayano, T., Buse, J.B., Burant, C.F., Takeda, J., Lin, D., Fukumoto, H., Senio, S. (1990) Molecular biology of mammalian glucose transporters. *Diabetes Care*, 13(3): 198-208.

Benecke, H., Flier, J.S., Moller, D.E. (1992) Alternatively spliced variants of the insulin receptor protein - expression in normal and diabetic human tissues. *Journal of Clinical Investigation*, 89(6): 2066-2070.

Benedict, M.R., Richman, R.A. (1991) Ontogenesis of insulin processing in foetal rat hepatocytes. *Diabetologia*, 34(12): 868-876.

Bennett, C.A., Franklin, N.L. (1954) Statistical analysis in chemistry and the chemical industry (1st ed.). New York: Wiley.

Bernier, M., Laird, D.M., Lane, M.D. (1987) Insulin activated tyrosine phosphorylation of a 15-kilodalton protein in intact 3T3-L1 adipocytes. *Proceedings of the National Academy of Sciences of the United States of America*, 84: 1844-1848.

Blazquez, E., Rubalcava, B., Montesano, R. (1976) Development of insulin and glucagon binding and the adenylate cyclase response in liver membranes of the perinatal, postnatal and adult rat: evidence of glucagon "resistance". *Endocrinology*, 94: 1014-1023.

Blundell, T., Dodson, G., Hodgkin, D., Mercola, D. (1972) Insulin: The structure in the crystal and its reflection in chemistry and biology. *Advances in Protein Chemistry*, 26: 279-403.

Boeynaems, J.M. (1976) Comparison of the effects of chemical and isotopic dilution on the dissociation of bound labelled ligands. *Analytical Biochemistry*, 70: 366-376.

Bolander, F.F., Nicholas, K.R., Van Wyk, J.J., Topper, Y.J. (1981) Insulin is essential for accumulation of casein mRNA in mouse mammary epithelial cells. *Proceedings of the National Academy of Sciences of the United States of America*, 78: 5682-5684.

Bolander, F.F., Topper, Y.T. (1981) Loss of differentiation potential in the mammary gland in ovariectomized mice: identification of a biochemical lesion. *Endocrinology*, 108: 1649-1655.

Bollag, G.E., Roth, R.A., Beaudoin, J., Mochly-Rosen, D., Koshland, D.E. (1986) Protein kinase C directly phosphorylates the insulin receptor in vitro and reduces its protein-tyrosine kinase activity. *Proceedings of the National Academy of Sciences of the United States of America*, 83: 5822-5824.

Bonischnetzler, M., Rubin, J.B., Pilch, P.F. (1986) Structural requirements for the transmembrane activation of the insulin receptor kinase. *Journal of Biological Chemistry*, 261: 15281-15287.

Boulton, T.G., Nye, S.H., Robbins, D.J., Ip, N.Y., Radziejewska, E., Morgenbesser, S.D., Depinho, R.A., Panayotatos, N., Cobb, M.H., Yancopoulos, G.D. (1991) ERKs - A family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell*, 65(4): 663-675.

Brange, J., Langkjaer, L. (1992) Chemical stability of insulin; 3. Influence of excipient, formulation, and pH. *Acta Pharmacology Nordica*, 4(3): 149-158.

Breiner, M., Weiland, M., Becker, W., Mullerwieland, D., Streicher, R., Fabry, M., Joost, H. G. (1993) Heterogeneity of insulin receptors in rat tissues as detected with the partial agonist B29,B29'-suberoyl-insulin. *Molecular Pharmacology*, 44(2): 271-276.

Buchanan, T.A., Metzger, B.E., Freiknel, N., Bergman, R.N. (1990) Insulin sensitivity and B-cell responsiveness to glucose during later pregnancy in lean and moderately obese women with normal glucose tolerance or gestational diabetes. *American Journal of Obstetrics and Gynaecology*, 162: 1008-1014.

Buchanan, T.A. (1991) Glucose metabolism during pregnancy: Normal physiology and implications for diabetes mellitus. *Israel Journal of Medical Sciences*, 27: 432-441.

Burant, C.F., Treutelaar, M.K., Block, N.E., Buse, M.G. (1986a) Structural differences between liver- and muscle-derived insulin receptors in rats. *Journal of Biological Chemistry*, 261(31): 14361-14364.

Burant, C.F., Treutelaar, M.K., Buse, M.G. (1986b) Diabetes induced functional and structural changes in insulin receptors from rat skeletal muscle. *Journal of Clinical Investigation*, 77: 260-270.

Burnol, A.F., Leturque, A., Ferre, P., Kande, J., Girard, J.R. (1986) Increased insulin sensitivity and responsiveness during lactation in rats. *American Journal of Physiology*, 251(5 Pt. 1): E537-E541.

Burnol, A.F., Ferre, P., Leturque, A., Girard, J.R. (1987) Effect of insulin on in vivo glucose utilisation in individual tissues of anesthetized lactating rats. *American Journal of Physiology*, 252(2 Pt. 1): E183-E188.

Burnol, A.F., Ebner, S., Kande, J., Girard, J.R. (1990a) Insulin resistance of glucose metabolism in isolated brown adipocytes of lactating rats. Evidence for a post-receptor defect in insulin action. *Biochemical Journal*, 265: 511-517.

Burnol, A.F., Loizeau, M., Girard, J. (1990b) Insulin receptor activity and insulin sensitivity in mammary gland of lactating rats. *American Journal of Physiology*, 259: E828-E834.

Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Kapellar, R., Soltoff, S. (1991) Oncogenes and signal transduction. *Cell*, 64: 281-302.

Caro, J.F., Dohm, L.G., Pories, W.J., Sinha, M.K. (1989) Cellular alterations in liver, skeletal muscle and adipose tissue responsible for insulin resistance in obesity and type II diabetes. *Diabetes and Metabolism Reviews*, 5: 655-689.

Caron, M., Picard, J., Kern, P. (1978) Involvement of glycoconjugates in insulin-receptor interactions. *Biochimica et Biophysica Acta*, 512: 29-40.

Carrascosa, J., Vogt, B., Ullrich, A., Haring, H-U. (1991) Activation of phosphatidylinositol-3-kinase by insulin is mediated by both A and B human insulin receptor types. *Biochemical and Biophysical Research Communications*, 174(1): 123-127.

Chatwin, A.L., Linzell, J.L., Setchell, B.P. (1969) Cardiovascular changes during lactation in the rat. *Journal of Endocrinology*, 44: 247-254.

Cherqui, G., Caron, M., Capeau, J., Picard, J. (1981) Further characterisation of the insulin receptor glycosidic moiety in rat adipocytes. *Molecular and Cellular Endocrinology*, 23: 297-310.

Cherqui, G., Caron, M., Capeau, J., Picard, J. (1982) Carbohydrate determinants involved in both the binding and action of insulin in rat adipocytes. *Molecular and Cellular Endocrinology*, 28: 627-643.

Chomczynski, P., Qasba, P., Topper, Y.J. (1984) Essential role of insulin in transcription of the rat 25,000 molecular weight casein gene. *Science*, 226: 1326-1328.

Chomzynski, P., Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry*, 162: 156-159.

Chou, C.K., Dull, T.J., Russell, D.S., Gherzi, R., Lebwohld, D., Ullrich, A., Rosen, O.M. (1987) Human insulin receptors mutated at the ATP-binding site lack protein tyrosine kinase activity and fail to mediate postreceptor effects of insulin. *Journal of Biological Chemistry*, 262(4): 1842-1847.

Chou, P.Y., Fasman, G.D. (1978) Empirical predictions of protein conformations. *Annual Reviews in Biochemistry*, 47: 251-276.

Christiansen, K., Tranum-Jensen, J., Carlsen, J., Viten, J. (1991) A model for the quaternary structure of the human placental insulin receptor deduced from electron microscopy. *Proceedings of the National Academy of Sciences of the United States of America*, 88: 249-252.

Chu, Y.-C., Zong, L., Burke, G.T., Katsoyannis, P.G. (1992) The A14 position of insulin tolerates considerable structural alterations with modest effects on the biological behaviour of the hormone. *Journal of Protein Chemistry*, 11(5): 571-577.

Ciaraldi, T.P., Maisel, A. (1989) Role of guanine nucleotide regulatory proteins in insulin stimulation of glucose transport in rat adipocytes. Influence of bacterial toxins. *Biochemical Journal*, 264: 389-396.

Cohen, P., Parker, P.J., Woodgett, J.R. (1985) Molecular basis of insulin action. New York: Plenum Press.

Collet, C., Joseph, R., Nicholas, K. (1992) Molecular characterisation and in-vitro hormonal requirements for expression of two casein genes from a marsupial. *Journal of Molecular Endocrinology*, 8(1): 13-20.

Collier, E., Gorden, P. (1991) O-linked oligosaccharides on insulin receptor. *Diabetes*, 40: 197-203.

Corin, R. A., Donner, D.B. (1982) Insulin receptors convert to a higher affinity state subsequent to hormone binding. *Journal of Biological Chemistry*, 257(1): 104-110.

Cowie, A.T., Forsyth, I.A., Hart, I.C. (1980) Hormonal control of lactation. Berlin: Springer-Verlag.

Cuatrecasas, P. (1972) Affinity chromatography and purification of the insulin receptor of liver cell membranes. *Proceedings of the National Academy of Sciences of the United States of America*, 69: 1277-1281.

Czech, M., Massague, J (1982) Subunit structure and dynamics of the insulin receptor. *Federation Proceedings*, 41: 2719-2723.

Danho, W., Sasaki, A., Bullesbach, E., Gattner, H-G, Wollmer, A (1980) (A14-phenylalanine)insulin: a new synthetic analogue. *Hoppe-Seyler's Z Physiological Chemistry*, 361: 747-754.

Davidson, M.B., Kaplan, S.A. (1976) Increased in insulin binding by hepatic plasma membranes from diabetic rats: normalisation by insulin therapy. *Journal of Clinical Investigation*, 59: 22-30.

Davis, H W., McDonald, J.M. (1990) Insulin receptor function is inhibited by guanosine 5'-[gamma-thio] triphosphate (GTP[S]). *Biochemical Journal*, 270: 401-407.

