THE SELECTION, ISOLATION, AND CHARACTERIZATION OF A CHINESE HAMSTER LUNG FIBROBLAST CELL LINE RESISTANT TO AN INSULIN-DIPHTHERIA A-CHAIN TOXIC CONJUGATE MOLECULE

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Abstract

The objective of this study was to determine how alterations in the insulin-receptor trafficking pathway affect ligand-dependent cellular action in Chinese hamster lung fibroblasts. Cell lines altered in their trafficking pathway were selected using a toxic insulin-diphtheria A-chain molecule (DTaI) which specifically interacts with the insulin receptor. One cell variant (IV-A1-j) which exhibited decreased insulin binding and enhanced insulin degradation did not demonstrate insulin or IGF-1 dependent cell proliferation. IV-A1-j cells also failed to produce an insulin-stimulated tyrosine phosphorylated protein (pp175). IGF-1 binding, insulin-stimulated hexose uptake, epidermal growth factor and thrombin stimulated cell proliferation were unaltered in IV-A1-j cells. These results suggest that the insulin and IGF-1 receptor may share a common pathway post-ligand binding for cell proliferation which does not involve EGF or thrombin, and that enhanced insulin degradation and loss of pp175 may be responsible for the loss of insulin-stimulated cell proliferation.

Résumé

Dans cette étude, nous tentons de comprendre de comment les changements dans le transport intracellulaire des complexes insuline-récepteur affectent le mécanisme d'action des ligands intracellulaires. Pour ce faire, nous avons utilisé comme modèle les fibroblastes de hamster chinois. En couplant la chaîne A de la toxine diphthérique (DTaI) au récepteur de l'insuline, nous avons sélectionné un clone, IV-A1 j, dont les récepteurs attachent faiblement l'insuline et dans lequel le taux de dégradation de l'insuline est augmenté. Nous démontrons d'une part que ce clone ne peut être induit à proliférer en présence d'insuline et d'IGF-1, et d'autre part que la présence d'insuline est incapable d'induire la phosphoprotéine pp175. Nous observons de plus que l'attachement de l'IGF-1, l'incorporation d'hexose induite par l'insuline et la réponse prolifératrice induite par la thrombine et le EGF restent normales. Nos résultants suggèrent que les récepteurs de l'insuline et IGF-1 partagent les mêmes voies métaboliques après attachement à un ligand autre que EGF et thrombine, et que l'augmentation de la dégradation de l'insuline et la perte de pp175 résulte probablement de la perte de toute réponse prolifératrice à l'insuline

This work is dedicated to my parents for their understanding, encouragement, support, and love.

"The important thing is to not stop questioning."

- Albert Einstein

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I would like to acknowledge my sincere gratefulness to Dr. Ralph Germinario, my Research Supervisor for his guidance and support through out the course of this work, as well as for his expertise and criticism in many related topics.

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Abbreviations

2-DG	2-deoxy-D-glucose (sugar analogue for hexose transport studies)
3-OMG	3-O-methyl-glucose (sugar analogue for hexose transport studies)
α-IR3	Insulin-like growth factor receptor antiboby for the α-chain (not insulin receptor)
A/K1018	cell line expressing an amino acid change of lysine (A) to phenyl-alanine (K) at position 1018 of the β-subunit of the insulin receptor
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
cDNA	Complementary deoxyribonucleic acid
CHL	Chinese hamster lung fibroblasts
CHO	Chinese hamster ovary cells
DT	Diphtheria toxin
DTa	A-cha n of diphtheria toxin
DTaI	Insulin diphtheria toxin A-chain toxic molecule (SPDP-linked)
DTb	B-chain of diphtheria toxin
EDAC	Ethyl-3-(dimethylaminopropyl) carbodiimide-hydrochloride
EF-2	Elongation factor - 2
EGF	Epidermal growth factor
EGF-DTa	A-chain of diphtheria toxin A-chain linked to epidermal growth factor
EGF-RTa	A-chain of ricin toxin linked to epidermal growth factor
EMS	Ethyl-methyl sulfonate
FGF	Fibroblast growth factor
GAP	Guanine nucleoside triphosphatase-activating protein
Glc	Glucose
GlcNac	N-Acetyl-glucosamine
GTP	Guanidine triphosphate
hIR	Human insulin receptor
IGF-I	Insulin-like growth factor type 1
IGF-II	Insulin-like growth factor type II
IP	Insulin protease (insulinase)
kDa	Kilo-dalton

MAP	Microtubule-associated protein kinase
Mr	Relative molecular weight (as based on molecular weight standards)
mRNA	Messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
PAGE	Polyacrylamide gel electrophoresis
PAO	Oxophenylarsine
PDGF	Platelet-derived growth factor
ppXXX	Tyrosine phosphorylated protein of XXX,000 molecular weight (Mr or Da)
RTa	A-chain of ricin toxin
SPDP	n-succinimidyl-3-(2-pyridyldithio)-propionate
TGF	Transforming growth factor
THR	Thrombin growth factor
TK	Tyrosine kinase

Preface

The thesis presented herein, is written in the form of original papers. The provision from the Thesis Guidelines reads as follows: "The candidate has the option, subject to the approval of the Department, of including as part of the thesis of the text, or duplicated published text (see below), of an original paper, or papers. In this case the thesis must still conform to all other requirements explained in Guidelines Concerning Thesis Preparation. Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail (e.g. appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported. The thesis should be more than a mere collection of manuscripts published or to be published. It must include a general abstract, a full introduction, and literature review and a final overall conclusion. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interests of cohesion."

"It is acceptable for theses to include as chapters authentic copies of papers already published, provided these are duplicated clearly on regulation thesis stationary and bound as an integral part of the thesis. Photographs or other materials which do not duplicate well must be included in their original form. In such instances, connecting texts are mandatory and supplementary explanatory material is almost always necessary."

"The inclusion of manuscripts co-authored by the candidate and others is acceptable but the candidate is required to make an explicit statement on who contributed to such work and to what extent, and supervisors must attest to the accuracy of the claims, e.g. before the Oral Committee. Since the task of the Examiners is made more difficult in these cases, it is in the canidates interest to make the responsibilities of the authors perfectly clear. Candidates following this option must inform the Department before it submits the thesis for review."

As a result, three original papers are presented here, all of which have been submitted for publication. Chapters 2 to 4 which contain the manuscripts include abstracts, introductions, materials and methods, results, and discussions. In accordance to McGill University's thesis guidelines this thesis also includes a general abstract (résumé), claim for original work, introduction (review of the liturature), and general discussion.

References and figures appear at the end of each chapter and are grouped in the order that they appear in the text. The three original papers are as follows:

Chapter 2: Leckett, B. and Germinario, R.J. (1991) Construction of a highly specific toxic insulin molecule: Selection and partial characterization of cells Resistant to its killing effects. Submitted to *Biochim. Biophys Acta* , July 1991.

Chapter 3: Leckett, B. and Germinario, R.J. (1991) The growth promoting effects of insulin and IGF-1 occur at a step post-ligand binding and are independent of other growth factors in a Chinese Hamster lung fibroblast cell line. Submitted to *Exp. Cell Res.*, July 1991.

Chapter 4: Leckett, B. and Germinario, R.J. (1991) Tyrosine phosphorylated substrate pp175 and insulin degradation may be involved in insulin and IGF-1 dependent cell growth in chinese hamster lung fibroblasts Submitted to *J. Biol. Chem.*, July 1991.

The candidate was responsible for all the work described in this thesis Use of the terms "we" and "our" refers to myself and my supervisor. Funding for this work was from the Medical Research Council of Canada. All work was performed in the laboratory of Dr. Ralph J. Germinario.

Claim For Original Work

The following elements of this thesis represent claims for original work.

1. SPDP was used as a crosslinking reagent to construct a unimolecular DTaI molecule. The highly specific nature of this molecule towards the insulin receptor permits its use in cell systems containing both insulin and IGF-1 receptors.

2. The isolation and characterization of DTaI-resistant/DT-sensitive cell variants which have a defect in their ability to grow in serum containing medium suggests that the insulin-receptor[1] pathway has components (regulators) which are essential for the functioning of other growth factor receptors.

3. The isolation of a single DTaI-resistant mutant (IV-A1-j) and its characterization in a hormonally-defined medium gives clear indication that insulin and IGF-1 share a common pathway post-receptor binding, and that other growth factors such as EGF and THR are not related to this pathway. Previous reports employing a hormonally-defined system have supported these conclusions based on speculations in cell lines which show additive or synergistic effects with different hormones. IV-A1-j cells show a complete inability to grow with either insulin or IGF-1.

4. In conjunction with statement 3, we have isolated a unique and naturally occurring mutant, which maintains its insulin modulated metabolic pathway, but has a disruption in its insulin-mediated mitogenic pathway. No such mutant has been previously isolated.

6 Our studies of this mutant show that a 175,000 molecular weight insulin-inducible endogenous tyrosine phosphorylated protein is not associated with insulin-stimulated hexose uptake, but may be involved in eliciting a mitogenic response specific to insulin and IGF-1 and not EGF or THR.

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[1] *Insulin-receptor* describes both insulin and the insulin receptor.

