INVESTIGATION OF INSULIN-LIKE RECEPTOR SYSTEMS



THE UNIVERSITY OF ADELAIDE AUSTRALIA

THIS THESIS IS SUBMITTED IN PART OF THE REQUIREMENTS FOR THE DEGREE OF DOCTORATE OF PHILOSOPHY IN THE SCHOOL OF MOLECULAR AND BIOMEDICAL SCIENCE.

> ERIC RICHARD BONYTHON B.SC.(HON)

> > **JUNE 2005**

SUMMARY

W Trop

The insulin and insulin-like growth factor receptor (IR and IGF-1R respectively) networks are ancient and fundamental systems that control growth and metabolism in multicellular organisms. This thesis has examined several aspects of this field focusing on mammalian receptor biology and a comparison of the similarities and differences between the insulin and IGF receptor signalling systems.

The insulin receptor family of proteins consist of eleven structural domains, of which the extracellular domains contain all the ligand binding and specificity determinants. The insert domain, within the extracellular region is the least understood of all the domains, and it has no similarity to any other protein sequence. It does however contain the cleavage site which separates the receptor into two subunits and also a small stretch of residues shown to directly contact bound ligand and which is absolutely required for ligand binding in short recombinant forms of the receptor. In addition, the human insulin receptor, expressed as one of two isoforms, A and B, results in the exclusion or inclusion of 12 amino acids directly adjacent to the ligand contacting amino acids in the insert domain. The A isoform lacking exon11 is expressed ubiquitously and the B isoform containing exon11 is co-expressed mainly in the traditional insulin responsive tissues of liver, muscle, adipocytes and kidney, where it is the dominant isoform.

In this thesis recombinant insert domain was expressed in a bacterial system in an attempt to purify folded protein suitable for NMR structural analysis. The results of the expression studies indicated that the insert domain was unstructured in isolation and was unable to be adequately refolded by all conditions tried, although hydrophobic conditions appeared to partially stabilize the structure. The overall conclusions of this project were that the Insert domain is likely to have limited structure, and probably buried within the receptor, and therefore requires the presence of the rest of the extracellular domains to adopt its correct structure.

A comparison of the ligand binding and phsophorylation potential between the two human isoforms of the insulin receptor was made. A competition binding assay using europium labelled insulin was developed, that found that both IGF-1 and IGF-2 had an increased affinity for the hIR-A, but insulin had a slightly reduced affinity. These results differ from the established literature in the raw values, however the relative ratios of binding strength are consistent. The

most likely reason for this is that the europium labelled insulin has a different mode of binding the receptors due to the location of the europium chelate. Interestingly, using europium labelled IGF-1 produced results nearly identical to those of conventional competition assays.

Phosphorylation assays indicated that the hIR-B isoform was more responsive than hIR-A. Even though IGF-2 and IGF-1 had improved affinity for hIR-A, the level of phosphorylation was not as high. The ability of each growth factor to promote cellular proliferation correlated well with the relative strength of binding and activation of the receptor.

The regions of the IR and IGF-1R involved in binding substrates and regulators are predominantly found in the juxtamembrane domain and the C-terminal domain, which contain several potential tyrosine and serine phosphorylation target sequences. In this study the effect of mutations in unique tyrosine residues and other residues in the C-terminal domain of the hIGF-1R was investigated. Results of time-course phosphorylation assays showed that mutation of Tyrosine¹²⁵¹ to phenylalanine caused hyperphosphorylation of the receptor and increased proliferation, which was caused by deregulation of a tyrosine phosphatase. A Tyrosine¹²⁵⁰ to phenylalanine nutation had altered kinetics of phosphorylation, displaying an unchanging rate of phosphorylation over time after ligand stimulation. However, proliferation was unaltered, indicating that even under extended exposure to ligand, the initial strength of receptor activation is more critical to affecting the biological response.

The *Caenorhabditis elegans* insulin-like peptide family is a very large family consisting of possibly 38 peptides likely to be both agonists and antagonists of Daf-2 Receptor (IR homologue) signalling. Comparative modelling of all 38 peptides was performed based on the known structures of mammalian peptides. The overall results indicated that good quality models of ins peptides could be made despite the low sequence similarity with the templates. This suggested that it is the conformational shape of the molecule allowable by the individual residues that is most important when modelling and not having a perfect sequence match.

CHAPTER 1 INTRODUCTION	2
1.1 COMPONENTS OF THE INSULIN/IGF SYSTEM	2
1.2 STRUCTURAL BIOLOGY OF INSULINIGF RECEPTORS	4
1.2.1 Receptor Gene Structure	5
I.2.2 Domain Organization	6
1.2.3 Receptor Biosynthesis	6
1.2.3.1 Hybrid Receptors	7
1.2.4 Three-dimensional Structure of the Receptor	8
1.2.4.1 Anti-receptor Antihodies 1.2.5 Molecular basis of ligand binding	9 10
1.2.5.1 Role of the L1 Domain	10
1.2.5.2 Role of the Cys-Rich Domain	10
1.2.5.3 Role of the L2 Domain	11
1.2.5.4 Role of the Fibronectin Type-III Domains	12
1.2.5.5 Role of the Insert Domain	12
1.2.5.6 Different Mechanisms of Binding between the Insulin Receptor and IGF-IR 1.2.5.7 Minimized Ligand Binding Receptor	13
1.2.6 Structure and Function of Insulin-like Proteins	15
1.2.7 Insulin-like Growth Factor II Receptor	16
1.2.8 Insulin-like Growth Factor Binding Proteins	16
1.3 RECEPTOR SIGNAL TRANSDUCTION: SIGNALLING AND SPECIFICITY	17
1.3.1 Tyrosine Kinase Activation	17
1.3.2 Phosphorylation and Activation of Common Receptor Substrates	17
1.3.2.1 Activation of Pathways Controlling Biological Effects	18
1.3.2.1.1 Phosphatidylinositol 3-kinase Pathway 1.3.2.1.2 Mitogen Activated Kinase Pathway	18 2
1.3.2.2 Separating the IR and IGF-IR Specific Pathways and Substrates	20
1.4 ROLE OF INSULIN-LIKE PROTEINS IN DISEASE	22
1.4.1 Diabetes	22
1.4.1.1 Normal Regulation of Glucose Metabolism	22
 1.4.1.2 Molecular Basis of Insulin Dependent Diabetes Mellitis 1.4.1.3 Molecular Basis of Non-Insulin Dependent Diabetes Mellitis 	22 23
1.4.1.3.1 Is there a Role for Altered Expression of the IR Isoforms in NIDDM?	23
1.4.2 Cancer	25
1.4.2.1 Evidence of a Role for the IGF-IR in Transformation and Maintenance of Tumours	25
1.4.2.2 Breast Cancer	26
1.4.2.2.1 Is there a Role for Altered Expression of IR Isoforms in Cancer?	26 27
1.4.3 Targeting the Insulin/IGF system 1.5 SUMMARY AND PROJECT AIMS	27
CHAPTER 2 MATERIALS AND METHODS	31
2.1 ABBREVIATIONS	
2.2 MATERIALS	31 32
2.2.1 General Materials	32
2.2.2 Chemicals and Reagents	32
2.2.3 Enzymes	34
2.2.4 Antibodies	34
2.2.5 Bacterial Strains	34
2.2.6 Tissue Culture Cell Lines	35
2.2.7 Bacterial Cloning and Protein Expression Vectors	35
2.2.8 Manunalian Cell Culture Expression and Reporter Vectors	35
2.2.9 PCR Primers	35
2.2.10 Commercial Kits	36
2.2.11 Molecular Weight Standards	36
2.2.11.1 DNA Markers 2.2.11.2 Protein Markers	36 37
2.2.12 Solutions	37
2.2.12 Online and Computing Resources	38
2.3 METHODS	40
2.3.1 Bacterial Methods	40
2.3.1.1 Making Glycerol Stocks	40
2.3.1.2 Calcium Chloride Competent Cells	40
2.3.1.3 Transformation of Competent Cells by Heat Shock	40
2.3.1.4 Large Scale Midiprep Kit	40
2.3.1.5 Medium Scale Plasmid Preparation 2.3.1.6 Small scale kit	41 41
2.3.1.7 Small Scale Plasmid Preparation	42