Dawson, P.A., Hofmann, S.L., van der Westhuyzen, D.R., Sudhof, T.C., Brown, M.S., Goldstein, J.L. (1988) Sterol-dependent repression of low density lipoprotein receptor promoter mediated by 16-base pair sequence adjacent to binding site for transcription factor Sp1. *Journal of Biological Chemistry*, 263: 3372-3379.

Dayhoff, M.D. (1969) Atlas of protein sequence and structure. Silver Springs, Maryland: National Biochemical Research Foundation.

de Meyts, P., Roth, J., Neville, D.M., Gavin, J.R. (1973) Insulin interactions with its receptors: experimental evidence for negative cooperativity. *Biochemical Biophysical Research Communications*, 55(1): 154-161.

de Meyts, P., Bianco, A.R., Roth., J. (1976) Site-site interactions among insulin receptors. *Journal of Biological Chemistry*, 251(7): 1877-1888.

Deleo, D.T., Helmerhorst, E. (1992) Purification of A14-tyrosyl^{125}-I iodoinsulin using C18 reverse -phase cartridges. *Analytical Biochemistry*, 206: 207-210.

Denton, R.M., Brownsey, R.W., Belsham, G.J. (1981) A partial view of the mechanism of insulin action. *Diabetologia*, 21: 347-362.

De Pablo, F., Scott, L.A., Roth, J. (1990) Insulin and insulin-like growth factor-1 in early development: peptides, receptors and biological events. *Endocrinology Reviews*, 11: 558-577.

Deutsch, P.J., Wan, C.F., Rosen, O.M., Rubin, C.S. (1983) Latent insulin receptors and possible receptor precursors in 3T3-L1 adipocytes. *Proceedings of the National Academy of Sciences of the United States of America*, 80: 133-136.

Devaskar, S.U., Ganguli, S., Devaskar, U.P., Sperling, M.A. (1982) Glucocorticoids and hypothyroidism modulate foetal development of foetal lung insulin receptors. *American Journal of Physiology*, 242: E384-E391.

Diamond, M.I., Miner, J.N., Yoshinaga, S.K., Yamamoto, K.R. (1990) Transcription factor interactions: Selectors of positive or negative regulation from a single DNA element. *Science*, 24: 1266-1272.

DiFiore, M.S.H. (1981) Atlas of human histology (5th ed.). Philadelphia: Lea and Febiger.

Dijane, J., Durand, P., Kelly, P.A. (1977) Evolution of prolactin receptors in rabbit mammary gland during pregnancy and lactation. *Endocrinology*, 100: 1348-1356.

Donner, D., Casadei, J., Hartstein, L., Martin, D., Sonenberg, M. (1980) Characterisation of the slowly dissociable human growth hormone binding component of isolated rat hepatocytes. *Biochemistry*, 19: 3293-3300.

Dynan, W.S., Tjian, R. (1983) Isolation of transcription factors that discriminate between different promoters recognised by RNA polymerase II. *Cell*, 32: 669-680.

Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J-H., Masiarz, F., Kan, Y.W., Goldfine, I.D., Roth, R.A., Rutter, W.J. (1985) The human receptor cDNA: The structural basis of hormone-activated transmembrane signalling. *Cell*, 40: 747-758.

Ebina, Y., Araki, E., Taira, M., Shimada, F., Mori, M. Craik, C.S., Siddle, K., Pierce, S.B., Roth, R.A., Rutter, W.J. (1987) Replacement of lysine residue 1030 in the putative ATP-binding region of the insulin receptor abolishes insulin- and antibody-stimulated glucose uptake and receptor kinase activity. *Proceedings of the National Academy of Sciences of the United States of America*, 84: 704-708.

Ellis, L., Morgan, D.O., Clauser, E., Edery, M., Jong, S-M., Wang, L-H., Roth, R.A., Rutter, W.J. (1986) Mechanisms of receptor-mediated transmembrane communication. *Cold Spring Harbor Symposia on Quantitative Biology*, LI: 773-781.

Espinal, J. (1989) Understanding insulin action: principles and molecular mechanisms. Chichester: Ellis Horwood Limited.

Fernig, D.G., Mayer, R.J. (1989) Insulin processing in primary endosomes is not responsible for insulin resistance observed in parametrial adipocytes from lactating rats. *Biochimica et Biophysica Acta*, 1010: 237-245.

Fletcher, J.A. (1987) Immunogenetics of insulin-dependent diabetes. Lancaster: MTP Press Limited.

Flint, D., Sinnett-Smith, P., Clegg, R., Vernon, R. (1979) Role of insulin receptors in the changing metabolism of adipose tissue during pregnancy and lactation in the rat. *Biochemical Journal*, 182: 421-427.

Flint, D. (1980) Changes in the number of insulin receptors of isolated rat hepatocytes during pregnancy and lactation. *Biochimica et Biophysica Acta*, 628: 322-327.

Flint, D. (1982) Insulin binding to rat mammary gland at various stages of cell isolation and purification. *Molecular and Cellular Endocrinology*, 26: 281-294.

Flores-Riveros, J.R., Sibley, E., Kastelic, T., Lane, M.D. (1989) Substrate phosphorylation catalysed by the insulin receptor tyrosine kinase. Kinetic correlation to autophosphorylation of specific sites in the beta-subunit. *Journal of Biological Chemistry*, 264: 21557-21572.

Florke, R., Klein, H., Reinauer, H. (1990) Structural requirements for the signal transduction of the insulin receptor. *European Journal of Biochemistry*, 191: 473-482.

Frank, B.H., Beckage, M.J., Willey, K.A. (1983) High-performance liquid chromatographic preparation of single-site carrier-free pancreatic polypeptide hormone radiotracers. *Journal of Chromatography*, 266: 239-248.

Freinkel, N., Metzger, B.E., Nitzan, M. (1974). Facilitated anabolism in late pregnancy: Some novel maternal compensations for accelerated starvation. In W. J. Malaisse Pirart, J. (Ed.), VII Congress of the International Diabetes Federation, (pp. 474-488). Amsterdam: Exerpta Medica.

Freinkel, N. (1980) The Banting Lecture 1980. Of pregnancy and progeny. *Diabetes*, 29: 1023-1035.

Fujita-Yamaguchi, Y., Choi, S., Sakamoto, Y., Itakura, K. (1983) Purification of insulin receptor with full binding activity. *Journal of Biological Chemistry*, 258(8): 5045-5049.

Gammeltoft, S., Gliemann, J. (1973) Binding and degradation of ^{125}I-labelled insulin by isolated rat fat cells. *Biochimica et Biophysica Acta*, 320: 16-32.

Gammeltoft, S. (1984) Insulin receptors: Binding kinetics and structure-function relationship of insulin. *American Physiological Society*, : 1321-1364.

Garnier, J., Osguthorpe, D.J., Robson, B (1978) Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *Journal of Molecular Biology*, 120: 97-120.

Gawler, D., Milligan, G., Speigel, A.M., Unson, C.G., Houslay, M.D. (1987) Abolition of the expression of inhibitory guanine nucleotide regulatory protein G_i activity in diabetes. *Nature*, 327: 229-232.

Giddings, S.J., Carnaghi, L. (1989) Insulin II gene expression by extraplacental membranes: a non-pancreatic source for foetal insulin. *Journal of Biological Chemistry*, 264: 9462-9469.

Giddings, S.J., Carnaghi, L. (1990) Selective expression and developmental regulation of the ancestral rat insulin II gene in foetal liver. *Molecular Endocrinology*, 4: 1363-1369.

Giddings, S.J., Carnaghi, L.R. (1992) Insulin receptor gene expression during development: Developmental regulation of insulin receptor mRNA abundance in embryonic rat liver and yolk sac, developmental regulation of insulin receptor gene splicing, and comparison to abundance of insulin-like growth factor 1 receptor mRNA. *Molecular Endocrinology*, 6: 1665-1672.

Girard, J.R., Kervran, A., Soufflet, E. (1973) Factors affecting the secretion of insulin and glucagon in the rat foetus. *Diabetes*, 23: 310-317.

Gliemann, J., Sonne, O., Linde, S., Hansen, B. (1979) Biological potency and binding affinity of monoiodoinsulin with iodine in tyrosine A14 or tyrosine A19. *Biochemical and Biophysical Research Communications*, 87: 1183-1190.

Gold, G. (1989) Insulin Secretion. New York: Alan R. Liss Inc.

Goldstein, B., Dudley, A. (1990) The rat insulin receptor: Primary structure and conservation of tissue-specific alternative messenger RNA splicing. *Molecular Endocrinology*, 4(2): 235-244.

Goldstein, B.J. (1992) Protein-tyrosine phosphatases and the regulation of insulin action. *Journal of Cellular Biochemistry*, 48(1): 33-42.

Goren, H.J., White, M.F., Kahn, C.R. (1987) Separate domains of the insulin receptor contain sites of autophosphorylation and tyrosine kinase activity. *Biochemistry*, 26: 2374-2382.

Gottschalk, W.K. (1991) The pathway mediating insulin's effects on pyruvate dehydrogenase bypasses the insulin receptor tyrosine kinase. *Journal of Biological Chemistry*, 266: 8814-8819.

Grigorescu, F., White, M., Kahn, C.R. (1983) Insulin binding and insulin-dependent phosphorylation of the insulin receptor solubilised from human erythrocytes. *Journal of Biological Chemistry*, 258(22): 13708-13716.

Grunberger, G., Zick, Y., Gorden, P. (1984) Defect on phosphorylation of insulin receptors in cells from an insulin-resistant patient with normal insulin binding. *Science*, 223: 932-934.

Gruppuso, P.A. (1989) Effects of foetal hyperinsulinaemia on foetal hepatic insulin binding in the rat. *Biochimica et Biophysica Acta*, 1010: 270-273.

Gruppuso, P.A., Boylan, J.M., Carter, P.A., Madden, J.A., Raven, T. (1992) Hepatic insulin and EGF receptor phosphorylation and dephosphorylation in foetal rats. *American Journal of Physiology*, 262(1): E7-E13.

Gruppuso, P.A., Boylan, J.M., Bienieki, T.C., Curren, T.R. (1994) Evidence for a direct hemotrophic role for insulin in foetal rat: implications for the impaired hepatic growth seen in foetal growth retardation. *Endocrinology*, 134(2): 769-775.