Chapter 1.
Review of Literature and Introduction

1.1 Preface to Chapter 1

The purpose of this project was to study the various pathways that may be involved in insulin action. For this, the following questions were addressed:

> The trafficking pathway for the insulin receptor (i.e. receptor expression ligand binding, internalization and intracellular routing) is well established, yet what parts of this pathway are important in insulin action?
>
> Can we derive cellular mutants that will have defects at the level of insulin receptor expression, binding, internalization, or intracellular routing?
>
> In what way is the insulin response pathway affected in cells exhibiting alterations in the insulin-receptor trafficking pathway? Can we determine what parts of the insulin-receptor trafficking pathway are important for eliciting insulin-dependent metabolic and mitogenic cellular action?

The trafficking of insulin and its receptor are fairly well characterized, as well as the observations seen for insulin-dependent action. Yet understanding how the insulin-receptor trafficking pathway[2] is coupled to insulin-dependent cellular effects is lacking. In order to better understand the relationship between the insulin-receptor pathway and cellular action, an approach was taken which involved the construction of an insulin-diphtheria A-chain (DTaI)[3] toxic molecule. In order for this molecule to prove toxic to the cell, it must first enter through the insulin receptor pathway from which the diphtheria A-chain (DTa) can be released into the cytoplasm and inhibit protein synthesis. Thus, the DTaI molecule can be used as a selection agent to isolate DTaI-resistant cell variants. With regards to the questions cited above, the hypothesis is that cells which are DTaI-resistant will have a defect in the insulin receptor pathway either at the level of insulin receptor expression, insulin binding, internalization, or compartmentalization, and that a defect at one of these levels would disrupt at least one insulin-dependent cellular effect. Hence, cell variants of this nature can be used in dissecting out the various mechanisms involved in insulin action as well as to study the relationships involved between the insulin-receptor pathway and insulin action.

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[2] Note that the term *pathway* refers to the expression, internalization, and fate of the receptor and/or its ligand. This should not be confused with the term *response pathway* which involves second messengers and ligand-dependant cellular resposes.

[3] All abbreviations can be found on page xiv of this thesis.

This chapter covers a review of the current concepts regarding insulin-receptor trafficking and insulin action. A section on insulin-like growth factor type one (IGF-1) and other growth factors, and their receptors is also included (chapter 3). Section 1.7, *Toxic Insulin Molecules and Insulin Action* contains a review on diphtheria toxin (DT) (the toxin used in this project) and toxic ligand molecules used for studying ligand-dependent action. When possible, review articles have been cited rather than individual papers.

1.2 Insulin

1.2.1 Introduction

Insulin structure is presented in this section, since it is crucial to understanding the procedure used in constructing the toxic conjugate molecule[4]. In addition to this a brief history towards the isolation and characterization of the insulin molecule has been included.

1.2.2 History

The first purification of insulin was obtained by Banting and Best in 1925 [1]. This preparation was far from being pure and it was not until 1935 that the first successful X-ray photographs of air-dried insulin crystals were studied [54]. These studies along with Svedberg's calculation of molecular weight using an ultracentrifuge gave insulin a mass of 35,100 - 37,600 [55]. The crystals showed a trigonal symmetry, which divided insulin into a molecular weight of 12,000. Only 25 years later did additional evidence lead to the discovery of the amino acid sequence which then showed that insulin was actually a 6,000 molecular weight molecule [55]. By the early 1960's, insulin could be synthesized. Although this eventually led to helping in the treatment of diabetic individuals, it did not help with elucidating the structure of the molecule. It was only between the years of 1960 and 1975 with improved X-ray crystallography and electron density map analysis, that insulin's structure was finally determined [55,76,174]. Below is a brief description of what we know about the structure of the insulin molecule.

1.2.3 Insulin Molecule Structure

Human insulin is synthesized as a high molecular weight peptide (pre-proinsulin), processed as an intermediatory precursor (proinsulin), and then cleaved in two [215,216]. Though insulin is solely secreted from β-cells located in the islands of Langerhans of the pancreas [297], there is supporting evidence which suggests that insulin can be

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[4] Author's note: Pay particular attention to the location of the amino-terminals and lys residue in the insulin molecule with regards to the insulin binding domain. These contain amino groups which were chemically modified for conjugation to the toxin molecule.

synthesized in lung, intestine, and the central nervous system [233,243]. Human insulin has a molecular weight of approximately 6,000 (there are small differences between species) and consists of two chains held together by two sulfhydryl bonds (Figure 1). The A chain consists of 21 amino acids and possesses an intra-sulfhydryl bridge between amino acids 6 and 11. This area is responsible for the differences observed in the amino acid composition between species. The B-chain is comprised of 30 amino acids. Species differences for the B-chain occur only at the carboxy-terminal end. The only difference between human insulin and porcine insulin lies in the substitution of a threonine for an alanine amino acid at the carboxy-terminal end of the B-chain.

The bio-active region of the insulin molecule has been determined by studying receptor binding by using a number of insulin analogues. These include insulins from different animal species [101], chemically or enzymatically altered insulins [28], synthetic insulins [109], mutated insulins from insulin-resistant patients [238,260,273], as well as proinsulin, its intermediates and other peptides from the same family (i.e. insulin like growth factors (IGF) one and two, somatomedins, and relaxin) [40]. Insulin molecules modified in the carboxy-terminal region of the B-chain show that the aromatic amino acids from B24 to B26 are important in binding to its receptor [28,109,268]. This also holds true for the amino-terminal sequence of the A-chain. The glycine at position A1 is directly involved in receptor binding and maintenance of the insulin molecule's confirmation [19,85]. Figure 2 shows the structure of the insulin molecule with its binding recognition site based on the studies above. In addition to this, insulin also appears to have specific residues (region) that induce both a positive cooperative interaction between receptors [73] and negative cooperativity [72]. Another region appears to have an alternate receptor binding region for growth promoting activity [99]. Further information on insulin-receptor interaction is discussed in section 1.4.3.

1 2 4 Summary

Insulin, secreted from the pancreas, was first isolated by Banting and Best in 1925 [1]. After nearly half a century, the structure of this insulin molecule was elucidated Its 6,000 molecular weight mass is comprised of an A-chain (21 amino acids) and a B-chain (30 amino acids) held together by two intra- and one inter-sulfhydryl bonds. Lastly, studies involving the use of insulin analogues have helped in locating the receptor binding region of the insulin molecule. These studies have shown that amino acids 24 to 26 of the B-chain and the amino terminal of the A-chain are extremely important in maintaining the molecule's structural integrity and hence, binding capabilities

1.3 Insulin Action

1.3.1 Introduction

This section involves an overview of the type of effects that insulin can elicit on a cell. It should be pointed out that this section only deals with observed effects and not the mechanisms of insulin action. Mechanisms of insulin action are covered in section 1.6. Additionally, not all cells generate the same insulin response characteristics. A fat cell will act differently from a liver cell, where in turn, both will exhibit responses not observed in insulin non-target tissues such as fibroblasts. In order for insulin to produce an effect it must first specifically bind to its receptor. Information dealing with insulin binding to its receptor can be found in section 1.4.3. Once insulin is bound, the insulin-receptor complex will undergo a series of steps to produce a number of physiological responses. These are extremely complex and can occur from within a few minutes to periods of up to 24 hours. Nonetheless, one way in which these responses can be classified is at the cellular level. For this, insulin responses can be divided into two categories; (1) short-term metabolic or maintenance responses, and (2) long-term mitogenic or growth promoting responses [46,53,99,182]. These two classifications of insulin-dependent cellular responses are discussed below.

1.3.2 Metabolic Effects of Insulin Action

One of the first observations made while studying insulin's effect on the cell was carbohydrate metabolism. This is due to three observations: (1) an increase in glucose metabolism, (3) a decrease in blood glucose, and (3) an increase in glycogen stores of various tissues [120]. The most popular and most studied of these aspects was the ability of insulin to increase the rate of glucose uptake into the cell [96,103,161]. The time for this to occur after insulin is recognized by its receptor can vary from 3 to 5 minutes for adipocytes [96,161] to 15 to 30 minutes for human skin fibroblasts [56,103]. Even though methods for studying this particular response initially proved to be difficult due to the rapid rate at which glucose became phosphorylated and lost into the cell as other intermediates, sugar analogues such as 2-deoxy-D-glucose (2-DG) and 3-O-methyl-D-glucose (3-OMG) have been constructed to overcome this problem [56,96,103,161,251]. Other insulin-dependent effects involve the phosphorylation and dephosphorylation of

various proteins within the cell. This phosphorylation occurs on serine, threonine, and to a lesser degree, tyrosine groups, the general consensus being that insulin would activate protein phosphatases and inhibit protein kinases. Nonetheless, the type and degree of phosphorylation that occurs for cellular proteins will largely depend on the cell type. For example, insulin in the liver is known to cause an increase in glycogen synthetase by a dephosphorylation of the enzyme [251], where the phosphorylation of phosphorylase causes a feedback in the degree of phosphorylation, which in turn causes the breakdown of glycogen [170]. Other phosphorylated proteins are acetyl-CoA carboxylase [23], ATP-citrate lyase [3], and S-6 ribosomal protein [204]. Pyruvate dehydrogenase is yet another example of an insulin-dependent protein dephosphorylation [143,305]. These are described in section 1.5.2.