2.3.2 Molecular Methods 2 2.3.2 J Agrate Gel Electrophoresis 2 2.3.2 Purification of Linear DNA fragments 2 2.3.3 Restriction Endorucleus Digestion of Rumid DNA 2 2.3.3 Restriction Endorucleus Digestion OR 2 2.3.3 Provide Sequencing of Rusand DNA 2 2.3.3 I Culture of Mannalana Cultis 45 2.3.3 Culture of Mannalana Cultis 46 2.3.3 Culture of Mannalana Cultis 47 2.3.3 Culture of Mannalana Cultis 47 2.3.3 Culture Cultisting assay 46 2.3.3 Culture Cultisting assay 46 2.3.3 Culture Cultisting assay 47 2.3.3 Culture Culture Cultisting assay 47 2.3.3 Culture Cult		
 2.3.21 Agrone Gel Electrophresis 2.3.2 Particiation Structure DNA fragments 2.3.2 Restriction Endonuclesse Digestion of Planid DNA 2.3.2 Structure Construction from which c. edgoart RNA 2.3.2 Structure Transcription 2.3.2 Restructure Transcription 2.3.2 Protection Structure Construction from which c. edgoart RNA 2.3.2 Primer Design 2.3.3 Projection Structure Transcription 2.3.4 Primer Design aging systematics 2.3.5 Cell future of Manualian C.Itis 2.3.3 Cell Locating structure aging systematics 2.3.3 Cell Locating Structure aging systematics 2.3.3 Cell Locating Structure aging systematics 2.3.3 Cell Locating Constructure aging systematics 2.3.4 Restructure aging systematics 2.3.5 Cell Inter GIO assay 2.3.4 Prophysical Constructure aging systematics 2.3.4 Prophysical Constructure aging aging systematics 2.3.4 Prophysical Constructure aging aging systematics 2.3.4 Prophysical Constructure aging aging aging systematics 2.3.4 Prophysical Constructure aging agi		
2.3.2 Purification of Linear DNA fragments 42 2.3.2.3 Restriction Endonuclease Digestion of Hannid DNA 42 2.3.2.4 Lightion Reactions 43 2.3.2.5 RNA purification from whole c. elegons RNA 43 2.3.2.6 mRAN Purification from whole c. elegons RNA 43 2.3.2.6 mRAN Purification from whole c. elegons RNA 44 2.3.2.8 priving Design 44 2.3.2.8 priving Design 44 2.3.2.8 priving Design 44 2.3.3.1 Culture of Manmalian Cells 43 2.3.3.1 Culture of Manmalian Cells 43 2.3.3.3 Transforming get yolong Lipofectamine + ¹⁷⁶ 46 2.3.3.4 Scaliture of Manmalian Cells 47 2.3.3.5 Cell ture Gio assay 47 2.3.3.6 Cell Lysis 47 2.3.3.7 Basic FACS analysis 47 2.3.3.7 Basic FACS analysis 47 2.3.3.7 Basic FACS analysis 47 2.3.4.7 Protein Methods 49 2.3.4.7 Protein Methods 49 2.3.4.8 Transforming Colong Lipofectamine + 174 50 2.3.4.4 Teuropium -lisbelling proteins 49 2.3.4.4 Teuropium -lisbelling proteins 49 2.3.4.4 Teuropium -lisbelling proteins 50 2.3.4.5 Competition Asay 51 2.3.4.6 Competition Asay 51 <		
 2.3.2 Restriction Endonucless Digetters of Plasmid DNA 2.3.2 Ignor Reactions 3.2.3 RNA parification from whole, <i>e.loguns</i> RNA 3.2.3 RNA parification from whole, <i>e.loguns</i> RNA 3.2.3 Priver Design 44 3.2.3 Cellure of Mammalian Cells 3.3 Cellure of Mammalian Cells 3.3 Cellure of Mammalian Cells 3.3 Cellure of Cellus anig LyopOctamine et TM 46 3.3 Transfection of cellus anig LyopOctamine et TM 3.3 Cellure Science Private P		
2.12.4 Ligation Reactions 43 2.12.5 INA purification from whole c. elegans RNA 43 2.12.5 Reverse Transcription 44 2.12.8 Project Design 44 2.12.9 Reverse Transcription 44 2.12.9 Reverse Transcription 44 2.12.10 Cycle Sequencing of Plosmid DNA 45 2.13.11 Culture of Mammalane Cdls 45 2.13.21 Culture of Mammalane Cdls 46 2.13.3 Transfection of cell suing Lipofectamine - I ^M 46 2.13.3 Cell Units of Mammalane Cdls 47 2.13.3 Cell Units 47 2.13.3 Cell Units 47 2.13.3 Cell Units 47 2.13.3 Transfection of cell suing Lipofectamine - I ^M 46 2.13.3 Transfection of cell suing Lipofectamine - I ^M 47 2.13.3 Transfection of cell suing Lipofectamine - I ^M 47 2.13.3 Transfection of cell suing Lipofectamine - I ^M 48 2.14.1 Weither Blot 49 2.14.2 Protein-Methods 49 <		
 2.12.5 RNA purification from wholes. <i>elegons</i> RNA 2.12.7 Reverse Transcription 44 2.12.8 Thiore Design 44 2.12.9 Hole Design 44 2.12.9 Hole Design 44 2.12.9 Hole Design 44 2.12.9 Loging Statement of PERSING DAA 45 2.3.10 Vitro Methods 2.3.11 Nitro Methods 2.3.3 Callure of Marmabian Cells 2.3.3 Transfection of cells using Lipofectamine +TM 46 2.3.3 Transfection of cells using Lipofectamine +TM 47 2.3.3 Cell tire Glo assiy 48 2.4 Pretein Methods photophation Assay 3.4 Reprint Photometry Company Company 3.4.4 Nutriem Blo 2.4.4 Pretein Methods photophation Assay 3.4.4 Descent ELISA 4.4 Insuin Receptor ELISA 4.4 Descent Elisa Company Assay 4.3.4 Descent ELISA 4.4 Descent Elisa Company Analysis 4.3.4 Descent Elisa 4.3.5 Cemputational Methods for Comparative Modeling 4.3.4 Descent Aligned proteins 4.3.4 Descent Aligned proteins 4.3.5 Cemputational Methods for Comparative Modeling 4.3		
 2.3.2 mRNA Purification from whole <i>c. elegans</i> RNA 2.3.2 more Design 4.4 2.3.2 Polymerse Chain Reaction (PCR) 4.4 2.3.2 Polymerse Chain Reaction (PCR) 4.4 2.3.2 In Cycle Sequencing of Plusmid DNA 4.5 2.3.3 Liture of Mammalian Cells 4.6 2.3.3 Transfection of cell suing Lipofectamie 1^{NL} 4.6 2.3.3 Transfection of cell suing Lipofectamie 1^{NL} 4.6 2.3.3 Cell Costing using cytometer 4.6 2.3.3 Cell Live GN analysis 4.7 2.3.3 Cell Live GN analysis 4.7 2.3.3 Resployed and the cell vability assay 4.6 2.3.4 Methylene Bluc cell vability assay 4.6 2.3.4 Resployed analysis 4.7 2.3.3 Resployed and cost analysis 4.8 2.3.4 Protein Methods 4.9 2.3.4 Justine Cost Phase Methods 2.3.4 Justine Recorese Phase Method growth factors 2.3.4 Justine Recorese Phase Method growth factors 2.3.4 Justine Recorese Musicity Phase Method factors 2.3.4 Justine Recorese Mission proteins 3.3.4 Justine Recorese Mission proteins 3.3.4 Justine Records Recorese Mission proteins 3.		
2.2.7 Revise Transcription 44 2.2.3 Prioric Design 44 2.2.3 Prioric Design 44 2.2.3 In vitre Methods 45 2.3.1 In vitre Methods 45 2.3.3 In vitre Methods 45 2.3.3 In vitre Methods 46 2.3.3 Collute of Mannahae Cidls 46 2.3.3 Transfection of cells using Lupofectamine + ¹⁴ 46 2.3.3 Coll Upsis 47 2.3.3 Thestop FACS analysis 47 2.3.3 Thestop FACS analysis 47 2.3.4 Protein Methods 49 2.3.4 Protein Methods 49 2.3.4 Protein Methods 49 2.3.4 Protein Methods 49 2.3.4 Protein Methods 50 2.3.4 Protein Methods 50 2.3.4 Protein Methods 50 2.3.4 Protein Methods 50 2.3.4 Suppoint-labeling proteins 50 2.3.4 Throtein Methods 50 2.3.4 The Method of Throin Proteins		
2.3.2 Promer Design 44 2.3.2 Projences Chara Reaction (PCR) 44 2.3.3 In vitro Methods 45 2.3.3 In vitro Methods 45 2.3.3 In vitro Methods 45 2.3.3 Transfection of cells using Lipofectamine + ¹⁴⁰ 66 2.3.3 Cell counting using cytometer 46 2.3.3 Cell cutting using cytometer 46 2.3.3 Cell thre Gio assay 47 2.3.3 Cell thre Gio assay 47 2.3.3 Cell thre Gio assay 47 2.3.3 Phosphorylation Assay 48 2.3.3 Phosphorylation Assay 48 2.3.4 Western Blot 49 2.3.4 Ustern Blot 49 2.3.4 Sequencing and the develop with the south anti-receptor antibody 50 2.3.4 Ustern Blot 50 2.3.4 Sequention of 96-well Plates with anti-receptor antibody 50 2.3.4 Sequention of 96-well Plates with anti-receptor antibody 51 2.3.4 Sequence Eligit Chronatography 52 2.3.4 Detection of Protein oncentration 52 2.3.4 Detection of Protein oncentration 53 2.3.4 Detection of Protein oncentration 53 2.3.4 Detection of Prote	*	
2.3.2 Projumense Chain Reaction (PCR) 44 2.3.10 vitro Methods 45 2.3.3.10 vitro Methods 45 2.3.3.10 vitro Methods 45 2.3.3.1 Culture of Mammalian Cells 46 2.3.3.1 Transferior of cells sargly Lopotextarine - 1 ⁷⁴ 46 2.3.3.1 Transferior of cells sargly Lopotextarine - 1 ⁷⁴ 46 2.3.3.5 Cell tire (Go asay 47 2.3.3.6 Cell tor (Go asay 47 2.3.3.7 Basic FACS analysis 47 2.3.3.7 Basic FACS analysis 47 2.3.3.8 Trinscreious Phosphorylation Asay 48 2.3.4 Protein Methods 49 2.3.4 Suppoint-labelling proteins 49 2.3.4 Suppoint-labelling proteins 49 2.3.4 Suppointion of 95-well Plates with anti-receptor antibody 50 2.3.4 Suppointion of 95-well Plates with anti-receptor antibody 50 2.3.4 Suppointion of 95-well Plates with anti-receptor antibody 51 2.3.4 Suppointion of 95-well Plates with anti-receptor antibody 51 2.3.4 Protein of Prostine Phosphorylation using cu-labeled anti-phosphotyrosine Antibody 51 2.3.4 Suppointion of Prostine Phosphorylation using cu-labeled anti-phosphotyrosine Antibody 52 <tr< td=""><td></td><td></td></tr<>		