Gustafson, T., Rutter, W. (1990) The cysteine-rich domains of the insulin and insulin-like growth factor 1 receptors are primary determinants of hormone binding specificity. *Journal of Biological Chemistry*, 265(30): 18663-18667.

Haft, D. E. (1968) Studies of the metabolism of isolated livers of normal and alloxan-diabetic rats perfused with insulin. *Diabetes*, 17: 244-250.

Hamlin, J.R., Arquilla, E.R. (1974) Monoiodoinsulin. Preparation, purification and characterisation of a biologically active derivative substituted predominantly on tyrosine A14. *Journal of Biological Chemistry*, 249: 21-32.

Hanks, S.K., Quinn, A.M., Hunter, T. (1988) The protein kinase family: Conserved features and deduced phylogeny of the catalytic domains. *Science*, 241: 42-52.

Hansen, T., Bjorbaek, C., Vestergaard, H., Gronskov, K., Bak, J. F., Pedersen, O. (1993) Expression of insulin receptor spliced variants and their functional correlates in muscle from patients with non-insulin-dependent diabetes mellitus. *Journal of Clinical Endocrinology and Metabolism*, 77(6): 1500-1505.

Haring, H-U. (1991) The insulin receptor - Signalling mechanism and contribution to the pathogenesis of insulin resistance. *Diabetologia*, 34(12): 848-861.

Hauguel de-Mouzon, S., Peraldi, P., Alengrin, F., Van Obberghen, E. (1993) Alteration of phosphotyrosine phosphatase activity in tissues from diabetic and pregnant rats. *Endocrinology*, 132(1): 67-74.

Hayes, G.R., Livingston, J.N., Lockwood, D.H. (1991) Mapping of carbohydrate sites on the human insulin receptor. *Biochemical and Biophysical Research Communications*, 174: 735-741.

Hedo, J.A., Kahn, C.R., Hayoshi, M., Yamada, K.M., Kasuga, M. (1983) Biosynthesis and glycosylation of the insulin receptor. Evidence for a single polypeptide precursor of the two major subunits. *Journal of Biological Chemistry*, 258: 10020-10026.

Heidenreich, K.A., Zahniser, N.R., Berhanu, P., Brandenburg, D., Olefsky, J.M. (1983) Structural differences between insulin receptors in the brain and peripheral target tissues. *Journal of Biological Chemistry*, 258: 8527-8530.

Heidenreich, K.A., Brandenburg, D. (1986) Oligosaccharide heterogeneity of insulin receptors. Comparison of N-linked glycosylation of insulin receptors in adipocytes and brain. *Endocrinology*, 118: 1835-1842.

Helmerhorst, E., Ng, D.S., Moule,M. and Yip, C.C. (1986) High molecular weight forms of the insulin receptor. *Biochemistry*, 25(8): 2060-2065.

Helmerhorst, E. (1987) The insulin-receptor interaction: Is the kinetic approach for inferring negative-cooperative site-site interactions valid? *Biochemical and Biophysical Research Communications*, 147(1): 399-407.

Helmerhorst, E., Yip, C. (1993) Insulin binding to rat liver membranes predicts a homogeneous class of binding sites in different affinity states that may be related to a regulator of insulin binding. *Biochemistry*, 32(9): 2356-2362.

Herzberg, V., Boughter, J.M., Carlisle, S., Ahmad, F., Hill, D. (1980) [125I]iodoinsulin receptor binding to cord blood erythrocytes of varying gestational age and comparison with adult values. *Paediatric Research*, 14: 4.

Herzberg, V.L., Grigorescu, F., Edge, A.S.B., Spiro, R.G., Kahn, C.R. (1985) Characterisation of insulin receptor carbohydrate by comparison of chemical and enzymatic deglycosylation. *Biochemical and Biophysical Research Communications*, 129: 789-796.

Heyworth, C.M., Grey, A.M., Wilson, S.R., Lianski, E., Houslay, M.D. (1986) The action of islet activating protein (pertussis toxin) on insulin's ability to inhibit adenylate cyclase and activate cyclic AMP phosphodiesterases in hepatocytes. *Biochemical Journal*, 235: 145-149.

Hoet, J.J. (1991) A hope for people with diabetes. *World Health*, May-June: 4-5.

Horikoshi, M., Hai, T., Lin, Y.S., Green, M.R., Roeder, R.G. (1988) Transcription factor ATF interacts with the TATA factor to facilitate establishment of a preinitiation complex. *Cell*, 54: 1033-1042.

Howell, S.L. (1972) Role of ATP in the intracellular translocation of proinsulin and insulin. *Nature New Biology*, 235: 85-87.

Howell, S.L., Tyhurst, M., Green, I.C. (1977) Direct effects of progesterone on the islets of Langerhans *in vivo* and in tissue culture. *Diabetologia*, 13: 579-583.

Howell, S.L. (1988) Regulation and mechanism of insulin secretion. London: Gower Medical.

Howell, S.L., Bird, G. St. J. (1989) Biosynthesis and secretion of Insulin. *British Medical Bulletin*, 45(1): 19-36.

Hresko, R.C., Bernier, M., Hoffman, R.D., Flores-Riveros, J.R., Liao, K., Laird, D., Lane, M.D. (1988) Identification of phosphorylated 422(aP2) protein as pp15, the 15 kilodalton target of the insulin receptor tyrosine kinase in 3T3-L1 adipocytes. *Proceedings of the National Academy of Sciences of the United States of America*, 85: 8835-8839.

Hunter, T. (1984) The proteins of oncogenes. *Scientific American*, 251: 70-79.

Inagaki, Y., Kohmoto, K. (1982) Changes in Scatchard plots for insulin binding to mammary epithelial cells from cycling, pregnant and lactating mice. *Endocrinology*, 110(1): 176-182.

Ishii, S., Xu, Y-H., Stratton, R.H., Roe, B.A., Merlino, G.T., Pastan, I. (1985) Characterisation and sequence of the promotor region of the human epidermal growth factor receptor gene. *Proceedings of the National Academy of Sciences of the United States of America*, 82: 4920-4924.

Itoh, M., Okamoto, H. (1980) Translational control of proinsulin synthesis by glucose. *Nature*, 283: 100-102.

Jacobs, S., Cuatrecasas, P. (1981) Insulin receptor: Structure and function. *Endocrinology Reviews*, 2: 251-263.

Jameson, B.A., Wolf, H. (1988) The antigenic index: A novel algorithm, for predicting antigenic determinants. *Computer Applications in the Biosciences*, 4: 181-186.

Jirasek, J.E. (1980) Human foetal endocrines. The Hague: Martinus Nijhoff Publishers.

Jo, H., Cha, B.Y., Davis, H.W., Mcdonald, J.M. (1992) Identification, partial purification, and characterisation of 2 guanosine triphosphate-binding proteins associated with insulin receptors. *Endocrinology*, 131(6): 2855-2862.

Kadowaki, T., Kasuga, M., Akanuma, Y., Ezaki, O., Takaku, F. (1984) Decreased autophosphorylation of the insulin receptor-kinase in streptozotocin-diabetic rats. *Journal of Biological Chemistry*, 259: 14208-14216.

Kadowaki, T., Kasuga, M., Tobe, K., Takaku, F., Nishida, E., Sakai, H., Korasu, S., Yahara, I., Toyoshima, K., Yamamoto, T. (1987) A Mr = 190, 000 glycoprotein phosphorylated on tyrosine residues in epidermal growth factor stimulated KB cell is the product of the c-erbB-2 gene. *Biochemical and Biophysical Research Communications*, 144: 699-704.

Kahn, C.R., Hedo, J.A., Kasuga, M. (1984). Antibodies to the insulin receptor: Studies of receptor structure and function. In G. Litwack (Eds.), Biochemical actions of hormones (pp. 127-162). New York: Academic Press Inc.

Kahn, C.R., White, M.F. (1988) The insulin receptor and the molecular mechanism of insulin action. *Journal of Clinical Investigation*, 82(Oct 88): 1151-1156.

Kakita, K., Giddings, S., Rotwein, P., Permutt, M.A. (1983) Insulin gene expression in the developing rat pancreas. *Diabetes*, 32: 691-696.

Karnieli, E., Hissin, P.J., Simpson, I.A., Salans, L.B., Cishman, S.W. (1981) A possible mechanism of insulin resistance in the rat adipose cell in streptozotocin-induced diabetes mellitus. Depletion of intracellular glucose transport systems. *Journal of Clinical Investigation*, 68: 811-814.

Kasuga, M., Akanuma, Y., Iwamoto, Y., Kosaka, K. (1978) Insulin binding and glucose metabolism in adipocytes of streptozotocin-diabetic rats. *American Journal of Physiology*, 235: E175-E182.

Kasuga, M., Karlsson, F.A., Kahn, C.R. (1982a) Insulin stimulates the phosphorylation of the 95,000-Dalton subunit of its own receptor. *Science*, 215(8): 185-186.

Kasuga, M., Zick, Y., Blithe, D.L., Crettaz, M., Kahn, C.R. (1982b) Insulin stimulates tyrosine phosphorylation of the insulin receptor in a cell-free system. *Nature*, 298: 667-669.

Kasuga, M., Tetsuro, I., Kazuyuki, T., Shiba, T., Monomurra, K., Tashiro-Hashimoto, Y., Kadowaki, T. (1990) Substrates for insulin-receptor kinase. *Diabetes Care*, 13(3): 317-326.

Kato, H., Faria, T. N., Stannard, B., Levy-Toledano, R., Taylor, S.I., Roberts, C.T., LeRoith, D. (1993) Paradoxical biological effects of over expressed insulin-like growth factor-1 receptors in Chinese hamster ovary cells. *Journal of Cellular Physiology*, 156: 145-152.

Kellerer, M., Sesti, G., Seffer, E., Ullrich, A., Haring, H-U (1992a) Insulin receptor isoform expression in skeletal muscle membranes of NIDDM patients. *Diabetologia*, 35(Supplement 1): A75 Abstract.

Kellerer, M., Lammers, R., Ermel, B., Tippmer, S., Vogt, B., Obermaier-Kusser, B., Ullrich, A., Haring, H-U. (1992b) Distinct a-subunit structures of human insulin receptor A and B variants determine differences in tyrosine kinase activities. *Biochemistry*, 31: 4588-4596.