1.3.3 Mitogenic Effects of Insulin Action

Insulin has long been known to be an important growth factor for the maintenance of cells in culture [137,140,203,220,270,306,324]. The way in which insulin may do this can be separated into three categories. One, the addition of insulin to the cell causes an increase in amino acid transport [139,140]; two, there is increased translation of messenger RNA [137]; and three, insulin causes an increase in DNA synthesis, which in turn, further increases the level of RNA and protein synthesis [137,220,234,270,306]. One of many possible ways in which insulin may mediate these effects is through the phosphorylation of various proteins (i.e. ribosomal-S6 kinase [58], microtubule associated protein (MAP) kinase [232], and small molecular weight peptides [88]). Section 1.5 covers this aspect in detail. Thus, not only is insulin an important peptide hormone for cell maintenance, but it is also appears valuable for regulating cell growth and differentiation.

1.3.4 Summary

Insulin is known to produce a complex number of cellular effects. These can be divided into metabolic and mitogenic cellular effects. The most commonly known metabolic (or maintenance) effect is increased insulin-dependent hexose uptake. In addition to this, there are a number of other metabolic effects which involve the phosphorylation and dephosphorylation of various cellular proteins. At the mitogenic level, phosphoryalation may also be involved for insulin-dependent protein, RNA, and DNA synthesis.

1.4 Insulin Receptor

1.4.1 Introduction

To better understand diabetes, one area of research has been to focus on the insulin receptor itself. This has been at the level of receptor structure, insulin-receptor interactions, internalization, and fate, in order to see if there are any alterations between normal and diabetic cells [36,92,126,142,150,160,225,263,269,277,287,290].

The study of the insulin receptor has not been easy, for the amount of receptor present in most cells constitutes less than 0.01% of the total protein [126]. Only within the last 10 to 15 years have a number of photoaffinity and chemical cross-linking methods been developed to selectively identify, purify, and study the insulin receptor [92,143]. In addition to this, a number of antibodies which bind either to the α or β subunits are available [290]. These antibodies can be used to detect, isolate, and quantitate the number of insulin receptors that are present in or on cells and tissue [126]. More recently molecular genetics has been employed in elucidating the sequence of the receptor which further aids in determining its structure [113].

1.4.2 Insulin Receptor Structure

The insulin receptor after processing and insertion into the plasma membrane consists of two subunits (Figure 3). The α-subunit (125,000 - 130,000 Mr) contains the insulin binding domain, where the β-subunit (90,000 Mr) contains putative phosphorylation sites and a tyrosine kinase activity. The α-subunit is located on the extracellular surface of the plasma membrane and the β-subunit transverses the cell membrane. A dimer of two α- and two β-subunits held together by an inter-α-α and two inter-α-β disulfide bonds makes up the insulin receptor. The α-subunit is known to possess 14 potential N-linked glycosylation sites, where the β-subunit has four such sites located on the extracellular portion of the molecule [83,287]. The insulin receptor is also known to contain at least one O-linked glycosylation site as well [48]. This model currently supports the idea that up to two insulin molecules can bind to each half of the insulin receptor giving the receptor a multivalent nature [126]. Nonetheless, it should be pointed out there exists other subunit stoichiometries of the insulin receptor as well [116].

1.4.3 The Insulin Receptor Trafficking Pathway

The insulin receptor pathway can be divided into four steps. These are: (1) synthesis of the receptor, (2) processing of the receptor and its expression at the plasma membrane, (3) internalization after ligand interaction, and (4) receptor compartmentalization, trafficking, and degradation.

The human insulin receptor (hIR) gene is located on the short arm of chromosome 19, spans a region of greater than 120,000 base pairs, and contains 22 exons [249,250,308. The 5'-flanking region of the gene contains no *TATA* or *CAAT* box sequences [179,186,249], yet does contain multiple G-C rich regions which could indicate Spl binding sites [179,186]. Spl binding sites are found in a number of constitutively expressed housekeeping genes indicating that the insulin receptor could be a housekeeping protein [113]. The cDNA of the insulin receptor gene encodes a single proreceptor that consists of the α-subunit and the β-subunit separated by four basic amino acids (ArgLysArgArg) which are proteolytically cleaved before undergoing fatty acylation, glycosylation and insertion into the plasma membrane [76,124,148] Glycosylation has been shown to be important in the expression of the insulin receptor at the cell surface. For example, a dolichol phosphate intermediate is necessary for the transfer of a $Glc_3Man_9GlcNac_2$ oligosaccharide to the asparagine of the insulin receptor protein. The $Glc_3Man_9GlcNac_2$ oligosaccharide undergoes a modification to a tertiary complex which initially involves the removal of three glucose moieties. If the removal of these glucose moieties is blocked, there is a dramatic reduction in the number of surface-associated insulin receptors [7,82]. The role that amide-linked and ester-linked fatty acids play in the processing or function of the insulin receptor is not known [123] Furthermore, little is known about the role of O-linked glycosylation on the insulin receptor, except that it is merely present [48,113].

In some instances diabetics that have extreme insulin resistance such as leprechaunism and Rabson-Medenhall syndrome, are known to have a decrease in the number of surface-associated insulin receptors [15,263,277,278]. This can occur from a decrease in the level insulin receptor mRNA, impaired transport of insulin receptors to the surface, nonsense mutations, increased receptor degradation, and/or impaired receptor recycling [206,263]. Decreased levels of mRNA could be attributed to mutations at the regulatory and intron exon regions which would affect the Spl binding and impair RNA splicing, respectively. Nonsense mutations and mutations which increase mRNA

degradation could also cause a decrease in insulin receptor mRNA levels [206,263]. It is even possible that structural defects in the insulin receptor could alter its trafficking mechanism after endocytosis resulting in increased degradation of itself and/or even insulin.

Once the insulin receptor is expressed on the surface of the cell it is capable of binding insulin in a saturable manner. Steady-state binding experiments and Scatchard analysis on IM-9 lymphocytes showed that insulin-receptor interactions give rise to two types of binding sites [72]; a high capacity, low affinity binding site and a low capacity high affinity binding site. An alternate model that supports this observation of different affinities is known as negative cooperativity [209], stating that the affinity for the receptor towards insulin is decreased as a function of its occupancy. Nonetheless, the exact role that this cooperativity or different receptor subtypes may play in insulin-dependent cellular effects has not been established [208,209].

After binding, the insulin-receptor complex will undergo a conformational change which leads to the autophosphorylation of the β-subunit of the insulin receptor [11,225]. Whether β-subunit autophosphorylation is a cause or an effect of the conformational change of the insulin-receptor complex is not known. At this step the complex is internalized into the cell [11,225]. At least two types of insulin-resistant patients have been reported as having defects in their insulin receptor function at the level of insulin binding [158,160]. The first involves a point mutation of a serine to an arginine for the last amino acid in the proteolytic cleavage site. This mutation does not allow for the cleavage of the proinsulin receptor in its respective α- and β-subunits. In this case the proinsulin receptor gets inserted into the plasma membrane, but its binding affinity is greatly decreased [160]. In the second case, cultured skin fibroblasts from a patient with a point mutation in the α-subunit of the insulin receptor, have also shown decreased insulin binding [158]. This mutation may structurally alter the insulin receptor itself or its insertion into the plasma membrane, since solubilized receptors bind insulin normally. Further study on this particular mutation has yet to be performed.

There are three possible locations where the insulin receptor can bind insulin before being internalized. The first location which is common for most cell types, is on microvilli (i.e. cultured human lymphocytes [35], primary rat hepatocytes [33], and 3T3-L1 adipocytes [90,92]). Other cell types (i.e. human fibroblasts [5,217]) exhibit hormone binding on a relatively flat surface (second location) in which the receptors may or may not get localized to the third location; clathrin coated pits. In most cell types insulin binds

preferentially to receptors inserted on microvillous structures, then redistributed to coated pits before being internalized. Insulin can also be internalized from non-coated pits, and it may be that this route of entry is subject to different intracellular processing. It has been suggested that there exists two separate pathways for the degradation of insulin. The first is known as the lysosomal, chloroquine-sensitive route, where the other is a chloroquine-insensitive route [18]. It has also been observed in liver cells that the concentration of insulin can affect the rate of insulin-receptor internalization, such that at high concentrations, the rate of internalization increases [27]. Whether this difference is due to binding to different subpopulations of insulin receptors or the distribution and localization of activated (ligand bound) insulin receptors is unknown. The next step involves internalization and endosomal compartmentalization which results in the formation of primary endocytic vesicles that are approximately 0.05 - 0.08 mm in diameter [141]. Thus, insulin receptor-mediated endocytosis is a cell-directed mechanism which allows the entry of insulin into the intracellular vesicular system [17,21,190]. Shortly after the formation of these coated vesicles, large smooth polymorphic structures appear [17,21,132,181]. These structures which are presumably the next step in endocytosis, have been classified by many terms (i.e. receptosomes [181,302], compartment of uncoupling receptor-ligand or CURL [105], pre-lysosomal organelles [271], endosomes [134], sorting vesicles [112], and non-coated vesicles [114]) and are involved in insulin-receptor complex disassociation. Uncoupling is a process that occurs over the next few steps by an acidification process which lowers the pH to 6.0. This alters the affinity of the receptor to bind insulin and/or by slight charge-related changes in the insulin molecule itself [189]. At this point, the receptor can either be degraded, sequestered within the cell, or recycled back to the plasma membrane [115,172]. After uncoupling of insulin from its receptor, it appears that the insulin molecule may or may not continue on into lysosomes, where the receptor remains either in multivesicular bodies or enters some other unidentified tubulovesicular compartment involved in recycling [83]. Studies by Doherty *et al.* which involved the developement of a cell-free system to assess the degradation of [^{125}I]-insulin, showed that endosomal insulin degradation was inhibited by acidotropic agents (see section 1.4.4) such as nigericin, monensin, and chloroquine or carboxylic ionophores (section 1.4.4) such as N-ethylmaleimide and dicyclohexylcarbodiimide [77]. Results also indicated that the endosomal protease(s) was insulin-specific, since both TCA precipitation and Sephadex G-50 chromatography where unable to detect any degradation of either internalized