2.3.1 In vitro Methods 45 2.3.1 Colture of Memmolian Cells 45 2.3.1 Colture of or Cells using Lypotectamine + TM 46 2.3.3 Transfered or Cells using Lypotectamine + TM 46 2.3.3 Transfered or Cells using Lypotectamine + TM 47 2.3.3 Cell true Glo sessy 47 2.3.3 Second Sessy 47 2.3.3 Cell true Glo sessy 47 2.3.3 Second Sessy 47 2.3.3 Cell true Glo sessy 47 2.3.3 Cell true Glo sessy 47 2.3.3 Proteomous Photopholian Assay 48 2.3.4 Protein Methods 49 2.3.4 I Western Blot 49 2.3.4 I Second To 75% 40 Pl Westers with anti-receptor antibody 50 2.3.4 I Methods 50 2.3.4 A Sequent Holds 50 2.3.4 Second True Photophorylation assay 50 2.3.4 Second True Interference Interference 50 2.3.4 Second Photophorylation using cu-labeled anti-phosphotyrosine Antibody 51 2.3.4 Second Photophorylation using cu-labeled anti-phosphotyrosine Antibody 51 2.3.4 Second Photon Concentration 52 2.3.4 I Big Performance Liquid Chronatography Analysis 53 2.3.4 I Directions of Protein Concentration 53 2.3.4 Second Photon Concentration 53 2.3.4 Second Photon Concentration 53 2.3.4 I Directions Concentration 53 2.3.5 Cemputational Methods for Comparative Modelling 54 2.3.5 Liptoperson of Thisredoxin-Insert Domain fusion protein 53 3.2.4 I Proteins Second Photophorean 55 3.2.4 Expression of Thisredoxin-Insert Domain fusion protein 53 3.2.2 Expression of Thisredoxin-Insert Domain fusio		44
2.3.1 Culture of Mammalian Cells 45 2.3.2 Call conting using cipotectamine + TM 46 2.3.3 Transfection of cells using Lipofectamine + TM 46 2.3.3 Solution Constant 47 2.3.3 Solution Constant 47 2.3.3 Coll time Conseasy 47 2.3.3 Data FACS analysis 47 2.3.3 Time-constent Methods 48 2.3.3 Time-constent Methods 49 2.3.4 Protein Methods 49 2.3.4 Protein Methods 49 2.3.4 Protein Methods 49 2.3.4 Sophon-Labelling proteins 49 2.3.4 Sophone Constant Methods 50 2.3.4 Sophone Charact Lipad Constant Constan	2.3.2.10 Cycle Sequencing of Plasmid DNA	
2.3.2 Cell counting using cytometer 46 2.3.3 Transferion of cells using Lipofectamine + ^{Trat} 46 2.3.3 Kell Lysis 47 2.3.3 Cell Lysis 47 2.3.3 Description of Cells using Lipofectamine + Trat 47 2.3.3 Description of Cells using Lipofectamine + Trat 47 2.3.3 Description of Cells using Lipofectamine + Trat 47 2.3.3 Description of Cells using Lipofectamine + Trat 47 2.3.3 Description - Lipote - Li		
2.3.3 Transfection of cells using Lipofectamine + TM 46 2.3.3 Cell tire Glo assay 47 2.3.3 Cell tire Glo assay 47 2.3.3 Cell tire Glo assay 47 2.3.3 Cell tire Glo assay 48 2.3.3 Phosphorylation Assay 48 2.3.4 Protein Methods 49 2.3.4 Protein Methods 49 2.3.4 Protein Methods 49 2.3.4 Spreparation of 96-well Plates with anti-receptor antibody 50 2.3.4 Spreparation of 95-well Plates with anti-receptor antibody 50 2.3.4 Spreparation of Protein Competition assay 51 2.3.4 Detection Assay with eurlabeled growth factors 50 2.3.4 Or Detection Of Tyrosine Phosphorylation using eurlabeled anti-phosphotyrosine Antibody 51 2.3.4 Or Detection Of Tyrosine Phosphorylation using eurlabeled anti-phosphotyrosine Antibody 52 2.3.4 Or Detection of Tyrosine Phosphorylation assay 51 2.3.4 Or Detection Assay with eurlabeled growth factors 52 2.3.4 Or Detection Assay with eurlabeled for Comparative Modelling 53 2.3.4 I High Feromance Liqued Chronatography Analysis 53 2.3.4 I Protein Sequence Aliguments 54 2.3.5 Envioten Sequence Aliguments <td></td> <td></td>		
2.3.3 Methylene Blue cell viability assay 47 2.3.3 Cell itro (Con assay 47 2.3.3 Cell itro (Con assay) 47 2.3.3 Pasic FACS analysis 47 2.3.3 Proteonause Phosphorylation Assay 48 2.3.3 Proteonause Phosphorylation Assay 48 2.3.4 Protein Methods 49 2.3.4 Protein Methods 49 2.3.4 Proparation of 96-well Plates with anti-receptor antibody 30 2.3.4 Protein Methods 50 2.3.4 Protein Assay with cu-labeled growth factors 50 2.3.4 Objection Assay with cu-labeled growth factors 50 2.3.4 Objection Assay with cu-labeled growth factors 50 2.3.4 Objection Of Tyrosine Phosphorylation using cu-labeled anti-phosphotyrosine Antibody 51 2.3.4 Objection Of Tyrosine Phosphorylation asing cu-labeled anti-phosphotyrosine Antibody 52 2.3.4 Objection Of Tyrosine Phosphorylation asing cu-labeled anti-phosphotyrosine Antibody 52 2.3.4 Difference Liquid Chronatography Analysis 53 2.3.4 Difference Liquid Chronatography Analysis 53 2.3.5 Computational Methods for Comparative Modeling 54 2.3.5 Simulation and Methods 54 2.3.5 Lipotics Signence Alignments		
2.3.3 Cell tire Glo assay 47 2.3.3 Cell tire Glo assay 47 2.3.3 Serie FACS analysis 47 2.3.3 Phosphorylation Assay 48 2.3.4 Protein Methods 49 2.3.4 Protein Methods 49 2.3.4 Protein Methods 49 2.3.4 Protein of 96-well Plates with anti-receptor antibody 50 2.3.4 Segregation of 96-well Plates with anti-receptor antibody 50 2.3.4 Segregation of 96-well Plates with anti-receptor antibody 50 2.3.4 Proparation of Provid Plates with anti-receptor antibody 50 2.3.4 Option Assay with eu-labeled growth factors 50 2.3.4 Option Assay with eu-labeled growth factors 50 2.3.4 Option Of Tyrosine Phosphorylation using eu-labeled anti-phosphotyrosine Antibody 51 2.3.4 Option Of Tyrosine Phosphorylation using eu-labeled anti-phosphotyrosine Antibody 51 2.3.4 Option Cleavage of fasion proteins 52 2.3.4 I Brotion asset KD bigstion 53 2.3.4 I Protein accleavage of fasion proteins 54 2.3.5 Simulational Methods for Comparative Modelling 54 2.3.5 Simulational Methods for Comparative Modelling 54 2.3.5 Simulation of Cloavagraphy Analysis		
2.3.3 Cell Lysis 47 2.3.3 J Baice PACS analysis 47 2.3.3 Phosphorylation Assay 48 2.3.4 Protern Methods 49 2.3.4 Protern Methods 49 2.3.4 Protern Methods 49 2.3.4 Vestern Biol 50 2.3.4 One Addition Assay with exclose and the costs 50 2.3.4 Objection of Tyrosine Phosphorylation using cu-labeled anti-phosphotyrosine Antibody 51 2.3.4 Objection of Tyrosine Phosphorylation using cu-labeled anti-phosphotyrosine Antibody 52 2.3.4 Disk Pacter Electrophoresis 52 2.3.4 Disk Pactern Scopy 52 2.3.4 Disk Pactern Scopy 52 2.3.4 Disk Pactern Scopy 52 2.3.5 Computational Methods for Comparative Modelling 54 2.3.5 Disting the Comparative Models 54 2.3.5 Model Evaluation<		
2.3.37 Basic FACS analysis 47 2.3.38 Phiosphorylation Assay 48 2.3.4 Protein Methods 49 2.3.4 Stroppum-labelling proteins 49 2.3.4 Stroppum-labelling proteins 50 2.3.4 Stroppum-labelling proteins 50 2.3.4 Optimical Assay with eu-labeled growth factors 50 2.3.4 Optimical Assay with eu-labeled growth factors 50 2.3.4 Optimical Concentration 52 2.3.4 Optimical Concentration 52 2.3.4 Optimical Concentration 52 2.3.4 I Dirpheromance Liggical Chronatography Analysis 53 2.3.4 I Dirpheromance Liggical Chromatography Analysis 53 2.3.4 I Dirpheromance Liggical Chromatography Analysis 53 2.3.4 I Dirpheromance Liggical Chromatography Analysis 53 2.3.5 I Proteins Sequence Alignments 54 2.3.5 Sequence Alignments 54 2.3.5 Sequence Alignments 54 2.3.5 Sequence Alignene	•	
2.3.3.8 Pine-course Phosphorylation Assay 48 2.3.4 Protein Methods 49 2.3.4 Proparation of 90-well Plates with anti-receptor antibody 50 2.3.4 A frequation of 90-well Plates with anti-receptor antibody 50 2.3.4 A frequation of 7 prosine Phosphorylation using eu-labeled anti-phosphotyrosine Antibody 51 2.3.4 Obvic flat factor competition assay 51 2.3.4 Obvic flat factor competition assay 51 2.3.4 Platinition of Protein concentration 52 2.3.4 Obvic flat factor competition assay 53 2.3.4 I Divic Africy Chronatography Analysis 53 2.3.4 I Divic Performance Liquid Chronatography Analysis 53 2.3.4 I Divic Africy Chromatography Analysis 53 2.3.4 I Divic Africy Chromatography 54 2.3.5 Dividing the Comparative Modelling 54 2.3.5 Dividing the Comparative Modells 54 2.3.5 Dividing the Comparative Models 54 2.3.5 Dividing the Comparative Models 54 2.3.5 A Model Evaluation <td< td=""><td></td><td></td></td<>		