Kellerer, M., Setsi, G., Seffer, E., Obermaier-Kusser, B., Pongratz, DE., Mosthaf, L., Haring, H-U. (1993) Altered pattern of insulin receptor isotypes in skeletal muscle membranes of type 2 (non-insulin-dependent) diabetic subjects. *Diabetologia*, 36(7): 628-632.

Kilgour, E., Vernon, R.G. (1988) Defect in signal transduction at the level of the plasma membrane accounts for inability of insulin to activate pyruvate dehydrogenase in white adipocytes of lactating rats. *Biochemical Journal*, 252: 667-672.

Kim, S.H., Moores, J.C., David, D., Respess, J.G., Jolly, D.J., Friedmann, T. (1986) Organisation of the human HPGRT gene. *Nucleic Acids Research*, 14: 3103-3118.

King, M.J., Sharma, R.P., Sale, G.J. (1991) Site-specific dephosphorylation and deactivation of the human insulin receptor tyrosine kinase by particulate and soluble phosphotyrosyl protein phosphatases. *Biochemical Journal*, 275: 413-418.

Klein-Hitpass, L., Tsai, S.Y., Weigel, N.L., Allan, G.F., Riley, D., Rodriguez, R., Schrader, W.T., Tsai, M.J., O'Malley, B.W. (1990) The progesterone receptor stimulates cell-free transcription by enhancing the formation of a stable preinitiation complex. *Cell*, 60: 247-257.

Kobayashi, M., Olefsky, J.M. (1979) Effects of streptozotocin-induced diabetes on insulin binding, glucose transport, and intracellular glucose metabolism in isolated rat adipocytes. *Diabetes*, 28: 87-95.

Koch, C.A., Anderson, D., Moran, M.F., Ellis, C., Pawson, T. (1991) SH2 and SH3 domains: Elements that control interactions of cytoplasmic signalling proteins. *Science*, 252: 668-674.

Kohanski, R.A., Lane, M.D. (1985) Homogeneous functional insulin receptors from 3T3-L1 adipocytes. Purification using N alpha B1-(biotinyl-epsilon-aminocaproyl)insulin and avidin-sepharose. *Journal of Biological Chemistry*, 260: 5014-5025.

Kosaki, A., Webster, N. (1993) Effect of dexamethasone on the alternative splicing of the insulin receptor mRNA and insulin action in HepG2 hepatoma cells. *Journal of Biological Chemistry*, 268(29): 21990-21996.

Kyte, J., Doolittle, R.F. (1982) A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology*, 157: 105-132.

Laakso, M., Edelman, S.V., Brechtel, G., Baron, A.D. (1990) Decreased effect of insulin to stimulate skeletal muscle blood flow in obese man. A novel mechanism for insulin resistance. *Journal of Clinical Investigation*, 85: 1844-1852.

Landreth, G.E., Shooter, E.M. (1980) Nerve growth factor receptors on PC12 cells: Ligand-induced conversion from low- to high-affinity states. *Proceedings of the National Academy of Sciences of the United States of America*, 77: 4751-4755.

Lee, J.-K., Tam, J.W., Tsai, M-J., Tsai, S.Y. (1992) Identification of cis- and trans-acting factors regulating the expression of the human insulin receptor gene. *Journal of Biological Chemistry*, 267(7): 4638-4645.

Levy, J.R., Hug, V. (1993) Nuclear protein binding analysis of a GC-Rich insulin-receptor promoter regulatory region. *Diabetes*, 42(1): 66-73.

Liao, K., Hoffman, R.D., Lane, M.D. (1991) Phosphotyrosyl turnover in insulin signalling. Characterisation of two membrane-bound pp15 protein tyrosine phosphates from 3T3-L1 adipocytes. *Journal of Biological Chemistry*, 266: 6544-6553.

Linde, S., Hansen, B. (1980) Monoiodoinsulin specifically substituted in Tyr A14 or Tyr A19. *International Journal of Peptide Research*, 15: 495-502.

Linde, S., Hansen, B., Sonne, O., Holst, J., Gleimann, J. (1981) Tyrosine A14[125I] monoiodoinsulin. Preparation, biologic properties and long-term stability. *Diabetes*, 30: 1-8.

Lioubin, M.N., Meier, M.D., Ginsberg, B.H. (1984) A rapid, high yield method of producing mono-[125I]A14 iodoinsulin. *Preparative Biochemistry*, 14(4): 303-311.

Lowry, O.H., Rosebrough, M.J., Farr, A.L., Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, 193: 265-275.

Ludwig, S., Muller-Wieland, D., Goldstein, B.J. and Kahn, C.R. (1988) The insulin receptor gene and its expression in insulin resistant mice. *Endocrinology*, 123: 594-600.

Luttrell, L., Kilgour, E., Larner, J., Romero, G. (1990) A pertussis toxin-sensitive G-protein mediates some aspects of insulin action in BC_3H-1 murine monocytes. *Journal of Biological Chemistry*, 265: 16873-16879.

Maegawa, H., McClain, D.A., Freidenberg, G., Olefsky, J.M., Napier, M., Lipari, T., Dull, T.J., Lee, J., Ullrich, A. (1988) Properties of a human insulin receptor with a COOH-terminal truncation II. Truncated receptors have normal kinase activity but are defective in signalling metabolic effects. *Journal of Biological Chemistry*, 263: 8912-8917.

Maller, J.L., Pike, L.J., Freidenberg, G.R., Cordera, R., Stith, B.J., Olefsky, J.M., Krebs, E.G. (1986) Increased phosphorylation of ribosomal protein S6 following micro injection of insulin receptor-kinase into xenopus oocytes. *Nature*, 320: 459-461.

Mamula, P.W., Wong, K.Y., Maddux, B.A., McDonald, A.R., Golfine, I.D. (1988) Sequence analysis of promoter region of human insulin receptor gene. *Diabetes*, 37(9): 1241-1246.

Margolis, R.N., Schell, M.J., Taylor, S.I., Hubbard, A.L. (1990) Hepatocyte plasma membrane ecto-ATPase (pp120/HA4) is a substrate for the tyrosine kinase activity of the insulin receptor. *Biochemical and Biophysical Research Communications*, 166: 562-566.

Martin, A., Baldwin, R. (1971) Effects of alloxan on lactational performance and mammary tissue metabolism in the rat. *Endocrinology*, 88: 863-867.

Martin, C.E., Cake, M.H., Hartmann, P.E., Cook, I.F. (1977) Relationship between foetal corticosteroids, maternal progesterone and parturition in the rat. *Acta Endocrinologia*, 84: 167-176.

McClain, D.A., Maegawa, H., Lee, J., Dull, T.J., Ullrich, A., Olefsky, J.M. (1987) A mutant insulin receptor with defective tyrosine kinase displays no biologic activity and does not undergo endocytosis. *Journal of Biological Chemistry*, 262: 14663-14671.

McClain, D.A. (1991) Different ligand affinities of the two human insulin receptor splice variants are reflected in parallel changes in insulin sensitivity for insulin action. *Molecular Endocrinology*, 5: 734-739.

McElduff, A., Comi, R., Grunberger, G. (1985) Structural difference of the insulin receptors from circulating monocytes and erythrocytes. *Biochemical and Biophysical Research Communications*, 133(3): 1175-1180.

McElduff, A., Poronnik, P., Baxter, R.C. (1987) The insulin-like growth factor II (IGF-II) receptor from rat brain is of lower apparent molecular weight than the IGF-II receptor from rat liver. *Endocrinology*, 121: 1306-1311.

McPherson, G.A. (1985) Analysis of radioligand binding experiments. *Journal of Pharmacological Methods*, 14: 213-228.

Melton, D.W., Konecki, D.S., Brennand, J., Caskey, C.T. (1984) Structure, expression and mutation of the hypoxanthine phosphoribosyl transferase gene. *Proceedings of the National Academy of Sciences of the United States of America*, 81: 2147-2151.

Moller, D.E., Yokota, A., Caro, J.F., Flier, J.S. (1989) Tissue-specific expression of two alternatively spliced insulin receptor mRNAs in man. *Molecular Endocrinology*, 3: 1263-1269.

Montague, W., Howell, S.L. (1974) Effects of insulin secretagogues on protein kinase activity in rat islets of Langerhans. *Biochemical Journal*, 134: 321-327.

Morgan, D.O., Roth, R.A. (1987) Acute insulin action requires insulin receptor kinase activity: Introduction of an inhibitory monoclonal antibody into mammalian cells blocks the rapid effects of insulin. *Proceedings of the National Academy of Sciences of the United States of America*, 84: 41-45.

Mortensen, E.R., Drachman, J., Guidotti, G. (1992) Guanosine nucleotides regulate hormone binding of insulin receptors. *Biochemical Journal*, 281(FEB): 735-743.

Mosthaf, L., Grako, K., Dull, T.J., Coussens, L., Ullrich, A., McClain, D.A. (1990) Functionally distinct insulin receptors generated by tissue-specific alternative splicing. *EMBO Journal*, 9(8): 2409-2413.

Mosthaf, L., Vogt, B., Haring, H-U., Ullrich, A. (1991) Altered expression of insulin receptor types A and B in the skeletal muscle of non-insulin dependent diabetes mellitus patients. *Proceedings of the National Academy of Sciences of the United States of America*, 88: 4728-4730.

Mosthaf, L., Eriksonn, J., Haring, H-U., Groop, L., Widen, E., Ullrich, A. (1992) Insulin receptor isotype expression correlates with risk of non-insulin-dependent diabetes. *Diabetologia*, 35(Supplement 1): A75 Abstract.

Mosthaf, L., Eriksson, J., Haring, H-U., Groop, L., Widen, E., Ullrich, A. (1993) Insulin receptor isotype expression correlates with risk of non-insulin-dependent

diabetes. *Proceedings of the National Academy of Sciences of the United States of America*, 90(7): 2633-2635.

Muglia, L., Locker, J. (1984) Extrapancreatic insulin gene expression in the foetal rat. *Proceedings of the National Academy of Sciences of the United States of America*, 81: 3635-3639.

Mulay, S., Giannopoulas, G., Solomon, S. (1973) Corticosteroid levels in the mother and the foetus of rabbits during gestation. *Endocrinology*, 93: 1342-1348.