[^{125}I]-epidermal growth factor or internalized [^{125}I]-prolactin. Furthermore, polyethylene glycol precipitation of insulin-receptor complexes showed that endosomal degradation enhanced the dissociation of insulin from its receptor and that only free insulin could serve as substrate to the endosomal protease(s). In some tissues a large amount of undegraded insulin can be recycled back to the surface [79], where in some cells the endosome contains the lysosomal enzyme Cathepsin D. Thus, even though these vesicles appear to be lysosomes, they are actually involved in receptor and membrane recycling [224]. The amount of degraded insulin within the cell is usually less than 5% for it is transported rapidly out of the cell and into the medium [108]. On the other hand, in some cells types (i.e. hepatocyctes) surface degradation may constitute up to more than 50% of the total amount of insulin degraded [79]. A number of potential enzymes have been isolated for the degradation of insulin and are described in section 1.6.3. Once insulin degradation is initiated in the endosomes, partially degraded molecules can then enter other vesicles (lysosomes?) for further degradation, where intact, partially degraded insulin, or completely degraded insulin can migrate to other parts of the cell such as the nucleus or endoplasmic reticulum [79,224]. Naturally occurring mutant alleles that cause a defect in receptor internalization and compartmentalization are extremely rare, thus a number of biochemical techniques have been employed which induce a wide variety of mutants at this stage of the pathway (see below). Nonetheless, accelerated receptor degradation has been seen in a patient with leprechaunism [263]. In this individual glutamic acid is substituted for lysine at position 460 in the α-subunit. *In vitro* the patient's cells exhibited a 5-fold increase in receptor binding affinity and showed normal stimulation of insulin-dependant tyrosine kinase activity. Yet *in vivo* this patient was known to be insulin insensitive. This is because the mutation made the receptor more stable to changes in pH such that fewer insulin molecules become uncoupled from its receptor. The end result was that more receptors enter the degradative pathway, rather than the recycling pathway [63,86,263].

1.4.4 Methods for Studying the Insulin Receptor Trafficking Pathway

One approach for studying this pathway has been to block insulin-receptor internalization and/or cellular routing through environmental or biochemical conditioning. Temperature shift experiments, one example of an environmental alteration, has been used quite extensively for studying insulin receptor binding and

internalization [32,104,112]. When cells are incubated at 37°C, insulin receptors are internalized rapidly into multivesicular bodies and lysosomes. Between temperatures of 14°C to 16°C, receptors are only internalized into endosomal compartments. At temperatures ranging from 0°C to 4°C, internalization is almost, if not completely, blocked. Thus for examining the binding kinetics of insulin receptors in different cell types, complications that arise from internalization are virtually eliminated by performing binding studies at temperatures between 0°C and 4°C. Environmental factors other than temperature also influence insulin receptor expression and must be taken into consideration when performing experiments which involve insulin binding and action. For example, cells placed in serum-free medium have elevated levels of surface-bound insulin receptors, where confluent and logarithmically growing cultures will differ with respect to insulin receptor expression [235,239].

Biochemical alterations are less favorable than environmental alterations since the former have more drastic effects on the cell's functional machinery. One example is bacitracin, a polypeptide antibiotic which inhibits membrane receptor internalization [269]. Although less preferred over temperature shift experiments, it has been used for studying binding kinetics [269]. Glycosylation inhibitors are another set of agents for analyzing receptor fate and recycling. The most popular chemical agent used in this category is tunicamycin, an antibiotic produced by *Streptomyces superificus* which inhibits the first reaction of the lipid-linked saccharide pathway [52,283]. There are many disadvantages to using tunicamycin. The foremost is that tunicamycins with different fatty acid side chains can be purchased. The result is that some derivatives will be more toxic than others and result in the inhibition of protein synthesis (A. Hercovics, per. commun.). Thus, observations thought to be a consequence of post-translational modification, may in fact be due to decreased protein synthesis of the receptor itself, or the inhibition of certain unknown regulatory proteins. There are many other glycosylation inhibitors (i.e. glucose and mannose analogues, bromoconduritol, and showdomycin) which are less toxic and more specific in inhibiting the various types (and steps) of glycosylation [87]. Yet a number of them work by unknown mechanisms and can quite possibly elicit other effects on the cell's physiological function. Inhibitors of vacuolar acidification have also successfully been used in studying the various steps of endosomal compartmentalization by producing blocks at the level of internalization. One set of vacuolar drugs known as

lysosomotropic or acidotropic agents[5] [69], increase the vacuolar pH by as much as one or two units. These drugs (i.e. ammonium chloride, chloroquine, and methylamine) are weak lipophilic bases which, when added to the culture medium, enter passively through the cell membrane and into vacuolar structures. Once within the acidic environment, they become protonated and are unable to diffuse back out. The use of acidotropic agents in examining membrane receptor fate is extremely difficult since drug concentrations, organelle acidity, and extracellular pH are all contributing factors to their effects [189]. Some of these effects include the inhibition of lysosomal degradation [69], disruption of exo or endocytosis [133], and alterations in endosomal recycling and sorting [281]. Thus, their actions on the cell's various biological mechanisms are not specific. Furthermore, a number of undesirable side-effects occur such as vacuolar swelling [310], inhibition of intracellular fusion events [66], and the activation or inhibition of various enzymes such as transglutaminase [62]. Carboxylic ionophores (i.e. monensin, nigericin, and X537A) have also been used as a means in elevating the vacuolar pH [14,275] by the exchange of potassium ions for protons [228]. However, these exchanges have a differential effect for each organelle (i.e. golgi function is more sensitive at lower ionophore concentrations than other organelles [276]) for no apparent reason. Thus, acidotropic agents are preferentially used over carboxylitic ionophores.

For quantifying insulin receptor expression two approaches have been used quite successfully. The first approach which has been extensively used in studying insulin-receptor interactions and binding kinetics, involves the use of an *insulin tracer* molecule. The second approach involves the use of antibodies and molecular probes which specifically bind to the insulin receptor and RNA, respectively. As for studying the fate of the insulin receptor, morphological methods in conjunction with the *insulin tracer* and biochemical methods which involve centrifugation techniques, have been used as successful tools. Each of these methods are discussed below.

The *insulin tracer* is an insulin molecule which has been modified to accommodate a marker which makes the hormone recognizable in small quantities. Markers that have been used are rhodamine-labeled lactalbumin-linked insulin (through insulin's lysine amino acid at position B29), ferritin (B29 linked), [^{14}C]-labelling, [^{3}H]-labelling,

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[5] These terms have been used interchangibly, but the correct term shoud be acidotropic agents rather than lysosomotrophic since the effects of chloroquine can also affect endosomal and not just lysosomal compartments

and [^{125}I]-labelling. The rhodamine-labeled insulin derivative has a decrease in binding when compared to native insulin [247], where ferritin-linked insulin appears to behave identically to native insulin both in potency and biological activity. The latter of the two is useful for studying the fate of the insulin receptor [150,225]. While radio-labelled insulin has been employed for studying insulin-receptor interactions, both [^{14}C]-labeled and [^{3}H]-labeled insulin are not able to achieve specific activities high enough to measure insulin binding, with the exception of cultured human lymphocytes [194,225] Thus, iodinated insulin derivatives are the key choice in studying insulin-receptor interaction and fate.

[^{125}I]-insulin has been used to study insulin receptor characteristics in two ways. First, experiments are employed which establish the optimal time for the insulin-receptor complex to reach equilibrium (steady-state conditions). These experiments are usually performed at temperatures between 0 and 22°C to keep [^{125}I]-insulin degradation at a minimum. Second, varying concentrations of insulin can be used to determine the affinity and number of surface exposed insulin receptors under steady-state conditions This is generally achieved by incubating cells with a fixed trace amount of [^{125}I]-insulin (ligand)[6] in the presence of different amounts of unlabeled insulin (ligand) and are interpreted by two types of graphic analyses; Competitive displacement profile analysis and Scatchard analysis. A detailed discussion of these analyses are beyond the scope of this text and can be reviewed elsewhere [159,241].

The use of antibodies directed towards the insulin receptor has greatly helped in quantitating the number of insulin receptors expressed extracellularly and intracellularly [290]. Quantifying the amount of insulin receptor antibody which binds to the receptor can either be analyzed by Western analysis [284] or by labeling the antibody with [^{125}I] [152,154,290]. Thus the advantages of using these antibodies are two-fold. First, tracer molecules need not be dependant on the receptor's ability to bind insulin (i.e. in some cases specific insulin binding can be as low as 3% [13,176]) and second, tracer molecules using anti-insulin receptor antibodies can be used to detect the β-subunit and examine its fate separately or with the α-subunit. It should be noted however, that these polyclonal antibodies do exhibit non-specific binding, yet with the availability of monoclonal

[6] It should be noted that insulin is only used as an example here Other ligands can be used in place of insulin. For example [^{125}I]-IGF-1 can be used with unlabeled IGF-1 to study IGF-1's receptor characteristics, or [^{125}I]-IGF-1 can be used with unlabeled insulin to study the ability of insulin to cross-react with the IGF-1 receptor.

antibodies this problem is virtually eliminated making them useful probes for studying both insulin receptor synthesis and degradation.