2.3.3 9 Time-course Phosphorylation Assay 48 2.3.4 Protein Methods 49 2.3.4 1 Western Blot 49 2.3.4 2 Europium-labelling proteins 49 2.3.4 3 Preparation of 96-well Plates with anti-receptor antibody 50 2.3.4 4 3 Preparation of 97 worse Phosphorylation using cu-labeled anti-phosphotytrosine Antibody 50 2.3.4 5 Competition Assay with cu-labeled growth factors 50 2.3.4 0 Protein concentration 52 2.3.4 10 Ni-Affinity Chromatography 52 2.3.4 10 Ni-Affinity Chromatography 53 2.3.4 11 Phyto Performance Liquid Chromatography Analysis 53 2.3.4 12 Thrombin Cleavage of Usion proteins 53 2.3.4 13 Proteinase K Digestion 53 2.3.4 14 NMK Spectroscopy 54 2.3.5 Computational Methods for Comparative Modelling 54 2.3.5 Simulated Annealing 54 2.3.5 A Model Evaluation 55 3.1 INTRODUCTION 56 3.2.1 Construction of recombinant insert domain expressing vectors 59 3.2.2 Repression of Thioredoxin-Insert Domain fusion protein 61 3.2.3 PROTEIN EXPRESSION 63 3.2.3 TRX-ID Purification by Resource Q chr		48
2.3.41 Western Blot 99 2.3.4.2 Europium-labeling proteins 99 2.3.4.3 Preparation of 96-well Plates with anti-receptor antibody 50 2.3.4.4 Insulin Receptor ELISA 50 2.3.4.5 Competition Assay with eu-labeled growth factors 50 2.3.4.6 ¹⁰¹ growth factor competition assay 51 2.3.4.7 Detection of Tyrosine Phosphorylation using eu-labeled anti-phosphotyrosine Antibody 51 2.3.4.7 Detection of Tyrosine Phosphorylation using eu-labeled anti-phosphotyrosine Antibody 52 2.3.4.9 DN-Affinity Chromatography 52 2.3.4.10 Ni-Affinity Chromatography 53 2.3.4.11 High Performance Liquid Chromatography Analysis 53 2.3.4.12 Thrombin Cleavage of fusion proteins 53 2.3.4.13 Proteinase K Digestion 53 2.3.5.1 Protein Sequence Alignments 54 2.3.5.2 Building the Comparative Models 54 2.3.5.3 Simulated Annealing 54 2.3.5.4 Model Evaluation 55 3.1.1 NTRODUCTION 56 3.2.1 Construction of recombinant insert domain expressing vectors 59 3.2.2 PROTEIN EVREXESION OF INSERT DOMAIN PROTEINS 56 3.1.1 NTRODUCTION 62	2.3.3.9 Time-course Phosphorylation Assay	48
2.3.4.2 Europium-labelling proteins 99 2.3.4.3 Preparation of 96-well Pitess with anti-receptor antibody 50 2.3.4.4 Inguin Receptor ELISA 50 2.3.4.5 Competition Assay with eu-labeled growth factors 50 2.3.4.6 ¹⁰ I growth factor competition assay 51 2.3.4.7 Detection of Tyrosine Phosphorylation using eu-labeled anti-phosphotyrosine Antibody 51 2.3.4.8 Quantitation of Protein concentration 52 2.3.4.9 DSi-PAGE Electrophoresis 52 2.3.4.9 DSi-PAGE Electrophoresis 53 2.3.4.10 Ni-Affinity Chromatography 53 2.3.4.11 High Performance Liquid Chromatography Analysis 53 2.3.4.13 Proteins elevence Alignments 53 2.3.5.2 Building the Comparative Modelling 54 2.3.5.2 Building the Comparative Models 54 2.3.5.3 Simulated Annealing 54 2.3.5.4 Model Evaluation 55 3.1 INTRODUCTION 56 3.1.1 Project Summary and Aims 58 3.2.2 Expression of Thioredoxin-Fibronetia - S-Insert Domain fusion protein 52 3.2.3 Throwbin Kanas digestion 53 3.2.1 Construction of recombinant insert domain fusion protein 52.3 PROTEIN EXPRESSION </td <td>2.3.4 Protein Methods</td> <td>49</td>	2.3.4 Protein Methods	49
2.34.3 Preparation of 96-well Plates with anti-receptor antibody 50 2.34.4 Insulin Receptor ELISA 50 2.34.6 ^{IM} growth factor competition assay 51 2.34.7 Detection of Tyrosine Phosphorylation using cu-labeled anti-phosphotyrosine Antibody 51 2.3.4.7 Detection of Tyrosine Phosphorylation using cu-labeled anti-phosphotyrosine Antibody 51 2.3.4.9 DSF-AQE Electrophoresis 52 2.3.4.10 Ni-Affinity Chromatography 52 2.3.4.10 Ni-Affinity Chromatography 53 2.3.4.13 Proteinase K Digestion 53 2.3.4.14 NMR Spectroacepy 54 2.3.5.1 Protein Sequence Alignments 54 2.3.5.2 Building the Comparative Modelling 56 3.1.1 Project Summary and Aims 58 3.2 RESULTS 59 3.2.1 Construction of recombinant insert domain expressing vectors 59 3.2.2 PROTEIN EXPRESSION 60 3.2.3 Thrombin Kinase digestion 51 3.2.3 Thrombin Kinase digestion 53 3.2.3 Thrombin Kinase digestio		49
2.3.4.1 insulin Receptor ELISA 50 2.3.4.5 Competition Assay with eu-labeled growth factors 50 2.3.4.6 ¹⁴] growth factor competition assay 51 2.3.4.7 Detection of Tyrosine Phosphorylation using eu-labeled anti-phosphotyrosine Antibody 51 2.3.4.8 Quantitation of Protein concentration 52 2.3.4.9 N-AITinity Chromance Liquid Chromatography 52 2.3.4.10 N-AITinity Chromance Juguid Chromatography Analysis 53 2.3.4.11 High Performance Liquid Chromatography Analysis 53 2.3.4.12 Thrombin Cleavage of fusion proteins 53 2.3.4.13 Proteinase K Digestion 53 2.3.4.14 NMR Spectroscopy 54 2.3.5.2 Gomputational Methods for Comparative Modelling 54 2.3.5.3 Simulated Annealing 54 2.3.5.4 Model Evaluation 56 3.1.1 Project Summary and Aims 58 3.2.1 Construction of recombinant insert domain fusion protein 60 3.2.2.1 Expression of Thioredoxin-Insert Domain fusion protein 61 3.2.3.1 RX-ID Purification by Ni-sifinity chromatography 62 3.2.3.1 TRX-ID Purification by Ni-sifinity chromatography 63 3.2.3.1 TRX-ID Purification by Ni-sifinity chromatography 63		
2.3.4.5 Competition Assay with ex-labeled growth factors 50 2.3.4.6 ¹⁴] growth factor competition assay 51 2.3.4.7 Detection of Trotsine Phosphorylation using ex-labeled anti-phosphotyrosine Antibody 51 2.3.4.8 Quantitation of Protein concentration 52 2.3.4.9 DEX-PAGE Electrophoresis 52 2.3.4.10 Ni-Affinity Chromatography 52 2.3.4.10 Ni-Affinity Chromatography Analysis 53 2.3.4.13 Proteinase K Digestion 53 2.3.4.13 Proteinase K Digestion 53 2.3.4.14 NMR Spectrascopy 54 2.3.5 Computational Methods for Comparative Modelling 54 2.3.5.1 Proteinas Coquence Alignments 54 2.3.5.2 Building the Comparative Models 54 2.3.5.3 Simulated Annealing 54 2.3.5.4 Model Evaluation 55 CHAPTER 3 EXPRESSION OF INSERT DOMAIN PROTEINS 3.2.1 Construction of recombinant insert domain expressing vectors 59 3.2.2 PROTEIN EXPRESSION 60 3.2.3.1 RX-ID Purification by Ni-affinity chromatography 62 3.2.3 TRX-ID Purification by Ni-affinity chromatography 63 3.2.3 TRX-ID Purification by Ni-affinity chromatography 63		
2.3.46 ¹²) growth factor competition assay 51 2.3.47 Detection of Tyrosine Phosphorylation using cu-labeled anti-phosphotyrosine Antibody 51 2.3.48 Quantitation of Protein concentration 52 2.3.49 DN-AGE Electrophoresis 52 2.3.410 NN-Affinity Chromance Liquid Chromatography 52 2.3.411 High Performance Liquid Chromatography Analysis 53 2.3.412 Thrombin Cleavage of fusion proteins 53 2.3.413 Proteinas K Digestion 53 2.3.414 NMR Spectroscopy 54 2.3.52 Computational Methods for Comparative Modelling 54 2.3.52 Building the Comparative Models 54 2.3.53 Simulated Annealing 54 2.3.54 Model 54 2.3.54 Model 55 3.1 INTRODUCTION 56 3.1 INTRODUCTION 56 3.2.1 Construction of recombinant insert domain expressing vectors 59 3.2.2 RESULTS 59 3.2.1 Construction of Thioredoxin-Insert Domain fusion protein 60 3.2.3 Simulated Annealing expressing vectors 59 3.2.2 RESULTS 50 3.2.1 Construction of recombinant insert domain fusion protein 60 3.2.		
2.3.47 Detection of Tyrosine Phosphorylation using cu-labeled anti-phosphotyrosine Antibody 51 2.3.4.8 Quantitation of Protein concentration 52 2.3.4.9 SDS-PAGE Electrophoresis 52 2.3.4.10 Ni-Affinity Chromatography 53 2.3.4.11 High Performance Liquid Chromatography Analysis 53 2.3.4.12 Thrombin Cleavage of fusion proteins 53 2.3.4.13 Proteinase K Digestion 53 2.3.4.14 NMR Spectroscopy 54 2.3.5.1 Protein Sequence Alignments 54 2.3.5.2 Building the Comparative Models 54 2.3.5.3 Building the Comparative Models 54 2.3.5.4 Model Evaluation 55 CHAPTER 3 EXPRESSION OF INSERT DOMAIN PROTEINS 3.2 RESULTS 59 3.2.1 Construction of recombinant insert domain expressing vectors 59 3.2.2 RESULTS 59 3.2.1 Construction of recombinant insert domain fusion protein 60 3.2.2 RESULTS 60 3.2.3 FRX-ID Purification by N-affinity chromatography 62 3.2.3 TRX-ID Purification by N-affinity chromatography 63 3.2.3 TRX-ID Purification by Resource Q chromatography 63 3.2.3 TRX-ID Purification by Resource	2.3.4.6 ¹²⁵] growth factor connectition assay	