Myers, M.G., Backer, J.M., Siddle, K., White, M.F. (1991) The insulin receptor functions normally in Chinese hamster ovary cells after truncation of the C-terminus. *Journal of Biological Chemistry*, 266: 10616-10623.

Naeye, R.L. (1965) Infants of diabetic mothers: A quantitative, morphologic study. *Paediatrics*, 35: 980-988.

Najjar, S.M., Accili, D., Philippe, N., Jernberg, J., Margolis, R., Taylor, S.I. (1993) pp120/ecto-ATPase, an endogenous substrate of the insulin receptor tyrosine kinase, is expressed as two variably spliced isoforms. *Journal of Biological Chemistry*, 268: 1201-1206.

Neufeld, N., Kaplan, S. (1980) Ontogeny of the mammalian insulin receptor. *Developmental Biology*, 78: 151-160.

Nielsen, J.H. (1982) Effects of growth hormone, prolactin and placental lactogen on insulin content, release and DNA synthesis in cultured pancreatic islets. *Endocrinology*, 110: 600-606.

Nir, U., Walker, M.D., Rutter, W.J. (1986) Regulation of rat 1 gene expression: Evidence for negative regulation in non pancreatic cells. *Proceedings of the National Academy of Sciences of the United States of America*, 83: 3180-3184.

Norgren, S., Zierath, J., Galuska, D., Wallberg-Henriksson, H., Luthman, H. (1993) Differences in the ratio of RNA encoding two isoforms of the insulin receptor between control and NIDDM patients. *Diabetes*, 42: 675-681.

Norgren, S., Li, L-S., Luthman, H. (1994a) Regulation of human insulin receptor RNA splicing in HepG2 cells: effects of glucocorticoid and low glucose concentration. *Biochemical and Biophysical Research Communications*, 199(1): 277-284.

Norgren, S., Zeirath, J., Wedell, A., Wallberg-Henriksson, H., Luthman, H. (1994b) Regulation of human insulin receptor RNA splicing *in vivo*. *Proceedings of the National Academy of Sciences of the United States of America*, 91: 1465-1469.

O'Brien, R., Granner, D. (1991) Regulation of gene expression by insulin. *Biochemical Journal*, 278: 609-619.

O'Keefe, E., Cuatrecasas, P. (1974) Insulin receptors in murine mammary cells: Comparison in pregnant and nonpregnant animals. *Biochimica et Biophysica Acta*, 343: 64-77.

Okabayashi, Y., Maddux, B.A., McDonald, A.R., Logsdon, C.D., Williams, J.A., Goldfine, ID. (1989) Mechanisms of insulin-induced insulin-receptor down regulation: decrease of receptor biosynthesis and mRNA levels. *Diabetes*, 38: 182-187.

Okamoto, M., White, M.F., Maron, R., Kahn, C.R. (1986) Autophosphorylation and kinase activity of insulin receptor in diabetic rats. *American Journal of Physiology*, 251(5 Pt. 1): E542-E550.

Olden, K., Parent, J.B., White, S.L. (1982) Carbohydrate moiety of glycoproteins: a re-evaluation of their function. *Biochimica et Biophysica Acta*, 650: 209-232.

Olson, T., Lane, M.D. (1987) Post-translational acquisition of insulin binding activity by the insulin proreceptor. *Journal of Biological Chemistry*, 262(14): 6816-6822.

Orci, L., Ravazzola, M., Amherdt, M., Madsen, O., Perrelet, A., Vassalli, J.D., Anderson, R.G. (1986) Conversion of proinsulin to insulin occurs coordinately with acidification of maturing secretory granules. *Journal of Cellular Biology*, 103(6 Pt. 1): 2273-2281.

Petruzzelli, L.M., Ganguly, S., Smith, C.J., Cobb, M.H., Rubin, C.S., Rosen, O.M. (1982) Insulin activates a tyrosine-specific protein kinase in extracts of 3T3-L1 adipocytes and human placenta. *Proceedings of the National Academy of Sciences of the United States of America*, 79: 6792-6796.

Phelps, R.L., Metzger, B.E., Freinkel, N. (1981) Carbohydrate metabolism in pregnancy. XVII. Diurnal profiles of plasma glucose, insulin, free fatty acids, triglycerides, cholesterol and individual amino acids in later normal pregnancy. *American Journal of Obstetrics and Gynaecology*, 140: 730-736.

Philippe, J. (1989) Glucagon gene transcription is negatively regulated by insulin in a hamster islet cell line. *Journal of Clinical Investigation*, 84: 672-677.

Pickup, J.C., Williams, G. (1991) Textbook of diabetes. Oxford: Blackwell Scientific Publications.

Pictet, R., Rutter, W.J. (1972) Development of the embryonic endocrine pancreas. In: Handbook of Physiology, Sect. &, Endocrinology 1. Baltimore: Williams and Wilkins.

Podlecki, D.A., Smith, R.M., Kao, M., Tsai, P., Huecksteadt, T., Brandenburg, D., Lasher, R.S., Jarett, L., Olefsky, J.M. (1987) Nuclear translocation of the insulin receptor: A possible mediator of insulins long term effects. *Journal of Biological Chemistry*, 262: 3362-3368.

Podskalny, J.M., McElduff, A., Gorden, P. (1984) Insulin receptors on chinses hamster ovary (CHO) cells: altered insulin binding to glycosylation mutants. *Biochemical and Biophysical Research Communications*, 125(1): 70-75.

Podskalny, J. M., Rouiller, D.G., Grunberger, G., Baxter, R.C., McElduff, A., Gorden, P. (1986) Glycosylation defects alter insulin but not insulin-like growth factor 1 binding to Chinese hamster ovary cells. *Journal of Biological Chemistry*, 261: 14076-14081.

Posner, B.I., Kelly, P.A., Shiu, R.P. (1974) Studies of insulin, growth hormone and prolactin binding: Tissue distribution, species variation and characterisation. *Endocrinology*, 96: 521-531.

Prigent, S.A., Stanley, K.K., Siddle, K. (1990) Identification of epitopes on the human insulin receptor reacting with rabbit polyclonal antisera and mouse monoclonal antibodies. *Journal of Biological Chemistry*, 265: 9970-9977.

Prives, J., Bar-Sagi, D. (1983) Effect of tunicamycin, an inhibitor of protein glycosylation, on the biological properties of acetylcholine receptor in culture muscle cells. *Journal of Biological Chemistry*, 258: 1775-1780.

Rastogi, G.K., Letarte, J., Fraser, T.R. (1970) Immunoreactive insulin content of 203 pancreases from foetuses of healthy mothers. *Diabetologia*, 6: 445-446.

Ray, T.K. (1970) A modified method for the isolation of the plasma membrane from rat liver. *Biochimica et Biophysica Acta*, 196: 1-9.

Redgrave, T.G., Callow, M.J. (1990) The effect of insulin deficiency on the metabolism of lipid emulsion models of triacylglycerol-rich lipoproteins in rats. *Metabolism and Clinical Experimentation*, 39(1): 1-10.

Regoeczi, E. (1984) Iodine-labelled plasma proteins. Boca Raton: CRC Press.

Robinson, A.M., Girard, J.R., Williamson, D.H. (1978) Evidence for a role of insulin in the regulation of lipogenesis in lactating rat mammary gland. *Biochemical Journal*, 176: 343-346.

Robson, N.A., Clegg, R.A., Zammit, V.A. (1984) Regulation of peripheral lipogenesis by glucagon. Inability of the hormone to inhibit lipogenesis in rat mammary acini in vitro in the presence or absence of agents which alter its effects on adipocytes. *Biochemical Journal*, 217: 743-749.

Ronnett, G.V., Lane, M.D. (1981) Post-translational glycosylation-induced activation of aglycoinsulin receptor accumulated during tunicamycin treatment. *Journal of Biological Chemistry*, 256: 4704-4707.

Ronnett, G.V., Knutson, V.P., Kohanski, R.A., Simpson, T.L., Lane, M.D. (1984) Role of glycosylation in the processing of newly translated insulin proreceptor in 3T3-L1 adipocytes. *Journal of Biological Chemistry*, 259: 4566-4575.

Rorsman, P., Abrahamsson, H., Gylfe, E., Hellman, B. (1984) Dual effects of glucose on the cytosolic Ca^{2+} activity of mouse pancreatic B-cells. *FEBS Letters*, 170(196): 200-205.

Rosen, O.M., Chia, G.H., Fung, C., Rubin, C.S. (1979) Tunicamycin-mediated depletion of insulin receptors in 3T3-L1 adipocytes. *Journal of Cell Physiology*, 99: 37-42.

Rosen, O.M., Herrera, R., Olowe, Y., Petruzzelli, L.M., Cobb, M.H. (1983) Phosphorylation activates the insulin receptor tyrosine protein kinase. *Proceedings of the National Academy of Sciences of the United States of America*, 80: 3237-3240.

Rosen, O.M. (1987) After insulin binds. *Science*, 237: 1452-1458.

Rosen, O.M. (1988). Protein tyrosine kinases, protein serine kinases, and the mechanism of action of insulin. In The Harvey Lectures (pp. 105-122). New York: Alan R. Liss Inc.

Roth, R.A., Beaudoin, J. (1987) Phosphorylation of purified insulin receptor by cAMP kinase. *Diabetes*, 36: 123-126.

Roth, R.A., Yonezawa, K., Pierce, S., Steele-Perkins, G. (1991) Substrates of the insulin and insulin-like growth factor I receptor tyrosine kinases. New York: Elsevier Science Publishing Corporation.

Roth, R.A., Zhang, B., Chin, J., Kovacina, K. (1992) Substrates and signalling complexes: The tortured path to insulin action. *Journal of Cellular Biochemistry*, 48: 12-18.

Rouiller, D.G., Sharon, N., McElduff, A., Podskalny, J.M., Gorden, P. (1986) Lectins as probes of insulin receptor carbohydrate composition: studies in glycosylation mutants of Chinese hamster ovarian cells with altered insulin binding. *Endocrinology*, 118(3): 1159-1165.

Ruderman, N.B., Kapeller, R., White, M.F., Cantley, L.C. (1990) Activation of phosphatidylinositol 3-kinase by insulin. *Proceedings of the National Academy of Sciences of the United States of America*, 87: 1411-1415.