More recently, with the construction of insulin receptor cDNA probes, mRNA levels of the insulin receptor have also be monitored by Northern analysis both in diabetic patients and with cells treated with various stimuli (glucocorticoids, growth hormones) in order to further understand receptor processing and expression [24,179,185,206,242]. Thus, from the use of both antibodies directed towards the insulin receptor and cDNA probes to insulin receptor mRNA, synthesis, expression, and degradation of the insulin receptor can be studied in cells that have undergone differentiation, exposed to various growth hormones, or derived from diabetic patients or animals [179,263]

To study internalization and endosomal compartmentalization [^{125}I]-labeled and photoreactive insulin analogues (i.e. [2-nitro-4-azidophenylacetyl^{B2}]-des-Phe^{B1}-insulin) have been combined with morphological and centrifugation techniques [34,36,142]. A major drawback with using these analogues is that results show conjugated tracer molecules entering lysosomal compartments before being recycled back to the surface [36]. Yet it has been well documented by other techniques that the receptor does not normally follow this route [17,69]. Thus, ligand-receptor covalent modifications may affect the normal processes of receptor recycling by by-passing a required route. A detailed review on using these techniques for studying insulin-receptor fate can be found in the reference of Posner *et al* [1985a].

1.4.5 Summary

The insulin receptor consists of two chains which are held together by disulfide bonds to form a tetrameric species. The α-subunit binds insulin, where the β-subunit is inserted into the membrane and contains, on the intracellular side, an intrinsic tyrosine kinase domain. Once insulin binds to its receptor, the complex is rapidly internalized. Insulin and/or its receptor can then be trafficked to different parts of the cell through a complex tubule-vesicular system. The receptor is then eventually recycled back to the cell surface or degraded. The route that insulin takes is even less clear. Insulin and/or degradated insulin products may go to different parts of the cell such as the nucleus, mitochondria, or Golgi before being extruded from the cell either as intact or degraded insulin.

Environmental conditioning such as temperature and biochemical reagents (the latter employing glycosylation inhibitors and/or acidification inhibitors) have been used to generate blocks in the insulin-receptor pathway. The effects produced by these blockage techniques have been analyzed by performing kinetic studies with [^{125}I]-insulin and other [^{125}I]-labeled ligands. Other techniques have involved the use of antibodies directed to various parts of the insulin receptor and molecular techniques have been used to study insulin receptor message RNA. Lastly, biochemical techniques involving labelled substrates in conjunction with cell fractionation methods have also aided in understanding the intracellular processing and targeting of insulin and its receptor

1.5 IGF-1 , Other Growth Factors, and Their Receptors

1 5 1 Introduction

There are many other key hormones that play crucial roles in regulating a cell's growth and differentiation process. These can be divided into many subclasses, however, this section will only deal with three sets of polypeptide growth factors. These are insulin-like growth factors (IGF), epidermal growth factor (EGF)/epidermal-like growth factors, and platelet-derived growth factor (PDGF)/fibroblast growth factor (FGF). In order to elicit a cellular response, these growth factors must first bind to specific cell surface receptors which transverse the plasma membrane. These receptors contain a highly glycosylated extracellular ligand-binding domain and an intracellular tyrosine-kinase (TK) catalytic domain similar to the insulin receptor [121,301]. The exception is the IGF type two receptor (IGF-II) which does not contain a tyrosine-kinase catalytic domain, yet does appear to serve as a substrate for a membrane-associated tyrosine kinase.

Growth factor receptors can be divided into 5 subclasses. The first subclass (i.e. EGF, transforming growth factor; TGF) involves a single protein molecule which passes through the plasma membrane and contains two cysteine-rich regions. The second subclass (i.e. insulin and IGF-1) have two protein molecules (α- and β-subunits) which form a tetramere (β–α–α–β). The β-subunit crosses the plasma membrane with a single cysteine-rich region on the extra-cellular surface of the plasma membrane The remaining subclasses (III-V) are single molecules which traverse the plasma membrane. Subclasses III (i.e. PDGFs, CSF) and IV (i.e. FGFs) have an interruption in their TK domain unlike the first two subclasses. Subclass III has five immunoglobulin-like repeats in its receptor where subclass IV receptors have only three repeats. Subclass III however, can exist in a dimeric form (i.e. PDGF-AA, AB, BB, see below). Subclass V (i.e. IGF-II, mannose-6-phosphate) contains 15 cysteine-rich regions on the external side of the plasma membrane which results in more than 90% of the receptor being extracellular. The significance of the cysteine-rich and immunoglobulin-rich regions is currently unknown.

The difficulty in understanding the mechanisms of how any particular growth factor works lies at two levels. First, each cell line studied may have different levels of expression for each class of growth factor receptors, which could result in differential cellular effects. The classic example involved studies on BALB/c3T3 cells

[171,222,299]. These studies showed that primary exposure to PDGF followed by IGF-1 or EGF allowed these cells to traverse the cell cycle. Results indicated that in this cell line, PDGF was a competence factor which then needed a progression factor such as IGF-1 or EGF to bring the cell through the entire cycle. In the case of human diploid fibroblasts both PDGF and EGF could bring the cells through the cell cycle, where IGF-1 could not [42]. It is entirely possible that, depending on the cell type, certain growth factors can induce the secretion of other growth factors or even down/up regulate other growth factor receptors [45,131. The second difficulty is that, because receptor dimerization occurs shortly after ligand binding, it is possible that receptors of the same subclass may interact with each other and become activated [286]. A prime example for this type of phenomenon would be interactions with the insulin and IGF-1 receptors as mentioned below (section 1.4.2).

Nonetheless, the interaction of growth factors with their receptors and the responses that they produce are extremely complex. Next is a brief description of some of the current concepts regarding IGF-1, other growth factors, and their receptors.

1.5.2 Insulin-like Growth Factors and Their Receptors

The structure of insulin-like growth factors type one (IGF-1) and type two (IGF-II) are remarkably similar. They are both composed of a single-chain polypeptide molecule of an approximate molecular weight of 7500 [145]. IGF-I has 70 amino acid residues while IGF-II has 67 amino acids [237]. The primary structure of both IGF-I and IGF-II can be grouped into four domains. Domains A and B are structurally similar to the insulin molecule's A and B chains, where domain C is representative of the polypeptide chain found in pro-insulin [145,237]. Domain D is not present in insulin. Site-directed mutagenesis of IGF-1 cDNA indicates that its binding region to the IGF-1 receptor is identical to that of insulin's and that binding is partly dependent on the aromatic amino acids located at positions 23, 24, and 25 [38], where amino acids between positions 49 and 51 appear to be involved in binding to the IGF-II receptor [37].

IGFs are of particular interest with regards to studying insulin action. The *in vitro* cellular effects of IGF are classified much like that of insulin as mentioned in section 1.2. IGFs are capable of producing both short-term metabolic effects (i.e. sugar and amino acid uptake) and long-term mitogenic effects (i.e. protein, RNA, and DNA synthesis) [144,293]. In comparison to insulin, it appears that insulin is potent in stimulating

metabolic responses by factors ranging from 5 (in muscle) to 100 (in adipose tissue) fold over the IGFs, where IGFs are more potent in stimulating mitogenic effects [95,293]. It should be noted however, that the degree of stimulation one growth factor has over the other will largely depend on the cell type and the degree in which their respective receptors are expressed. This is further complicated by three factors. First, competitive binding studies have shown that insulin and IGF-1 are capable of cross-reacting with each others receptors [95,99,232]. Second, a number of reports in the last five years suggest that there are many different subtypes of insulin and IGF-I receptors, and that these two receptors may even exist as hybrid complexes [200,252,267]. The third is that even though these growth factors bind specifically to their own receptors, cross-activation of the β-subunits could occur, which in turn leads to cellular responses [16]. The stage has been set where one can over express either insulin, IGF-1, or insulin/IGF-1 hybrid receptors in a number of different cell types by transfection [167,183]. This, coupled with the use of monoclonal antibodies which can specifically inhibit the binding of a ligand to its receptor (i.e. α-IR3 for the IGF-1 receptor [164]), have helped in partially understanding the importance of insulin and IGFs in mediating cellular responses [51,164,296].