2.3.4.8 Quantitation of Protein concentration 52 2.3.4.9 SDS-PAGE Electrophoresis 52 2.3.4.10 Ni-Affinity Chromatography 52 2.3.4.11 High Performance Liquid Chromatography Analysis 53 2.3.4.12 Thrombin Cleavage of fusion proteins 53 2.3.4.13 Proteinase K Digestion 53 2.3.4.14 NMR Spectroscopy 54 2.3.5.1 Protein Sequence Alignments 54 2.3.5.2 Building the Comparative Models 54 2.3.5.3 Simulated Annealing 54 2.3.5.4 Model Evaluation 55 CHAPTER 3 EXPRESSION OF INSERT DOMAIN PROTEINS Set Summary and Aims 3.1.1 Project Summary and Aims 58 3.2.2 RESULTS 59 3.2.1 Construction of Theoredoxin-Fibronectin 3-Insert Domain fusion protein 60 3.2.2.2 Expression of Thioredoxin-Fibronectin 3-Insert Domain fusion protein 61 3.2.3.1 TRX-ID Purification by Nearource Q chromatography 62 3.2.3.3 Thrombin Kinase digestion 63 3.2.3.3 Thrombin Kinase digestion 63 3.2.3.3 Thrombin Cleavage digestion 63 3.2.3.3 Thrombin Kinase digestion 63 3.2.3.4 MPUC Pu		
2.3.4.10 Ni-Affinity Chromatography 52 2.3.4.11 High Performance Liquid Chromatography Analysis 53 2.3.4.12 Thrombin Cleavage of fusion proteins 53 2.3.4.13 Proteinase K Digestion 53 2.3.4.14 NMR Spectroscopy 54 2.3.5.1 Protein Sequence Aliguments 54 2.3.5.2 Building the Comparative Models 54 2.3.5.3 Simulated Annealing 54 2.3.5.4 Model Evaluation 55 CHAPTER 3 EXPRESSION OF INSERT DOMAIN PROTEINS 3.1 INTRODUCTION 56 3.1.1 Project Summary and Aims 58 3.2.2 RESULTS 59 3.2.1 Expression of Thioredoxin-Insert Domain expressing vectors 59 3.2.1 Respective Summary and Aims 60 3.2.2.2 Expression of Thioredoxin-Insert Domain fusion protein 60 3.2.3.1 Expression of Thioredoxin-Insert Domain fusion protein 61 3.2.3.1 TRX-ID Purification by N=affinity chromatography 62 3.2.3.1 TRX-ID Purification by N=affinity chromatography 63 3.2.3.3 Thrombin Kinase digestion 63 3.2.3.4 REFOLDING STUDIES 65 3.2.4 LEXPOLUTIGN by Cele Filtration Chromatography 63		
2.3 4.11 High Performance Liquid Chromatography Analysis532.3.4.12 Thrombin Cleavage of fusion proteins532.3.4.13 Proteinase K Digestion532.3.4.14 NMR Spectroscopy542.3.5 Computational Methods for Comparative Modelling542.3.5 Computational Methods for Comparative Modelling542.3.5.1 Protein Sequence Alignments542.3.5.2 Building the Comparative Models542.3.5.3 Simulated Annealing542.3.5.4 Model Evaluation55CHAPTER 3 EXPRESSION OF INSERT DOMAIN PROTEINS563.1.1 NTRODUCTION563.1.1 Project Summary and Aims583.2 RESULTS593.2.1 Construction of recombinant insert domain expressing vectors593.2.2 Expression of Thioredoxin-Insert Domain fusion protein603.2.3.1 TRX-ID Purification by Ni-affinity chromatography623.2.3.2 TRX-ID Purification by Resource Q chromatography633.2.3.3 Thrombin Kinase digestion633.2.4 REFOLDING STUDIES643.2.3.4 REFOLDING STUDIES643.2.4 REFOLDING STUDIES653.2.4.1 Analysis of the State of ID by Gel Filtration Chronatography653.2.4.2 Proteinase K Assay653.2.4 REFOLDING REACTIVITY OF GROWTH FACTORS IN THE INSULIN SYSTEM704.1.1 Project Summary714.2.2 Construction of Full Length HIR Isoforms in Mammalian Expression Vectors724.2.1 Construction of Full Length HIR Isoforms in Mammalian Expression Vectors724.2.2 Constru		52
2.3.4 12 Thrombin Cleavage of fusion proteins 53 2.3.4 13 Proteinase K Digestion 53 2.3.4 14 NMR Spectroscopy 54 2.3.5 Computational Methods for Comparative Modelling 54 2.3.5 I Protein Sequence Alignments 54 2.3.5 I Protein Sequence Alignments 54 2.3.5 I Protein Sequence Alignments 54 2.3.5 J Building the Comparative Models 54 2.3.5 A Model Evaluation 55 CHAPTER 3 EXPRESSION OF INSERT DOMAIN PROTEINS 56 3.1 INTRODUCTION 56 3.1.1 Project Summary and Aims 58 3.2 RESULTS 59 3.2.1 Construction of recombinant insert domain expressing vectors 59 3.2.1 Expression of Thioredoxin-Insert Domain fusion protein 60 3.2.2 Expression of Thioredoxin-Fibronectin 3-Insert Domain fusion protein 61 3.2.3 TRX-ID Purification by N-affinity chromatography 62 3.2.3 TRX-ID Purification by N-affinity chromatography 62 3.2.3 TRX-ID Purification of ID 64		
2.34.13 Proteinase K Digestion 53 2.3.4.14 NMR Spectroscopy 54 2.3.5 Computational Methods for Comparative Modelling 54 2.3.5.1 Protein Sequence Alignments 54 2.3.5.2 Building the Comparative Models 54 2.3.5.3 Simulated Annealing 54 2.3.5.4 Model Evaluation 55 CHAPTER 3 EXPRESSION OF INSERT DOMAIN PROTEINS Set OLIPY COLSPAN Set OLIPY COLSPAN OLIPY COLSPAN Set Set OLIPY COLSPAN Set OLIPY COLSPAN OLIPY COLSPAN Set Set OLIPY COLSPAN Set OLIPY COLSPAN Set Set OLIPY COLSPAN Set Set Set Set Set Set <td></td> <td></td>		
2.3.4.14 NMR Spectroscopy542.3.5 Computational Methods for Comparative Modelling542.3.5 Computational Methods for Comparative Models542.3.5 Building the Comparative Models542.3.5 Mulated Annealing542.3.5 Mulated Annealing55CHAPTER 3 EXPRESSION OF INSERT DOMAIN PROTEINS563.1 INTRODUCTION563.1 INTRODUCTION563.1 Project Summary and Aims583.2 RESULTS593.2 1 Construction of recombinant insert domain expressing vectors593.2.2 PROTEIN EXPRESSION603.2.1 Construction of Thioredoxin-Fibronectin 3-Insert Domain fusion protein603.2.2.1 Expression of Thioredoxin-Fibronectin 3-Insert Domain fusion protein603.2.3 TRX-ID Purification by Ni-affinity chromatography623.2.3 TRX-ID Purification by Ni-affinity chromatography633.2.3 A Thrombin Kinase digestion633.2.3 A Thrombin Kinase digestion633.2.4 A HPLC Purification of ID643.2.3 SNR of Purified ID643.2.4 I Analysis of the State of ID by Gel Filtration Chronatography653.2.4.1 Analysis of the State of ID by Gel Filtration Chronatography653.2.4.2 Proteinase K Assay653.3 DISCUSSION67CHAPTER 4 CROSS-REACTIVITY OF GROWTH FACTORS IN THE INSULIN SYSTEM704.1.1 NTRODUCTION704.2.1 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors724.2.1 Construction of Full Length h		
2.3.5 Computational Methods for Comparative Modelling 54 2.3.5 Protein Sequence Alignments 54 2.3.5 Binulated Annealing 54 2.3.5 Simulated Annealing 54 2.3.5 Model Evaluation 55 CHAPTER 3 EXPRESSION OF INSERT DOMAIN PROTEINS 56 3.1 INTRODUCTION 56 3.1.1 Project Summary and Aims 58 3.2 RESULTS 59 3.2.1 Construction of recombinant insert domain expressing vectors 59 3.2.2 PROTEIN EXPRESSION 60 3.2.2.1 Expression of Thioredoxin-Insert Domain fusion protein 60 3.2.2.2 Expression of Thioredoxin-Fibronectin 3-Insert Domain fusion protein 61 3.2.3 TRX-ID Purification by Ni-affinity chromatography 62 3.2.3 Thrombin Kinase digestion 63 3.2.3 Thrombin Kinase digestion 63 3.2.3 Thrombin Kinase digestion 64 3.2.4 REFOLDING STUDIES 65 3.2.4 REFOLDING STUDIES 65 3.2.4 REFOLDING STUDIES 65 3.2.4 I Analysis of the State of ID by Gel Filtration Chroinatography 62 3.2.4 REFOLDING STUDIES 65 3.3 DISCUSSION 67		
2.3.5.1 Protein Sequence Alignments542.3.5.2 Building the Comparative Models542.3.5.3 Simulated Annealing542.3.5.4 Model Evaluation55CHAPTER 3 EXPRESSION OF INSERT DOMAIN PROTEINS563.1 INTRODUCTION57563.1 INTRODUCTION563.1.1 Project Summary and Aims583.2.2 RESULTS593.2.1 Construction of recombinant insert domain expressing vectors593.2.2 PROTEIN EXPRESSION603.2.1 Expression of Thioredoxin-Insert Domain fusion protein603.2.2.1 Expression of Thioredoxin-Insert Domain fusion protein613.2.3 PROTEIN PURIFICATION623.2.3 TRX-ID Purification by Ni-affinity chromatography633.2.3.1 TRX-ID Purification by Ni-affinity chromatography633.2.3.3 Thronbin Kinase digestion633.2.3.4 HPLC Purification of ID643.2.3.4 EPCD DING STUDIES653.2.4.1 Analysis of the State of ID by Gel Filtration Chromatography653.2.4.2 Proteinase K Assay653.2.4.3 Alteration of Refolding Conditions653.3 DISCUSSION704.1 INTRODUCTION704.1.1 Project Summary714.2.2 RESULTS AND DISCUSSION724.2.1 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors724.2.2 Construction of Recombinant hIR-B Ectedomain with a C-terminal Leucine Zipper72		