Russ, M., Reinauer, H., Eckel, J. (1992) Regulation of cardiac insulin receptor function by guanosine nucleotides. *FEBS Letters*, 314(1): 72-76.

Russ, M., Wichelhaus, A., Uphues, I., Koller, T., Eckel, J. (1994) Photoaffinity labelling of cardiac membrane GTP-binding proteins in response to insulin. *European Journal of Biochemistry*, 219(1-2): 325-330.

Sabatini, D.D., Kreibich, G., Morimoto, T., Adesnik, M. (1982) Mechanisms for the incorporation of proteins in membranes and organelles. *Journal of Cellular Biology*, 92: 1-22.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., Erlich, H.A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, 239: 487-491.

Sambrook, J., Fritsch, E.F., Maniatis, T. (1989) Molecular cloning: A laboratory manual (2nd ed.). Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Sara, V. R., Hall, K., Misaki, M., Fryklund, L., Christensen, N., Wetterberg, L. (1983) Ontogenesis of somatomedin and insulin receptors in the human foetus. *Journal of Clinical Investigation*, 71: 1084-1094.

Sawadogo, M., Roeder, R.G. (1985) Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region. *Cell*, 43: 165-175.

Scatchard, G. (1949) The attraction of proteins for small molecules and ions. *Annals of the New York Academy of Sciences*, 51: 660-672.

Schaefer, E.M., Erickson, H.P., Federwisch, M., Wollmer, A., Ellis, L. (1992) Structural Organisation of the Human Insulin Receptor Ectodomain. *Journal of Biological Chemistry*, 267(32): 23393-23402.

Secchi, L.A., Griffin, C.A., Grady, E.F., Grunfeld, C., Kalinyak, J.E., Schambelan, M. (1992) Tissue-specific regulation of insulin receptor mRNA levels in rats with STZ-induced diabetes mellitus. *Diabetes*, 41: 1113-1118.

Seger, R., Ahn, N.G., Boulton, T.G., Yancopoulos, G.D., Panayotatos, N., Radzeijewska, E., Ericsson, L., Bratlien, R.L., Cobb, M.H., Krebs, E.G. (1991) Microtubule-associated protein 2 kinases, ERK1 and ERK2, undergo autophosphorylation on both tyrosine and threonine residues: Implications for their mechanism of action. *Proceedings of the National Academy of Sciences of the United States of America*, 88: 6142-6146.

Seino, S., Seino, M., Nishi, S., Bell, G.I. (1989a) Structure of the human insulin receptor gene and characterisation of its promotor. *Proceedings of the National Academy of Sciences of the United States of America*, 86: 114-118.

Seino, S., Bell, G.I. (1989b) Alternative splicing of human insulin receptor messenger RNA. *Biochemical and Biophysical Research Communications*, 159(1): 312-316.

Sheldon, H. (1988) Boyds Introduction to the study of disease (10th ed.). Philadelphia: Lea & Feldger.

Shigeta, Y., Kobayashi, M., Olefsky, J. M. (1991) Recent Advances in Insulin Action and its Disorders. Proceedings of the International Symposium on Insulin Action and its Disorders, Shiga, 16 May 1990. Amsterdam: Elsevier Science Publishers B.V. (Biomedical Division).

Shoelson, S.E., Bonischnetzler, M., Pilch, P.F., Kahn, C.R. (1991) Autophosphorylation within insulin receptor beta-subunits can occur as an intramolecular process. *Biochemistry*, 30(31): 7740-7746.

Shoelson, S.E., Chatterjee, S., Chaudhuri, M., White, M.F. (1992) YMXM Motifs of IRS-1 define substrate Specificity of the insulin receptor kinase. *Proceedings of the National Academy of Sciences of the United States of America*, 89(6): 2027-2031.

Shyamala, G., Ferenczy, A. (1982) The non responsiveness of lactating mammary gland to estradiol. *Endocrinology*, 110: 1249-1256.

Singer-Sam, J., Keith, D.H., Tani, K., Simmer, R.L., Shively, L., Lindsay, S., Yoshida, A., Rigges, A.D. (1984) Isolation of a cDNA clone for Human X-linked 3-phosphoglycerate kinase by use of a mixture of synthetic oligodeoxyribonucleotides as a detection probe for insulin. *Gene*, 32: 409-417.

Slieker, L.J., Lane, M.D. (1985) Post-translational processing of the epidermal growth factor receptor. Glycosylation-dependent acquisition of ligand-binding capacity. *Journal of Biological Chemistry*, 260: 687-69 (Bolander, 1981)x, F., Goff, M.M., Zimmerman, A.E., Arquilla, E.R. (1975) [127I]- or carrier-free [125I]monoiodoinsulin: preparation, physical, immunological, and biological properties and susceptibility to "insulinase" degradation. *Journal of Biological Chemistry*, 250(11): 4268-4277.

Sonne, O., Gliemann, J. (1980) Insulin receptors of culture human lymphocytes (IM-9). Lack of receptor-mediated degradation. *Journal of Biological Chemistry*, 255: 7449-7454.

Sonne, O., Linde, S., Larsen, T.R., Gliemann, J. (1983) Monoiodoinsulin labelled in tyrosine residue 16 or 26 of the B-chain or 19 of the A-chain. *Hoppe-Seyler's Z. Physiological Chemistry*, Bd. 364: 101-110.

Spence, A. P., Mason, E.B. (1983) Human anatomy and physiology (2nd ed.) Menlo Park: Benjamin/Cummings Publishing Company.

Stanley, F. (1988) Stimulation of prolactin gene expression by insulin. *Journal of Biological Chemistry*, 263(26): 13444-13448.

Steiner, D.F., Kemmler, W., Clark, A.L., Oyer, P.E., Rubenstein, A.H. (1972) Handbook of physiology. Washington DC: American Physiology Society.

Steven, J., Whitsett, J.A. (1979) Insulin binding to neonatal human, guinea pig and rat myocardial membranes. *Paediatric Research*, 13: 482(abstract 938).

Sturgill, T., Wu, J. (1991) Recent progress in characterisation of protein kinase cascades for phosphorylation of ribosomal protein S6. *Biochimica et Biophysica Acta*, 1092(3): 350-357.

Sun, X.-J., Rothenberg, P., Kahn, C.R., Backer, J.M., Araki, E., Wilden, P.A., Cahill, D.A., Goldstein, B.J., White, M.F. (1991) Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. *Nature*, 352: 73-77.

Sung, C.K., Goldfine, I.D. (1992) Phosphatidylinositol-3-kinase is a non-tyrosine phosphorylated member of the insulin receptor signalling complex. *Biochemical and Biophysical Research Communications*, 189(2): 1024-1030.

Takata, Y., Webster, N.J., Olefsky, J.M. (1991) Mutation of the two carboxyl-terminus tyrosines result in an insulin receptor with normal metabolic signalling but enhanced mitogenic signalling properties. *Journal of Biological Chemistry*, 266: 9135-9139.

Tavare, J.M., Denton, R.M. (1988) Studies on the autophosphorylation of the insulin receptor from human placenta. Analysis of the sites phosphorylated by two-dimensional peptide mapping. *Biochemical Journal*, 252: 607-615.

Tewari, D. S., Cook, D.M., Taub, R. (1989) Characterisation of the promotor region and 3' end of the human insulin receptor gene. *Journal of Biological Chemistry*, 264(27): 16238-16245.

Tornqvist, H.E., Pierce, M.W., Frackelton, A.R., Nemenoff, R.A., Avruch, J. (1987) Identification of insulin receptor tyrosine residues autophosphorylated in vitro. *Journal of Biological Chemistry*, 262: 10212-10219.

Turk, J., Wolf, B.A., McDaniel, M.L. (1986) Glucose-induced accumulation of inositol triphosphates in isolated pancreatic islets. *Biochemical Journal*, 237: 259-263.

Ullrich, A., Bell, J.R, Chen, E.Y., Herrera, R., Petruzelli, L.M., Dull, T.J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P.H., Grunfeld, C., Rosen, O.M., Ramachandran, J. (1985) Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. *Nature*, 313: 756-761.

Valerio, D., Duyvesteyn, M.G.C., Dekker, B.M.M. Weeda, G., Berkvens, T.M., van der Voorn, L., van Ormondt, H., van der Eb, A.J. (1985) Adenosine deaminase - characterisation and expression of a gene with a remarkable promotor. *EMBO Journal*, 4: 437-443.

Van Obbergehn, E., Kowalski, A. (1982) Phosphorylation of the hepatic insulin receptor: Stimulating effect of insulin on intact cells and in a cell-free system. *FEBS Letters*, 143: 179-182.

Vinicor, F., Higdon, G., Clark, J.F. (1976) Development of glucagon sensitivity in neonatal rat liver. *Journal of Clinical Investigation*, 58: 571-578.

Vinicor, F., Kiedrowski, L. (1982) Characterisation of the hepatic receptor for insulin in the perinatal rat. *Endocrinology*, 110(3): 782-790.

Vogt, B., Carrascosa, J.M., Ermel, B., Ullrich, A., Haring, H-U. (1991) The two isotypes of the human insulin receptor (HIR-A and HIR-B) follow different internalisation kinetics. *Biochemical and Biophysical Research Communications*, 177(3): 1013-1018.

Walker, M. D., Edlund, T., Boulet, A.M., Rutter, W.J. (1983) Cell-specific expression controlled by the 5'-flanking region of insulin and chymotrypsin genes. *Nature*, 306: 557-561.

Walters, E., McLean, P. (1968) Effect of alloxan-diabetes and treatment with anti-insulin serum on pathways of glucose metabolism in lactating rat mammary gland. *Biochemical Journal*, 109: 407-417.

White, M.F., Haring, H.U., Kasuga, M., Kahn, C.R. (1984) Kinetic properties and sites of autophosphorylation of the partially purified insulin receptor from hepatoma cells. *Journal of Biological Chemistry*, 259: 255-264.

White, M.F., Shoelson, S.E., Keutmann, H., Kahn, C.R. (1988) A cascade of tyrosine autophosphorylation in the beta-subunit activates the phosphotransferase of the insulin receptor. *Journal of Biological Chemistry*, 263: 2969-2980.