Perhaps the most intriguing is IGF-II and its receptor. It was previously stated that IGF-II strongly resembles IGF-1. It is also known that IGF-II is capable of cross-reacting with the IGF-I receptor but not the insulin receptor [57]. Yet the structure and function of the IGF-II receptor appears to be different from that of any other growth factor receptor. First as mentioned above, the IGF-II receptor is structurally different from the IGF-I receptor. It is a single protein molecule which does not contain a TK region. The second observation is that it cannot really be classified as a *growth factor* as such, since IGF-II is not known to produce a mitogenic signal through its receptor, though it can serve as a substrate for a membrane-associated tryosine kinase [145,157]. Lastly, it appears that IGF-II receptors do not undergo ligand-dependent membrane internalization. Instead they are expressed and turned over in a constitutive manner [207]. The actual physiological relevance of IGF-II and its receptor is currently unknown. Yet there is reason to believe that IGF-II/receptor interactions may be involved in regulating the degradation of various proteins and growth hormones once they are brought into the cell [207]. It is known that insulin can induce the translocation of IGF-II receptors from an intracellular pool much like its ability to translocate insulin-sensitive glucose transporter pools [6]. Other growth factors such as IGF-I and EGF have also shown this

type of phenomenon to occur [22]. Furthermore, IGF-II/receptor complexes may also be involved in regulating insulin and/or insulin receptor degradation [163]. CHO cells transfected with IGF-II receptors, were found to inhibit protein degradation when exposed to either insulin or IGF-I [163]. It is postulated that the translocation of IGF-II receptor to the cell surface alters the normal "flow of lysosomal enzymes" within the cell which, in turn, inhibits protein degradation [163]. It would be interesting to see if the inhibition of protein degradation is general for all proteins taken into the cell, or whether the disruption of degradative enzymes occurs predominantly in certain vesicular pockets indigenous to growth regulatory proteins and/or their receptors (i.e. an intracellular compartment for the insulin-receptor versus an intracellular compartment for the EGF/receptor complex).

1.5.3 Other Growth Factors and Their Receptors

The EGF family of growth factors has been the most studied of all growth factors for its effects on cell growth and differentiation [30,93]. As a result it has provided the basis for the current knowledge and concepts of what is presently known about most polypeptide growth factors. The EGF family consists of a number of different growth factors which have been classified as EGF itself, transforming growth factor alpha (TGFα), amphiregulin, and certain pox virus growth factors [30,93]. Both EGF and TGFα are the major contributors in this family and are responsible for cell growth and differentiation. Amphiregulin on the other hand, has only recently been isolated [261]. The EGF precursor is the most sought after in terms of study, for it not only contains the native EGF sequence, but can also generate eight other EGF-like subunit structures. In addition, one of these EGFs contains a hydrophobic region which is characteristic of membrane binding proteins [202]. The way in which the EGF molecules are processed from the precursor molecule in currently unknown.

All the EGF-family proteins bind to a cell surface EGF receptor. This receptor is a single transmembrane protein of 170,000 Da [29,180]. The existence of other cell surface receptors for TGFα and amphiregulin has been proposed based on the evidence that these proteins can exhibit effects which differ from EGF. Also for TGFα, a monoclonal EGF-receptor antibody can effectively block TGFα binding, but not EGF binding in A431 cells [163,304]. Once the ligand binds to the EGF receptor, the receptor becomes phosphorylated and, in turn, is known to phosphorylate a number of cellular

substrates. Some of these substrates include phosphoproteins of 34,000 and 39,000 molecular weight (pp 34 and pp 39), phospholipase A2, phospholipase C-II, *raf* kinase, MAP kinase, and guanine nucleoside triphosphatase-activating protein (GAP) [71]. A number of these *primary substrates* also result in the formation and activation of other substrates such as the hydrolysis of phosphatidyl inositol 4,5-bisphosphate into inositol triphosphate and diaylglycerol (DAG), *ras* kinase by GAP, ribosomal-S6 kinase by MAP kinase, and even casein kinase II by *raf* kinase which in turn activates various topoisomerases within the nucleus [30,71]. This area of study is extremely complex and is known to not only involve the EGF receptor, but also other receptors which contain an intrinsic TK domain [29]. Whether all or some of these *secondary messengers* are responsible for cellular growth and differentiation remains a mystery.

PDGF is a cell mitogen that was originally isolated from platelets and is now known to exist in a number of other cell types [128]. PDGF exists as a dimeric protein where the two polypeptide chains designated as A and B can exist in three isoforms; AA, AB, and BB. These isoforms are known to bind two types of PDGF receptors; type A and type B [20,130]. The type A receptor binds all isoforms of PDGF, where the type B receptor binds the AA-isoform at a higher affinity than the AB-isoform. Binding of the AA-isoform to the type B receptor has not been demonstrated [20,130]. The role of PDGF *in vivo* is not known. Yet it is suggested that PDGF may be important in cell growth and differentiation during development and wound healing [128]. Human fibroblasts are known to have both receptor types and are known to produce a PDGF-dependent mitogenic response [130]. This effect probably occurs through the type A receptor since studies have shown that the type B receptor only causes chemotaxis and actin reorganization [262]. It should be noted however, that the PDGF type B receptor is not found in most normal tissues and that they are found only on fibroblasts once these cells are grown in culture [279]. The significance of this observation has yet to be elucidated. Once PDGF binds to its receptor, the TK domain becomes activated and causes various substrate phosphorylations similar to ligand-activated EGF receptors [127,188,199]. Another growth factor which largely mimics the actions of EGF and PDGF is α-thrombin (THR) [169,230]. THR is believed to have its own receptor for cellular activation in a number of cell types, including human fibroblasts [221,231]. It is widely accepted that the receptors for EGF, PDGF, and THR work through similar mechanisms to produce a variety of cellular responses that have been classified in cultured fibroblasts as class one mitogens and that the presence of different receptors and

different receptor subtypes leads to the belief that cell growth and differentiation is under a process that requires fine tuning [221].

1.5.4 Summary

In general, growth factor receptors appear to have an intrinsic tyrosine kinase suggesting the importance of cellular substrate phosphorylation to produce a growth response. The exception is the IGF-II receptor which can serve as a substrate for a membrane-associated tyrosine kinase [145], yet this receptor does not appear to be important in eliciting mitogenic actions. The IGF-I receptor strongly resembles the insulin receptor and has been shown to bind insulin. The affinity for IGF-I to bind to the insulin receptor, however, is not as great. All growth factor receptors are capable of producing short and long term metabolic and mitogenic effects. However, the way in which growth factor receptors can produce these effects is not well understood. This is further complicated by the fact that a number of responses are overlapped between different growth factors receptors and that not all cells have identical responses. Another complication is that a number of different growth hormones can cross react with different receptors. It is also possible that there exist small pools of unidentified receptors which can further complicate the roles that certain growth factors and their receptors play.

Lastly, it should be pointed out that the technical ability to produce large amounts of pure growth factors has only recently been accomplished. Thus, with a more stringently controlled environment, it is expected that within the next few years a clearer picture will be formed as to how growth factors work on creating the numerous cellular effects observed for maintenance, growth, and differentiation.

1.6 Mechanisms of Insulin Action

1.6.1 Introduction

The discovery of insulin occurred more than fifty years ago and since then a large amount of research has been directed at understanding how insulin elicits its effects. Later, the insulin receptor was discovered and insulin-dependent cellular effects were extensively studied. Yet the mechanisms involved in insulin action still remain a tantalizing mystery in today's study of cell biology. Only in the last 5 years, with the development of molecular techniques, has there been any significant findings into how insulin may mediate its effects through the insulin receptor. This section deals with the potential mechanisms involved after insulin and its receptor interact and produce the multitude of insulin-dependent effects seen in different cell types. As indicated in section 1.4.3 *The Insulin Receptor Trafficking Pathway*, after insulin binds to its receptor the latter becomes phosphorylated then internalized. It is somewhere during this process of binding and internalization that the insulin-receptor complex can activate a variety of substrates either on the surface of the cell or intracellularly. Thus, this section has been divided into three parts that look at the potential mechanisms involved in insulin action. The first involves the phosphorylation of the insulin receptor itself and the receptor's ability to phosphorylate the tyrosine, serine, and threonine sites of specific intracellular substrates. The second looks at the ability of the insulin-receptor complex to affect signal transduction by the activation of various substrates (i.e. cyclic nucleotides and ion channels). The activation of these substrates leads to the long believed theory that insulin mediates its effects through second messengers [245]. The third section describes the current theories on how intracellular routing of the insulin-receptor complex might lead to insulin-dependent cellular effects.