2.3.5.2 Building the Comparative Models542.3.5.3 Simulated Annealing542.3.5.4 Model Evaluation55CHAPTER 3 EXPRESSION OF INSERT DOMAIN PROTEINS563.1 INTRODUCTION51.1 Project Summary and Aims583.2 RESULTS593.2.1 Construction of recombinant insert domain expressing vectors593.2.2 PROTEIN EXPRESSION603.2.2 Expression of Thioredoxin-Insert Domain fusion protein603.2.2 Expression of Thioredoxin-Fibronectin 3-Insert Domain fusion protein613.2.3 PROTEIN PURIFICATION623.2.3 TRX-ID Purification by Ni-affinity chromatography633.2.3 TRX-ID Purification of ID643.2.3 Shorn of ID643.2.4 REFOLDING STUDIES653.2.4 REFOLDING STUDIES653.2.4 REFOLDING STUDIES653.2.4 REFOLDING STUDIES653.2.4 REFOLDING STUDIES653.2.4 REFOLDING STUDIES653.3 DISCUSSION67CHAPTER 4 CROSS-REACTIVITY OF GROWTH FACTORS IN THE INSULIN SYSTEM704.1 INTRODUCTION704.2.1 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors724.2.2 Construction of Recombinant HIR-B Ectedomain with a C-terminal Leucine Zipper72		
2.3.5.3 Simulated Annealing542.3.5.4 ModelEvaluation55CHAPTER 3 EXPRESSION OF INSERT DOMAIN PROTEINS563.1 INTRODUCTION563.1.1 Project Summary and Aims583.2.2 RESULTS593.2.1 Construction of recombinant insert domain expressing vectors593.2.2 PROTEIN EXPRESSION603.2.2.1 Expression of Thioredoxin-Insert Domain fusion protein603.2.2.2 Expression of Thioredoxin-Fibronectin 3-Insert Domain fusion protein613.2.3.1 TRX-ID Purification by Ni-affinity chromatography623.2.3.1 TRX-ID Purification by Resource Q chromatography633.2.3.3 Thrombin Kinase digestion633.2.3 A HPLC Purification of ID643.2.4 REFOLDING STUDIES653.2.4.1 Analysis of the State of ID by Gel Filtration Chroinatography653.2.4.3 Alteration of Refolding Conditions653.3 DISCUSSION67CHAPTER 4 CROSS-REACTIVITY OF GROWTH FACTORS IN THE INSULIN SYSTEM704.1 INTRODUCTION724.2.1 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors724.2.2 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors724.2.2 Construction of Full Length hIR-B Ectodomain with a C-terminal Leucine Zipper72		
2.3.5.4 Model Evaluation55CHAPTER 3 EXPRESSION OF INSERT DOMAIN PROTEINS563.1 INTRODUCTION563.1.1 Project Summary and Aims583.2 RESULTS593.2.1 Construction of recombinant insert domain expressing vectors593.2.2 PROTEIN EXPRESSION603.2.2.1 Expression of Thioredoxin-Insert Domain fusion protein603.2.2.2 Expression of Thioredoxin-Fibronectin 3-Insert Domain fusion protein613.2.3.1 TRX-ID Purification by Ni-affinity chromatography623.2.3.1 TRX-ID Purification by Resource Q chromatography633.2.3.3 Thrombin Kinase digestion633.2.3.4 REFOLDING STUDIES653.2.4.1 Analysis of the State of ID by Gel Filtration Chroinatography653.2.4.3 Alteration of Refolding Conditions653.2.4.3 Alteration of Refolding Conditions653.3 DISCUSSION67CHAPTER 4 CROSS-REACTIVITY OF GROWTH FACTORS IN THE INSULIN SYSTEM704.1 INTRODUCTION704.1.1 NTRODUCTION714.2 RESULTS AND DISCUSSION724.2.1 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors724.2.2 Construction of Full Length hIR-B Ectodomain with a C-terminal Leucine Zipper72		
3.1 INTRODUCTION563.1.1 Project Summary and Aims583.2 RESULTS593.2.1 Construction of recombinant insert domain expressing vectors593.2.2 PROTEIN EXPRESSION603.2.1 Expression of Thioredoxin-Fibronectin Jusion protein603.2.2 Expression of Thioredoxin-Fibronectin 3-Insert Domain fusion protein613.2.3 PROTEIN PURIFICATION623.2.3 Thrombin K inase digestion633.2.3 Thrombin K inase digestion633.2.3 Thrombin K inase digestion633.2.4 PIC Purification of ID643.2.3 S NMR of Purified ID643.2.4 REFOLDING STUDIES653.2.4.3 Alteration of Refolding Conditions653.3 DISCUSSION67CHAPTER 4 CROSS-REACTIVITY OF GROWTH FACTORS IN THE INSULIN SYSTEM704.1.1 Project Summary714.2.1 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors724.2.2 Construction of Recombinant hIR-B Ectodomain with a C-terminal Leucine Zipper72	2.3.5.4 Model Evaluation	55
3.1 INTRODUCTION563.1.1 Project Summary and Aims583.2 RESULTS593.2.1 Construction of recombinant insert domain expressing vectors593.2.2 PROTEIN EXPRESSION603.2.1 Expression of Thioredoxin-Fibronectin Jusion protein603.2.2 Expression of Thioredoxin-Fibronectin 3-Insert Domain fusion protein613.2.3 PROTEIN PURIFICATION623.2.3 Thrombin K inase digestion633.2.3 Thrombin K inase digestion633.2.3 Thrombin K inase digestion633.2.4 PIC Purification of ID643.2.3 S NMR of Purified ID643.2.4 REFOLDING STUDIES653.2.4.3 Alteration of Refolding Conditions653.3 DISCUSSION67CHAPTER 4 CROSS-REACTIVITY OF GROWTH FACTORS IN THE INSULIN SYSTEM704.1.1 Project Summary714.2.1 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors724.2.2 Construction of Recombinant hIR-B Ectodomain with a C-terminal Leucine Zipper72	CHAPTER 3 EXPRESSION OF INSERT DOMAIN PROTEINS	56
3.1.1 Project Summary and Aims583.2 RESULTS593.2.1 Construction of recombinant insert domain expressing vectors593.2.2 PROTEIN EXPRESSION603.2.2.1 Expression of Thioredoxin-Insert Domain fusion protein613.2.2.2 Expression of Thioredoxin-Fibronectin 3-lnsert Domain fusion protein613.2.3 PROTEIN PURIFICATION623.2.3.1 TRX-ID Purification by Ni-affinity chromatography623.2.3.2 TRX-ID Purification by Resource Q chromatography633.2.3.3 Thrombin Kinase digestion633.2.3.4 HPLC Purification of ID643.2.3.5 NMR of Purified ID643.2.4.1 Analysis of the State of ID by Gel Filtration Chromatography653.2.4.2 Proteinase K Assay653.2.4.1 Purification of Refolding Conditions653.2.4.1 Proteinase K Assay653.2.3 Alteration of Refolding Conditions653.3 DISCUSSION67CHAPTER 4 CROSS-REACTIVITY OF GROWTH FACTORS IN THE INSULIN SYSTEM704.1 INTRODUCTION724.2.1 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors724.2.1 Construction of Full Length hIR-B Ectodomain with a C-terminal Leucine Zipper72		
3.2 RESULTS593.2.1 Construction of recombinant insert domain expressing vectors593.2.2 PROTEIN EXPRESSION603.2.2.1 Expression of Thioredoxin-Insert Domain fusion protein603.2.2.2 Expression of Thioredoxin-Fibronectin 3-Insert Domain fusion protein613.2.3 PROTEIN PURIFICATION623.2.3.1 TRX-ID Purification by Ni-affinity chromatography623.2.3.2 TRX-ID Purification by Resource Q chromatography633.2.3.4 HPLC Purification of ID643.2.3.5 NMR of Purified 1D643.2.4 REFOLDING STUDIES653.2.4.1 Analysis of the State of ID by Gel Filtration Chromatography653.2.4.2 Proteinase K Assay653.2.4.3 Alteration of Refolding Conditions653.3 DISCUSSION67CHAPTER 4 CROSS-REACTIVITY OF GROWTH FACTORS IN THE INSULIN SYSTEM704.1.1 Project Summary714.2 RESULTS AND DISCUSSION724.2.1 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors724.2.2 Construction of Recombinant hIR-B Ectodomain with a C-terminal Leucine Zipper72		
3.2.1 Construction of recombinant insert domain expressing vectors593.2.2 PROTEIN EXPRESSION603.2.2.1 Expression of Thioredoxin-Fibronectin 3-Insert Domain fusion protein603.2.2.2 Expression of Thioredoxin-Fibronectin 3-Insert Domain fusion protein613.2.3 PROTEIN PURIFICATION623.2.3.1 TRX-ID Purification by Ni-affinity chromatography633.2.3.2 TRX-ID Purification by Resource Q chromatography633.2.3.3 Thrombin Kinase digestion633.2.3.4 HPLC Purification of ID643.2.3.5 NMR of Purified 1D643.2.4.1 Analysis of the State of ID by Gel Filtration Chromatography653.2.4.2 Proteinase K Assay653.2.4.3 Alteration of Refolding Conditions653.3 DISCUSSION67CHAPTER 4 CROSS-REACTIVITY OF GROWTH FACTORS IN THE INSULIN SYSTEM704.1 INTRODUCTION724.2.1 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors724.2.2 Construction of Recombinant hIR-B Ectodomain with a C-terminal Leucine Zipper72		
3.2.2 PROTEIN EXPRESSION603.2.2.1 Expression of Thioredoxin-Insert Domain fusion protein603.2.2.2 Expression of Thioredoxin-Fibronectin 3-Insert Domain fusion protein613.2.3 PROTEIN PURIFICATION623.2.3.1 TRX-ID Purification by Ni-affinity chromatography623.2.3.2 TRX-ID Purification by Resource Q chromatography633.2.3.3 Thrombin Kinase digestion633.2.3.4 HPLC Purification of ID643.2.3.5 NMR of Purified ID643.2.4.1 Analysis of the State of ID by Gel Filtration Chromatography653.2.4.2 Proteinase K Assay653.2.4.3 Alteration of Refolding Conditions653.2.4.3 Alteration of Refolding Conditions653.3 DISCUSSION67CHAPTER 4 CROSS-REACTIVITY OF GROWTH FACTORS IN THE INSULIN SYSTEM704.1.1 Project Summary714.2 RESULTS AND DISCUSSION724.2.1 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors724.2.2 Construction of Recombinant hIR-B Ectodomain with a C-terminal Leucine Zipper72		