World Health Organisation (1965) *World Health Organisation Technical Report* .

World Health Organisation (1985) *WHO study group report* .

World Health Organisation Expert Committee on Diabetes (1980) World Health Organisation Technical Report. .

Yamaguchi, Y., Flier, J.S., Yokota, A., Benecke, H., Backer, J.M., Moller, D.E. (1991) Functional properties of two naturally occurring isoforms of the human insulin receptor in Chinese hamster ovary cells. *Endocrinology*, 129(4): 2058-2066.

Yamaguchi, Y., Flier, J.S., Benecke, H., Ransil, B.J., Moller, D.E. (1993) Ligand-binding properties of the 2 isoforms of the human insulin receptor. *Endocrinology*, 132(3): 1132-1138.

Yamashita, S., Melmed, S. (1986) Insulin regulation of rat growth hormone gene transcription. *Journal of Clinical Investigation*, 78: 1008-1014.

Yang-Feng, T. L., Francke, U., Ullrich, A. (1985) Gene for human insulin receptor: Localised to site on chromosome 19 involved in pre-B cell leukemia. *Science*, 228: 728-731.

Yarwood, A. (1989). Protein sequencing: a practical approach. In J. B. C. Findlay Geisow, M.J. (Eds.),_Oxford: IRL Press.

Yip, C.C., Yeung, C.W., Moule, M.L. (1978) Photoaffinity labeling of insulin receptor of rat adipocyte plasma membrane. *Journal of Biological Chemistry*, 253(6): 1743-1745.

Yip, C.C., Moule, M.L., Yeung, C.W. (1980) Characterisation of insulin receptor subunits in brain and other tissues by photoaffinity labeling. *Biochemical and Biophysical Research Communications*, 96(4): 1671-1678.

Yip, C.C. (1984) Photoaffinity probes for hormone receptor characterisation. *Methods in Diabetes Research*, 1(B): 3-14.

Yip, C.C., Hsu, H., Patel, R.G., Hawley, D.M., Maddux, B.A., Goldfine, I.D. (1988) Localisation of the insulin-binding Site to the cysteine-rich region of the insulin receptor alpha subunit. *Biochemical and Biophysical Research Communications*, 157(1): 321-329.

Yonezawa, K., Roth, R.A. (1991) Assessment of the in situ tyrosine kinase activity of mutant insulin receptors lacking tyrosine autophosphorylation site 1162 and 1162. *Molecular Endocrinology*, 5: 194-200.

Zoppini, G., Kahn, C.R. (1992) Effect of phospholipase treatment on insulin receptor signal transduction. *Diabetologia*, 35(2): 109-115.

Purification of A14-Tyrosyl [^{125}I]Iodoinsulin Using C18 Reverse-Phase Cartridges[1]

D. T. De Leo[2] and E. Helmerhorst
School of Biomedical Sciences, Curtin University of Technology, Kent Street, Bentley 6102, Western Australia

Radioiodinated insulin is used in many applications including radioreceptor and radioimmunoassays. The validity of these applications often demands that the binding and biological properties of the radioiodinated insulin be essentially indistinguishable from the native hormone. A14-tyrosyl-monoiodinated insulin is an iodination product of insulin that best satisfies this criterion (1–4). Various purification procedures have been applied to the isolation of A14-tyrosyl [^{125}I]iodoinsulin from the other species of radiolabelled insulin and native insulin including HPLC (1,4), ion-exchange chromatography (5), and polyacrylamide gel electrophoresis (6). However, all these methods have significant limitations. First, they often require extensive handling of the iodination mixtures outside the safe confines of a fumehood. Second, because of the levels of radioactivity involved in an iodination, expensive equipment must be dedicated to the task. Third, HPLC and PAGE methods are relatively laborious. We have overcome these limitations using a method that entails the differential elution of free iodine, native insulin, and the various monoiodo- and diiodoinsulins on disposable cartridges packed with a C18 reverse-phase chromatographic support. This method is inexpensive and extremely simple and can be entirely confined to a fumehood environment, making it particularly safe.

Materials and methods: Zinc-free insulin. Zinc-free insulin was prepared according to the method outlined by

[1] This study was in part supported by grants from the Australian Research Council (A08931905) and Alphapharm/Diabetes Australia Research Trust.

[2] To whom correspondence and reprint requests should be addressed at School of Biomedical Sciences, Curtin University of Technology, GPO Box U1987, Perth 6001, Western Australia. Fax: 09 351 2342.

ANALYTICAL BIOCHEMISTRY **206**, 207–210 (1992)
0003-2697/92 $5.00
Copyright © 1992 by Academic Press, Inc.
All rights of reproduction in any form reserved.

Sodoyez *et al.* (7). The recovery of insulin was determined by protein analysis according to the method of Lowry *et al.* (8). Zinc-free porcine insulin was used as the insulin source in all experiments unless otherwise stated and was stored at −20°C until required.

Iodination of zinc-free porcine insulin. Zinc-free insulin (10 μg) in 10 μl of 0.4 M sodium phosphate at pH 7.4 was mixed with 1 mCi of $Na^{125}I$ (1890 Ci/mmol purchased from Amersham Australia Pty. Ltd., North Ryde, NSW, Australia) or 80 ng of $Na^{127}I$ in 0.1 mM NaOH. Oxidation of iodine in the presence of insulin was catalyzed by the addition of 10 μl of 15 mM chloramine-T in 0.4 M sodium phosphate at pH 7.4 with rapid mixing at room temperature. The reaction was stopped after 20 s by the addition of 50 μl of 15 mM sodium metabisulfite in 0.4 M sodium phosphate at pH 7.4. The iodination mixture was then immediately purified as described in the following sections.

Purification of chloramine-T-iodinated zinc-free porcine insulin. A 10-ml syringe was mounted on a Sep Pak C18 cartridge (Waters Chromatography Division, Millipore Corporation, MA) to facilitate safe washing, sample loading, and elution steps (between washes the syringe was separated from the cartridge to enable the plunger to be withdrawn). The cartridge was washed before sample application using 10 ml of 50% acetonitrile containing 50 mM triethylamine (TEA) which had been adjusted to pH 3 using orthophosphoric acid. The cartridge was then washed with 10 ml of distilled, deionized water and the iodination mixture was then applied to this prewashed cartridge. The sample was subsequently washed through the cartridge with 5 ml of the 0.4 M phosphate buffer. This was followed by 10 ml of 29% acetonitrile containing 50 mM TEA and 5 ml of 10% acetonitrile containing 0.2 M ammonium acetate, pH 5.5. A14-tyrosyl [^{125}I]iodoinsulin was eluted free from native insulin and unwanted iodinated products using 5 ml of 50% acetonitrile containing 0.2 M ammonium acetate, pH 5.5. All steps were carried out in a fume hood at room temperature.

Chromatography analysis. HPLC of the various iodination reaction mixtures and purified A14-tyrosyl [^{125}I]-iodoinsulin was carried out on a standard commercial system consisting of an LKB Bromma 2150 HPLC pump in conjunction with an LKB 2152 controller (Pharmacia-LKB Biotechnology, S-751 82 Uppsala, Sweden). The method employed a 0.46 × 25-cm Beckman ODS C18,5-μm column (Beckman Instrument Inc.).

N-terminal amino acid sequencing of purified A14-tyrosyl [^{125}I]iodoinsulin. A lyophilized aliquot of the iodoinsulin derivative was subjected to 26 successive Edman degradation cycles as outlined by Yarwood (9) to establish the amino acid residue(s) at which iodine was incorporated into the insulin molecule.

Competitive displacement binding studies. The binding potencies of the insulin derivatives were compared using placental microsomal membranes prepared according to the procedure outlined by Fujita-Yamaguchi *et al.* (10). A14-tyrosyl [^{127}I]iodoinsulin purified using a C18 cartridge or native porcine insulin (600–0.05 ng/50 μl) was incubated for 16 h at 4°C with 27 nCi (50 μl) of Amersham human A14-tyrosyl [^{125}I]iodoinsulin (1890 Ci/mmol) and placental microsomal membranes (70 μg protein). The membranes were recovered by the addition of 300 μl of an ice-cold bovine γ-globulin–polyethylene glycol solution. This solution of 0.07% bovine γ-globulin and 16.7% polyethylene glycol 6000 prepared in 0.05 M Hepes, pH 7.8, at 4°C containing 0.1 mM phenylmethylsulfonyl fluoride facilitated the quantitative recovery of the microsomal membranes. The membrane precipitate was recovered by centrifugation at 1800*g* for 30 min at 6°C. The supernatant was aspirated and the radioactivity in the remaining pellet was monitored in a Cobra auto-gamma counter. Data reduction was performed using the EBDA and LIGAND programs of McPherson and Cunningham-Smith (11) adapted from the Scatchard analysis program of Munson and Rodbard (12).

Results and discussion. Insulin was iodinated using chloramine-T as the oxidant at a 3:1 molar ratio of insulin to iodine and the products were separated by reverse-phase chromatography. Five major peaks of radioactivity were resolved (Fig. 1). On the basis of published data, peaks 1 to 5 corresponded to free iodine, A19-tyrosyl [^{125}I]iodoinsulin, B26-tyrosyl [^{125}I]iodoinsulin, B16-tyrosyl [^{125}I]iodoinsulin, and A14-tyrosyl [^{125}I]-iodoinsulin, respectively (1,4). Integration of the areas under peaks 1 to 5 indicated that 42% of the radioactivity was located on the A14-tyrosyl residue, 11% on the B26-tyrosyl residue, 13% on the B16-tyrosyl residue and 21% on the A19-tyrosyl residue.