1.6.2 Autophosphorylation and Protein Phosphorylation

The first studies which demonstrated that insulin could stimulate insulin receptor phosphorylation were in IM-9 lymphocytes [156] and rat hepatocytes [291]. This was performed by pre-incubating the cells with [^{32}P]-ortho-phosphate, lysing the cells, and partially purifying the insulin receptor using wheat-germ agglutinin-agarose. Immunoprecipitation with an antibody directed to the insulin receptor was then used to

further purify the insulin receptor. The insulin receptor was then subjected to polyacrylaminde gel electrophoresis (PAGE) and analyzed by autoradiography. The end result was a 95,000 molecular weight band whose phosphorylation was stimulated by insulin. Other studies followed using [γ-^{32}P]-ATP in a cell-free system of both rat liver and human placenta [10,289]. In each of these studies insulin could induce receptor phosphorylation. Phosphoamino acid analysis further showed that phosphorylation of the insulin receptor was occurring at serine, threonine, and tyrosine sites and that the phosphorylated receptor could in turn cause [^{32}P] incorporation into the tyrosine and serine amino acids of not only itself, but of other substrates such as casein, histone-B2, and synthetic tyrosine-containing peptides [102]. Later, it was discovered that highly purified insulin receptors were only capable of phosphorylating tyrosine-specific sites and that their ability to phosphorylate serine-specific sites occurred only in partially purified insulin receptor preparations [289]. This gave rise to the present day theory that the β-subunit of the insulin receptor is a tyrosine-specific protein kinase and that there exists an unidentified membrane bound serine kinase in close proximity to the insulin receptor [289]. The fact that insulin can cause the activation of two independent kinases (tyrosine kinase and serine/threonine kinase) gave rise to two models that may explain mechanisms involved in insulin action. These are the *dual signalling* hypothesis and the *sequential signalling* hypothesis. The former suggests that once insulin binds to its receptor, the receptor-associated tyrosine kinase is directly responsible for the promotion of cell growth. The tyrosine kinase can also act on the membrane-associated serine kinase which is then responsible for the activation of cell metabolism [289]. This is an attractive hypothesis since all kinases involved in the control of intermediary metabolism are serine and threonine specific [47]. Furthermore, more than 99.97% of the phosphorylated amino acids within the cell are either phosphoserine or phosphothreonine, where phosphorylated tyrosine amino acids make up only 0.03% [89]. With respect to the latter hypothesis, it suggests that both kinases are activated sequentially, such that in the final step, activation of the serine kinase is responsible for cell metabolism and growth [289]. With the knowledge that the insulin receptor acts as a tyrosine kinase capable of autophosphorylation and substrate phosphorylation, two separate approaches have been undertaken to understand how this phosphorylation ability can be tied into insulin-dependent growth and metabolic effects. One approach involves looking at the tyrosine domains of the β-subunit of the receptor, where the other examines the potential of tyrosine-kinase activated substrates being used as second messengers.

The previous section dealt with the possibility that kinase activation may be responsible for growth factor stimulation of cell growth and maintenance and that insulin and IGF-1 can mimic each others responses. Thus, it is not surprising to see that the highest degree of homology between the insulin receptor and the IGF-1 receptors lies in and around the tyrosine kinase domain between amino acids glycine-991 and proline-1257 (85% homology, Figure 3) [89]. Studies examining the autophosphorylation of mouse insulin receptors and the transfection of human insulin receptors (hIRs) altered by site-directed mutagenesis into CHO cells have yielded interesting results with regards to the importance of the tyrosine kinase domain [43,67,68,168,182,298]. Lane *et al.* discovered that insulin-dependent tyrosine phosphorylation of an endogenous substrate with a short half life required the activation (phosphorylation) of three neighboring tyrosine residues on the β-subunit of the mouse insulin receptor (Tyr-1148, Tyr-1152, and Tyr-1153) [168]. This endogenous substrate, termed pp15, was observed by using oxophenylarsine which was thought to block the turnover of potential phosphorylated intermediates. It is believed that pp15 resemoles the activated form of adipocyte-442(aP2), a protein which is important in up-regulating the glucose transport system [168]. Nonetheless, more work needs to be preformed to clarify pp15's exact role and importance in this system.

Site-directed mutagenesis and transfection studies have identified four regions on the β-subunit of human insulin receptor that may be important in inducing insulin-dependent cellular effects. The first region involves Tyr-1316 and Tyr-1322 [182,298]. Carboxy-truncated human insulin receptors (by 43 amino acids) exhibit normal ligand binding, insulin-receptor complex internalization, recycling, and targeting of insulin for degradation [125,178,182]. This receptor also retains its ability to autophosphorylate its β-subunit, but has lost its ability to produce any metabolic effects such as sugar uptake and glycogen synthesis [178]. An interesting observation in cells expressing this truncated receptor was the receptor's apparent enhanced ability to cause an insulin-dependent mitogenic effect when compared to normal receptors [182,280]. It has also been observed that there exists differences in the degree and type of endogenous substrates that are tyrosine phosphorylated between cells transfected with normal hIRs and truncated hIRs [182,280]. For example, pp170 was phosphorylated to a lesser extent in cells expressing the truncated hIR, where pp120 and pp140 proteins which normally undergo insulin-dependent dephosphorylation showed no significant changes in insulin treated carboxy-truncated hIR expressing cells [182].

The second region involves the tyrosine residues at positions 1146, 1150, and 1151 of the hIR β-subunit. Two approaches have been employed in studying this region; (1) truncating the insulin receptor by proteolysis which removes the last 70 amino acids (hIRΔCT) and (2) site-directed mutagenesis which converts the tyrosine groups to phenylalanine residues [178,298]. In both cases, it has been reported that the receptor undergoes normal endocytosis/recycling and retains its ability to produce an insulin-dependent mitogenic effect [178,298]. Yet like the hIRΔCT, an alteration in this region (1146-1151) affects insulin's ability to stimulate glucose transport and glycogen synthesis [178,298]. Also, it was shown from these studies that the insulin receptor was unable to phosphorylate exogenous substrates, suggesting that the receptor's protein kinase activity was abolished when this region was altered and that there exists a cryptic tyrosine kinase activity responsible for β-subunit autophosphorylation and mitogenic responses [67].

The third region is located at Tyr-953 and Tyr-960. This is a unique site that constitutes only about 10% of the total amount of phosphate that is incorporated into the receptor when it undergoes autophosphorylation [178]. Antibodies directed to this domain are able to inhibit protein tyrosine kinase activity [178], yet a mutation at Tyr-960 has illustrated no change in its ability to phosphorylate exogenous substrates [300]. A mutation at Tyr-960 has also been shown to decrease insulin's effect on glycogen synthesis, amino acid uptake, and DNA synthesis [298,300]. pp185 has also been shown to diminish with this mutation. Whether there is a correlation between pp185 and the mitogenic effects of insulin action still needs to be established.

The fourth region is the ATP binding domain. In this region lysine 1018 (Lys-1018) has been replaced with an uncharged amino acid (alanine) by site-directed mutagenesis and transfected into CHO cells [168]. These receptors (A/K1018) were virtually incapable of exhibiting any tyrosine kinase activity [43]. Alternately, they were able to exhibit normal insulin binding, but were not capable of insulin receptor autophosphorylation or insulin-receptor complex internalization [182,183]. These cells also lost insulin-dependent metabolic responses where the loss of a mitogenic response was both insulin-dependent and IGF-1 dependent. This suggests that only the mitogenic pathway, and not the metabolic pathway, is shared between the insulin and IGF-1 receptors [182]. Substrate phosphorylation was also examined in these cells and it was observed that the phosphorylation of two proteins, pp170 (which may be analogous to pp185 mentioned above [154]) and pp220, was inhibited when either insulin or IGF-1 are used. The phosphorylation of pp170 was not inhibited in cells transfected with normal

hIRs [182]. Based on this evidence it has been proposed that the kinase activity of the insulin receptor is important in: (1) the autophosphorylation of the β-subunit, (2) insulin receptor internalization, and (3) the phosphorylation of endogenous substrates pp170 and pp220 which are not responsible for glucose transport regulation (since IGF-1 dependent glucose transport appears to be normal), but may be responsible for eliciting a mitogenic response. Whether autophosphorylation of the insulin receptor is required for receptor internalization remains unclear, for antibodies directed toward the extracellular domain of the insulin receptor have been shown to induce receptor internalization and mimic insulin-dependent cellular actions without inducing insulin receptor autophosphorylation [110]. In addition, other ligand receptors such as the growth hormone receptor and the LDL receptor are not phosphorylated, yet are internalized [8]. Alternatively, Lai *et al.* studied internalization and ligand-mediated autophosphorylation activity of the epidermal growth factor receptor in the rat liver [165,166]. They showed that transient desensitization of cell surface autophosphorylation activity coincided with a diminished capacity for the endocytosis of [^{125}I]-EGF. They also showed that the rate of endocytosis returns to normal after the cell surface autophosphorylation activity is restored suggesting that EGF receptor internalization and trafficking are governed by ligand-dependent receptor phosphorylation. Thus, whether insulin receptor phosphorylation may be important (or the sole mechanism) for ligand-mediated internalization is still questionable.

Another approach to study the mechanisms involved between the insulin receptor's TK domain and insulin action has been to examine which substrates the insulin receptor can phosphorylate directly. In a cell-free system a number of substrates have been shown to be good substrates for the insulin receptor tyrosine kinase. Some of these are microtubule-associated protein (MAP) kinase, tubulin, calmodulin-dependent kinase, and calmodulin [154]. None of these have been found phosphorylated within the cell [154]. It may be that, as with pp15, the turnover rates of these potential second messengers are extremely rapid and that they have not been detected with conventional techniques.

It is known that insulin can stimulate a number of important proteins such as ATP citrate lyase and ribosomal-S6 protein at both serine and threonine sites [154,231]. Thus, one possible way in which insulin can activate these proteins is by first activating serine/threonine kinases through phosphorylations induced by the insulin-receptor tyrosine kinase [122]. One proposed mechanism on how insulin may activate protein

synthesis involves a sequential activation of a cascade with involves MAP kinase, S6 kinase and ribosomal-S6 protein [58,154]. Though the MAP kinase protein has shown insulin-dependent tyrosine phosphorylation, studies have not been successful in demonstrating that the insulin receptor is directly responsible for this activation [272]. Thus, there may be as of yet, an unidentified tyrosine kinase involved as an intermediary step.