3.2.2.1 Expression of Thioredoxin-Insert Domain fusion protein603.2.2.2 Expression of Thioredoxin-Fibronectin 3-Insert Domain fusion protein613.2.3.2 PROTEIN PURIFICATION623.2.3.1 TRX-ID Purification by Ni-affinity chromatography623.2.3.2 TRX-ID Purification by Resource Q chromatography633.2.3.3 Thrombin Kinase digestion633.2.3.4 HPLC Purification of ID643.2.3.5 NMR of Purified ID643.2.4 REFOLDING STUDIES653.2.4.1 Analysis of the State of ID by Gel Filtration Chromatography653.2.4.2 Proteinase K Assay653.2.4.3 Alteration of Refolding Conditions653.3 DISCUSSION67CHAPTER 4 CROSS-REACTIVITY OF GROWTH FACTORS IN THE INSULIN SYSTEM704.1.1 NTRODUCTION704.1.1 NTRODUCTION724.2.1 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors724.2.2 Construction of Recombinant hIR-B Ectodomain with a C-terminal Leucine Zipper72		
3.2.2 Expression of Thioredoxin-Fibronectin 3-Insert Donain fusion protein613.2.3 PROTEIN PURIFICATION623.2.3.1 TRX-ID Purification by Ni-affinity chromatography623.2.3.2 TRX-ID Purification by Resource Q chromatography633.2.3.3 Thrombin Kinase digestion633.2.3.4 HPLC Purification of ID643.2.3.5 NMR of Purified ID643.2.4.1 Analysis of the State of ID by Gel Filtration Chromatography653.2.4.2 Proteinase K Assay653.2.4.3 Alteration of Refolding Conditions653.3 DISCUSSION67CHAPTER 4 CROSS-REACTIVITY OF GROWTH FACTORS IN THE INSULIN SYSTEM704.1.1 Project Summary714.2 RESULTS AND DISCUSSION724.2.1 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors724.2.2 Construction of Recombinant hIR-B Ectodomain with a C-terminal Leucine Zipper72		
3.2.3 PROTEIN PURIFICATION623.2.3.1 TRX-ID Purification by Ni-affinity chromatography623.2.3.2 TRX-ID Purification by Resource Q chromatography633.2.3.3 Thrombin Kinase digestion633.2.3.4 HPLC Purification of ID643.2.3.5 NMR of Purified ID643.2.4.1 Analysis of the State of ID by Gel Filtration Chromatography653.2.4.1 Analysis of the State of ID by Gel Filtration Chromatography653.2.4.2 Proteinase K Assay653.2.4.3 Alteration of Refolding Conditions653.3 DISCUSSION67CHAPTER 4 CROSS-REACTIVITY OF GROWTH FACTORS IN THE INSULIN SYSTEM4.1 INTRODUCTION704.1.1 Project Summary714.2 RESULTS AND DISCUSSION724.2.1 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors724.2.2 Construction of Recombinant hIR-B Ectodomain with a C-terminal Leucine Zipper72		
3.2.3.1 TRX-ID Purification by Ni-affinity chromatography623.2.3.2 TRX-ID Purification by Resource Q chromatography633.2.3.3 Thrombin Kinase digestion633.2.3.4 HPLC Purification of ID643.2.3.5 NMR of Purified ID643.2.4 REFOLDING STUDIES653.2.4.1 Analysis of the State of ID by Gel Filtration Chromatography653.2.4.2 Proteinase K Assay653.2.4.3 Alteration of Refolding Conditions653.2.4.3 Alteration of Refolding Conditions653.3 DISCUSSION67CHAPTER 4 CROSS-REACTIVITY OF GROWTH FACTORS IN THE INSULIN SYSTEM4.1 INTRODUCTION704.1.1 Project Summary714.2 RESULTS AND DISCUSSION724.2.1 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors724.2.2 Construction of Recombinant hIR-B Ectodomain with a C-terminal Leucine Zipper72		
3.2.3.2 TRX-ID Purification by Resource Q chromatography633.2.3.3 Thrombin Kinase digestion633.2.3.4 HPLC Purification of ID643.2.3.5 NMR of Purified ID643.2.4 REFOLDING STUDIES653.2.4.1 Analysis of the State of ID by Gel Filtration Chromatography653.2.4.2 Proteinase K Assay653.2.4.3 Alteration of Refolding Conditions653.3 DISCUSSION67CHAPTER 4 CROSS-REACTIVITY OF GROWTH FACTORS IN THE INSULIN SYSTEM4.1 INTRODUCTION704.1.1 Project Summary714.2 RESULTS AND DISCUSSION724.2.1 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors724.2.2 Construction of Recombinant hIR-B Ectodomain with a C-terminal Leucine Zipper72		
3.2.3.3 Thrombin Kinase digestion633.2.3.4 HPLC Purification of ID643.2.3.5 NMR of Purified ID643.2.4 REFOLDING STUDIES653.2.4.1 Analysis of the State of ID by Gel Filtration Chromatography653.2.4.2 Proteinase K Assay653.2.4.3 Alteration of Refolding Conditions653.3 DISCUSSION67CHAPTER 4 CROSS-REACTIVITY OF GROWTH FACTORS IN THE INSULIN SYSTEM4.1 INTRODUCTION704.1.1 Project Summary714.2 RESULTS AND DISCUSSION724.2.1 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors724.2.2 Construction of Recombinant hIR-B Ectodomain with a C-terminal Leucine Zipper72		
3.2.3.4 HPLC Purification of ID643.2.3.5 NMR of Purified ID643.2.4 REFOLDING STUDIES653.2.4.1 Analysis of the State of ID by Gel Filtration Chronnatography653.2.4.2 Proteinase K Assay653.2.4.3 Alteration of Refolding Conditions653.3 DISCUSSION67CHAPTER 4 CROSS-REACTIVITY OF GROWTH FACTORS IN THE INSULIN SYSTEM4.1 INTRODUCTION704.1.1 Project Summary714.2 RESULTS AND DISCUSSION724.2.1 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors724.2.2 Construction of Recombinant hIR-B Ectodomain with a C-terminal Leucine Zipper72		
3.2.4 REFOLDING STUDIES653.2.4.1 Analysis of the State of ID by Gel Filtration Chroinatography653.2.4.2 Proteinase K Assay653.2.4.3 Alteration of Refolding Conditions653.3 DISCUSSION67CHAPTER 4 CROSS-REACTIVITY OF GROWTH FACTORS IN THE INSULIN SYSTEM4.1 INTRODUCTION704.1.1 Project Summary714.2 RESULTS AND DISCUSSION724.2.1 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors724.2.2 Construction of Recombinant hIR-B Ectodomain with a C-terminal Leucine Zipper72		
3.2.4.1 Analysis of the State of ID by Gel Filtration Chromatography653.2.4.2 Proteinase K Assay653.2.4.3 Alteration of Refolding Conditions653.3 DISCUSSION67CHAPTER 4 CROSS-REACTIVITY OF GROWTH FACTORS IN THE INSULIN SYSTEM704.1 INTRODUCTION4.1 INTRODUCTION704.1.1 Project Summary714.2 RESULTS AND DISCUSSION724.2.1 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors724.2.2 Construction of Recombinant hIR-B Ectodomain with a C-terminal Leucine Zipper72		64
3.2.4.2 Proteinase K Assay653.2.4.3 Alteration of Refolding Conditions653.3 DISCUSSION67CHAPTER 4 CROSS-REACTIVITY OF GROWTH FACTORS IN THE INSULIN SYSTEM4.1 INTRODUCTION704.1.1 Project Summary714.2 RESULTS AND DISCUSSION724.2.1 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors724.2.2 Construction of Recombinant hIR-B Ectodomain with a C-terminal Leucine Zipper72		65
3.2.4.3 Alteration of Refolding Conditions653.3 DISCUSSION67CHAPTER 4 CROSS-REACTIVITY OF GROWTH FACTORS IN THE INSULIN SYSTEM4.1 INTRODUCTION704.1.1 Project Summary714.2 RESULTS AND DISCUSSION724.2.1 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors724.2.2 Construction of Recombinant hIR-B Ectodomain with a C-terminal Leucine Zipper72		
3.3 DISCUSSION67CHAPTER 4 CROSS-REACTIVITY OF GROWTH FACTORS IN THE INSULIN SYSTEM4.1 INTRODUCTION704.1.1 Project Summary714.2 RESULTS AND DISCUSSION724.2.1 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors724.2.2 Construction of Recombinant hIR-B Ectodomain with a C-terminal Leucine Zipper72		
CHAPTER 4 CROSS-REACTIVITY OF GROWTH FACTORS IN THE INSULIN SYSTEM 70 4.1 INTRODUCTION 70 4.1.1 Project Summary 71 4.2 RESULTS AND DISCUSSION 72 4.2.1 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors 72 4.2.2 Construction of Recombinant hIR-B Ectodomain with a C-terminal Leucine Zipper 72		
4.1 INTRODUCTION704.1.1 Project Summary714.2 RESULTS AND DISCUSSION724.2.1 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors724.2.2 Construction of Recombinant hIR-B Ectodomain with a C-terminal Leucine Zipper72		
4.1.1 Project Summary714.2 RESULTS AND DISCUSSION724.2.1 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors724.2.2 Construction of Recombinant hIR-B Ectodomain with a C-terminal Leucine Zipper72		
4.2 RESULTS AND DISCUSSION724.2.1 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors724.2.2 Construction of Recombinant hIR-B Ectodomain with a C-terminal Leucine Zipper72		-
4.2.1 Construction of Full Length hIR Isoforms in Mammalian Expression Vectors724.2.2 Construction of Recombinant hIR-B Ectodomain with a C-terminal Leucine Zipper72		
4.2.2 Construction of Recombinant hIR-B Ectodomain with a C-terminal Leucine Zipper 72		
	4.2.3 Creation of Cell Lines Expressing Full Length hIR Isoforms	73