Since the A14-tyrosyl [^{125}I]-insulin derivative is adsorbed more strongly to C18 supports than the other

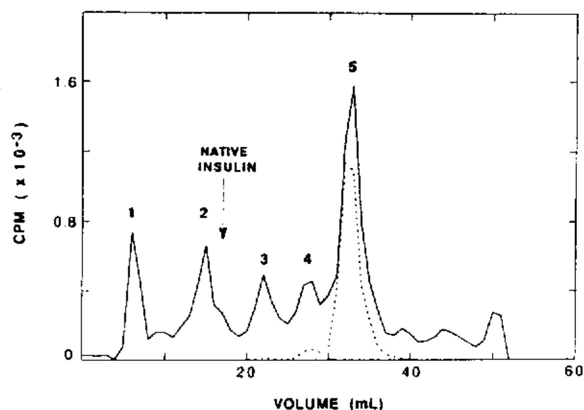

FIG. 1. High-performance liquid chromatography of an insulin iodination mixture. An insulin iodination mixture was chromatographed using a mobile phase of 29% acetonitrile in 0.2 M ammonium acetate, pH 5.5, at a flow rate of 1 ml/min (—). Rechromatography of the A14-tyrosyl [^{125}I]iodoinsulin purified using a C18 cartridge was performed using the same conditions (---).

monoiodinated forms (1,4) we evaluated small, disposable cartridges packed with a C18 matrix for selectively purifying the A14-tyrosyl [^{125}I]iodoinsulin derivative. A Na[^{127}I]iodination mixture of insulin spiked with commercial A14-tyrosyl [^{125}I]iodoinsulin, free iodine or native insulin were each applied to a C18 cartridge. A 0.4 M sodium phosphate buffer removed the free iodine from the cartridge. More than 96% of the native insulin eluted off the cartridge using 29% acetonitrile in 50 mM TEA, pH 3. A subsequent wash with 10% acetonitrile containing 0.2 M ammonium acetate, pH 5.5, facilitated the removal of TEA from the system. This was necessary as TEA had an adverse effect on the stability of the purified insulin derivative during prolonged storage. The A14-tyrosyl [^{125}I]iodoinsulin derivative was eluted quantitatively in 5 ml with 50% acetonitrile in 0.2 M ammonium acetate, pH 5.5, at room temperature. Less than 4% of unmodified insulin coeluted with the product. Thus the specific activity of our product was estimated from the known specific activity of the Na^{125}I assuming that one iodine molecule was incorporated per molecule of insulin and accounting for a 4% dilution of the specific activity by native insulin. On this basis the A14-tyrosyl [^{125}I]iodoinsulin purified from the C18 column was estimated to have a specific activity of 1800–2000 Ci/mmol.

A14-tyrosyl [^{125}I]iodoinsulin was purified from a Na^{125}I iodination mixture of insulin using the protocol described above. It resolved as a single peak and coeluted with commercial A14-tyrosyl [^{125}I]iodoinsulin using HPLC (Fig. 1). Furthermore, N-terminal amino acid sequencing data from the analysis of the Sep Pak-purified A14-tyrosyl [^{125}I]iodoinsulin indicated that the predominant release of radioactivity occurred in the fourteenth cycle (Fig. 2). Carryover of the radioactivity in the subsequent cycles was due to incomplete coupling associated with each cycle (1) and was an unavoidable limitation of carrying out this procedure manually. As no radioactivity would be expected in the fifteenth cycle, the carryover due to incomplete coupling was estimated to be approximately 50–75%. On the basis of this calculation the radioactivity appearing in the sixteenth cycle, which corresponds to the B16-tyrosyl residue, was completely accounted for by carryover from previous cycles. No radioactivity was released in the nineteenth and twenty-sixth cycles. The profile obtained using a commercially purchased A14-tyrosyl [^{125}I]iodoinsulin was virtually superimposable with the profile obtained for the A14-tyrosyl [^{125}I]iodoinsulin prepared using the C18 cartridge. Thus the product eluted from the C18 cartridge was unambiguously identified as A14-tyrosyl [^{125}I]iodoinsulin.

The binding affinity of the Sep Pak-purified A14-tyrosyl [^{127}I]iodoinsulin for placental microsomal membranes was directly compared to native insulin by Scatchard analysis as detailed under Materials and Methods. No difference was observed ($K_D = 9.3 \times 10^{-10}$ M). The stability of the purified tracer was estimated by monitoring the binding affinity over a 2-month period. We found that the C18 cartridge-purified tracer was stable for at least up to 1 month when stored lyophilized at −20°C or at least 2 months when stored in 50 mM Tris buffer, pH 7.4, containing 0.1% BSA, 5% glycerol, and 100 U/ml of bacitracin.

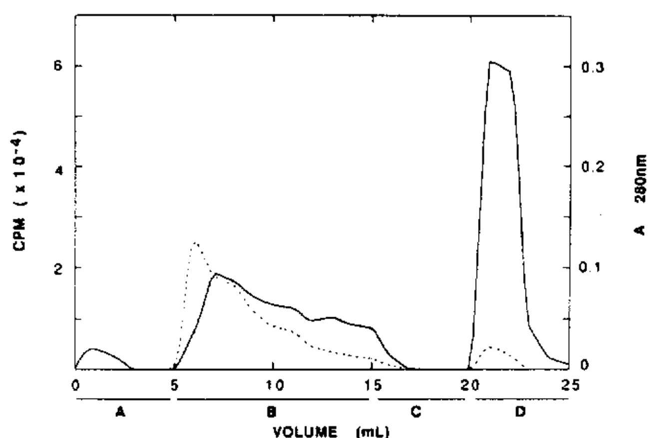

FIG. 2. N-terminal amino acid sequencing of the purified iodoinsulin derivatives. A14-tyrosyl [^{125}I]iodoinsulin purified using a cartridge packed with a C18 support (■) and commercially purchased A14-tyrosyl [^{125}I]iodoinsulin (▨) were sequenced as described under Materials and Methods.

We report a simple, safe, rapid, and inexpensive method for the purification of A14-tyrosyl [^{125}I]-iodoinsulin from an iodination mixture. The product prepared using this procedure displayed binding potency and stability with storage comparable to commercially available preparations.

Acknowledgment. We thank Annette Degebrodt for her research assistance.

REFERENCES

1. Frank, B. H., Beckage, M. J., and Willey, K. A. (1983) *J. Chromatogr.* **266**, 239–248.
2. Linde, S., and Hansen, B. (1974) *Int. J. Pept. Protein Res.* **6**, 157–163.
3. Gliemann, J., Sonne, O., Linde, S., and Hansen, B. (1979) *Biochem. Biophys. Res. Commun.* **87**, 1183–1190.
4. Lioubin, M. N., Meier, M. D., and Ginsberg, B. H. (1984) *Prep. Biochem.* **14**, 303–311.
5. Linde, S., Sonne, O., Hansen, B., and Gliemann, J. (1981) *Hoppe-Seyler's Z. Physiol. Chem. Bd.* **362**, 573–579.
6. Linde, S., Hansen, B., Sonne, O., Holst, J., and Gliemann, J. (1981) *Diabetes* **30**, 1–9.
7. Sodoyoz, J. C., Sodoyez-Goffaux, F., Goff, M., Zimmerman, A. E., and Arquilla, E. R. (1975) *J. Biol. Chem.* **250**, 4268–4277.
8. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 261.
9. Yarwood, A. (1989) *in* Protein Sequencing: A Practical Approach (Findlay, J. B. C., and Geisoe, M. J., Eds.), IRL Press, Oxford.
10. Fujita-Yamaguchi, Y., Choi, S., Sakamoto, Y., and Itakura, K. (1983) *J. Biol. Chem.* **258**, 5045–5049.
11. McPherson, G. A. (1985) *in* Kinetic, Ebda, Ligand, Lowry: A Col-

lection of Radioligand Binding Analysis Programs. BIOSOFT, Cambridge.

12. Munson, P. J., and Rodbard, D. (1980) *Anal. Biochem.* **107,** 220–239.

SP57

THE STRUCTURE OF THE MAMMARY INSULIN RECEPTOR

Domenica De Leo, Adil Vakil, Nalini Swaminathan, and Erik Helmerhorst.

School of Biomedical Sciences, Curtin University of Technology, Kent St., Bentley, WA 6102

During pregnancy and lactation insulin acts on the mammary gland to promote fat, lactose and casein synthesis for export in milk. In addition, insulin promotes mammary gland growth. Proliferation of the mammary gland results in its becoming one of the primary insulin sensitive organs of the body. Thus the mammary gland partitions a significant proportion of the body's nutrients for milk synthesis during lactation. This state of nutrient partitioning also is promoted by concomitant decreases in circulating insulin levels (Flint *et.al.*, 1980) and diminished insulin-sensitivity of other major insulin sensitive organs such as the adipose and liver tissues (Vernon *et.al.*, 1988).

The first event in insulin action is the interaction of insulin with a specific plasma membrane receptor. This insulin receptor is a transmembrane glycoprotein which is a disulphide-linked, heterodimeric complex composed of two insulin binding subunits and two tyrosine kinase subunits. Several reports have appeared describing alterations in the size of the insulin binding subunits and the tyrosine kinase subunits of the insulin receptor in different tissues (reviewed by Goldstein *et.al.*, 1990). We have specifically radiolabelled the insulin binding subunits of the rat mammary and liver insulin receptors using photoaffinity labelling methods. The insulin binding subunit of the mammary and liver insulin receptors had an apparent molecular mass of 125 kDa and 130 kDa respectively on SDS polyacrylamide gels. This 125-kDa insulin binding subunit of the mammary insulin receptor did not result from proteolysis of a larger form, and was shown to be present during all stages of lactation. Treatment of the photoaffinity labelled liver and mammary tissues with neuraminidase resulted in comigration of the insulin binding subunits inferring that the variation in the size of the insulin binding subunit between these two tissues is in part due to the degree of receptor glycosylation. Additionally, we have obtained preliminary evidence of enhanced tyrosine kinase activity of the mammary insulin receptor following insulin binding.

We are currently sequencing the mRNA of the rat mammary insulin receptor to compare it with the known sequence of the rat liver insulin receptor. This approach will determine whether the differences observed in the size of the insulin binding subunits between the mammary and the liver tissues is correlated with differences in the primary amino acid sequence in addition to the extent of receptor glycosylation. Interestingly, recent studies have shown that tissue-specific alternative splicing of the rat insulin receptor pre-mRNA may account for the insulin receptor heterogeneity evident between tissues (Goldstein *et.al.*, 1990).

Flint D.J., Clegg R.A. and Vernon R.G. (1981) *Mol Cell Endocrinol.*, 22, 265-275
Vernon R.G. and Finley E., (1988) *Biochem. J.*, 256, 873-878
Goldstein B.J. and Dudley A., (1990) *Molecular Endocrinology*, 4, 235-244