n Maria		
	2.4 Creation of a Cell Line Expressing Recombinant hIR-B EDZIP Isoform	74
4.	2.5 Development of a Receptor Competition Assay using Europium Labeled Ligands	75
	4.2.5.1 Europium Labelling Insulin, IGF-1 and IGF-2 4.2.5.2 Optimization of the Europium Competition Assay	75 76
4.	2.6 Comparison of Ligand Binding between Full-length hlR Isoforms in the Europium Competition	
	ssay	77
	4.2.6.1 The C and D domains of IGF-1 and IGF-2 Determine Receptor Specificity	79
	2.7 Comparison of Ligand Binding between Recombinant Soluble Ectodomain Isoforms in the Europic	
	ompetition Assay. 2.8 Comparison of Ligand Binding between Recombinant Soluble Ectodomain ZIP Isoforms in the	80
	uropium Competition Assay.	80
	2.9 Phosphorylation Assays	81
	2.10 Proliferation Assays	83
	2.11 Anti-Apoptosis Assays	84
4,	2.12 Creation of antibody that can differentiate between the insulin receptor isoforms 4.2.12.1 Characterisation of the polyclonal anti-exon 1 serum	85
435	UMMARY AND CONCLUSION	85 87
CHAP	TER 5 C-TERMINAL MUTANTS OF THE IGF-1R	89
	NTRODUCTION	89
	1.1 Project Summary and Aims RESULTS AND DISCUSSION	90
	2.1 Construction of hIGF-IR Cytoplasmic Mutant Plasmids	91 91
		91
	2.3 Competition Assays on Solubilised Receptors with IGF-1, 2	94
	2.4 Mutant Receptor Complexes Show Altered Amounts of Tyrosine Phosphorylation in Response to	
		95
	2.5 Effect of Phosphatase Inhibition on Tyrosine Phosphorylation2.6 Effect of Mutations on the Proliferative Potential of the Receptor	98 99
		.01
		02
CHAPT	TER 6 COMPARATIVE MODELLING OF <i>C. ELEGANS</i> INSULAN-LIKE PEPTIDES	\$ 3
6.1 II	NTRODUCTION	03
6.		05
		06
		06
6,		06 107
		107
	6.2.2.3 Building the Models	109
		111
		112
6.		14
		16
6.3 S	UMMARY AND CONCLUSION	18
CHAPT	FER 7 FINAL DISCUSSION1	19
		19
		21
	LOLE OF TYROSINE 1250 AND 1251 IN IGF-1R PHOSPHORYLATION 1 THE RELATIONSHIP OF INSULIN-LIKE PEPTIDES IN <i>C. ELEGANS</i> WITH MAMMALIAN	22
		23
		25
APPEN		39