Recently, one group used okadaic acid, a specific protein phosphatase inhibitor, to look at the possibility that insulin may differentially phosphorylate key serine/threonine kinases and phosphatases required for insulin action [136]. These studies showed that okadaic acid could inhibit insulin-stimulated lipogenesis and glycogen synthesis in rat hepatoma cells as well as antilipolysis in rat adipocytes. Okadaic acid, however, had no effect on insulin-stimulated amino acid uptake and even potentiated insulin-stimulated glucose transport (unpublished observation by Hess *et al* [136]) This would suggest that the mechanisms for insulin-dependent glucose and amino acid uptake may not require certain protein phosphatases that are required for other metabolic effects such as glycogen and lipid synthesis. This further substantiates the idea that insulin may regulate at least two sets of pathways. One involving the activation of serine/threonine kinases and another which activates serine/threonine phosphatases. It has further been suggested that the activation of these pathways may involve compartmentalization and trafficking mechanisms which could help explain why some proteins are differentially phosphorylated over others [136].

Lastly, it should be mentioned that there are a number of clinical cases in which patients with non-insulin dependent diabetes mellitus possess reduced insulin-dependent tyrosine kinase activity (i.e. a single mutation in the ATP-binding region of the β-subunit [203] and a heterozygous mutation in which the entire tyrosine-kinase domain has been omitted [274]). Receptor-mediated insulin resistance may be a consequence of various factors including increased serine/threonine phosphorylation of the receptor with decreased tyrosine phosphorylation, which leds to receptor desensitization Thus, it would appear that insulin receptor phosphorylation and insulin receptor-associated tyrosine kinase activity are important steps in eliciting some of the effects observed in insulin action. Nonetheless, the functions mediated by the various phosphorylated proteins observed during insulin-dependent cellular activation are currently not known It may be that a number of these phosphorylated proteins have no significant relevance in mediating insulin-dependent responses and that they are phosphorylated only due to their

relative proximity to the β-subunit of the insulin receptor. In order to fully understand the significance of these proteins they will need to be isolated so they can be examined more closely.

1.6.3 Transmembrane Signalling and Insulin Mediators

The above section dealt with the importance of the insulin receptor having a functional tyrosine kinase domain for its ability to produce insulin-dependent cellular effects. However, evidence supports the idea that phosphorylated substrates and tyrosine-activated regions of the insulin receptor are not the only means in which the activated receptor can elicit its effects. It has already been stated that antibodies directed towards the insulin receptor are capable of mimicking insulin-dependent cellular effects without the phosphorylation of the insulin receptor's β-subunit [12,110]. Thus, it may be that phosphorylation of the insulin receptor is the result of a confirmational change which is induced by the binding of insulin to the α-subunit. It can further be speculated that this conformational change causes perturbations in the plasma membrane which in turn, activate membrane-associated proteins important in eliciting insulin-dependent cellular effects. Within the past few years, a number of secondary mediators have been discovered by conventional chromatography techniques. These mediators regulate a variety of insulin-dependent enzymes such as glucose-6-phosphate dehydrogenase and phosphodiesterase [149]. A number of these potential secondary messengers have been classified as having distinct inositol phosphate derivatives which are capable of attaching to the intracellular and extracellular surface of the plasma membrane [245]. The purification of substances known as glycosyl-phosphatidylinositides, have been shown to modify a number of *in vitro* insulin-sensitive enzymes ranging from adenylate cyclase to pyruvate dehydrogenase [77,178,253 for review]. Thus a proposed mechanism of action is as follows: insulin binds to its receptor to produce a conformational change which activates an insulin-specific phospholipase C, possibly through a G-binding protein. This has been supported by the observation that pertussis toxin or antibodies directed towards the GTP-binding ras-p21 protein can inhibit a number of insulin-dependent cellular effects [118,162]. Phospholipase C then cleaves specific phosphatidyl inostitol molecules located on the intracellular surface of the plasma membrane. The cleavage of phosphatidyl inostitol then leads to the formation of unique diacylglycerol (DAG) derivatives which can selectively activate various pools of protein kinase C. The ability

for these protein kinase C molecules to produce differential insulin-dependent cellular effects would depend largely on the cell type, localization and intracellular compartmentalization for activation, and substrate specificity. Additionally, phosphatidyl inositol cleavage leads to the formation of inositol glycans. Inositol glycans are as unique as the DAG derivatives and, when purified, have been shown to mimic some of insulin's actions on pyruvate kinase [4], specific protein phosphorylation [2], and lipogenic action [246]. Perhaps the most striking observation is that once the insulin receptor is activated, extracellularly anchored phosphatidyl inositols are cleaved from the plasma membrane and act as autocrine growth regulators by binding to specific sites on the plasma membrane which are then internalized [147,245,266]. It is currently unknown whether the release of these regulators is due to phospholipase C, since phospholipase C is thought to reside solely on the intracellular portion of the plasma membrane [94]. It may be that their release is dependent upon a separate mechanism from intracellular phosphatidyl inositols which involves an enzyme termed as *phospholipase D* [175]. Nonetheless, in order to establish whether these putative secondary messengers are important in producing insulin-dependent cellular effects *in vivo*, these proteins will need to be isolated and produced in large quantities in order to study each of their mechanisms separately.

1.6.4 Internalization and Degradation

The role that insulin internalization and degradation plays in eliciting insulin-dependent cellular effects is not well known. Is insulin degradation and internalization important in insulin action? Evidence supports the idea that internalization, degradation, and cellular routing are important. For example, the use of insulin-ricin B-chain and insulin-cholera B-chain toxin molecules have been able to produce insulin-dependent responses in cells that do not possess any extra-cellular insulin receptors [134] Nonetheless, insulin degradation and cellular routing is an extremely complex issue, for which little is understood. One of the major reasons for not fully understanding how insulin internalization and degradation may be involved in insulin action stems from the problems involved in isolating the various degraded insulin products, as well as purifying the enzymes that are responsible for insulin (and insulin receptor) degradation. This is only the first step in understanding the role that intracellular insulin and/or its receptor

may play in insulin action. Just as important is the understanding and dissection of the various vesicles that carry insulin and its receptor to various parts of the cell.

The first step at understanding the significance of insulin degradation and cellular routing in insulin action has been to isolate and study insulin degrading enzymes. There are a number of insulin degrading enzymes located within each cell type. The most studied has been a neutral thiol metalloproteinase which has been called on occasion, insulin protease (IP) or insulinase [80,192]. IP, despite knowledge of its existence since 1957 [192], has eluded investigations into its properties. This is because the insulinase is extremely sensitive to purification techniques and is extremely unstable. As a result, literature on IP is very confusing. Conflicting results on IP's properties include differences in its pH optima, molecular weight, requirements for certain metals, and isoelectric point [44,223,254]. Nonetheless, a number of laboratories have reported the isolation of the IP enzyme and have examined its ability to degrade insulin. In one instance, which employs a cell-free system, it appears that IP has a selective effect on degrading insulin and IGF-II over IGF-1 and proinsulin [81]. Results also indicate that IP might have a selective ability to degrade the B-chain of insulin over insulin in general [254]. However, this specificity towards the B-chain changes with the concentration of IP employed. For example, at high concentrations of IP the B-chain is a better substrate than insulin, where the opposite is true at low IP concentrations [254]. It may be that this difference is actually due to contaminating substances in the purified IP preparation and not due to any kinetic properties of the IP enzyme itself. In addition to this, another insulin degrading enzyme with a molecular weight similar to that of IP exists, which is capable of degrading insulin and proinsulin at the same rate [79]. However unlike IP, this enzyme consists of a number of small catalytic proteins ranging in molecular weights from 20,000 to 30,000 daltons, and does not show a pH optima for its activity [309]. This multicatalytic IP is also believed to be responsible for insulin degradation via the non-lysosomal pathway [79]. Multi-catalytic IP's ability to degrade IGF-1 however, has not been extensively studied.

An *in vivo* approach to studying the importance of IP has been to place antibodies raised against IP into the cytoplasm of hepatocytes [253]. These antibodies were able to inhibit more than 50% of the total amount of insulin that is intracellularly degraded. It may be that a larger degree of inhibition could occur, but due to a number of factors such as instability of the antibodies by intracellular proteases or the inability of the antibodies to reach specific intracellular compartments, this is not possible.

Glutathione insulin transhydrogenase was thought to be another important insulin degrading enzyme, as it was believed to be responsible for reducing the insulin molecule to its separate A and B chains allowing proteolysis by IP [78]. However, more recent evidence illustrates that proteolysis of insulin can occur with the disulfide bonds still intact [25] and that antibodies directed towards the glutathione insulin transhydrogenase enzyme are unable to render the insulin molecule resistant to degradation [253] Nonetheless, glutathione insulin transhydrogenase may play another role where it indirectly stabilized the IP enzyme or insulin receptor structure [294].

One approach to studying the importance of insulin degradation in insulin action has been to look at the degradation products that occur with insulin degrading enzymes A number of reports have looked at how IP degrades insulin both in a cell-free system and *in vivo* [9,64,80,193]. *In vitro* studies with purified IP have shown that the insulin B-chain is broken apart in at least seven different sites, where the A-chain is broken at one to two sites [9,64,80]. The first cleavage involves the breakage of the insulin B-chain around the B-16 to B-18 region and occurs before endosomal acidification [193]. Studies using a modified insulin molecule which is resistant to this first cleavage have shown that this molecule is still susceptible to further degradation by IP, suggesting that even though the first step may be important in the degradation pathway, it is not crucial [295] After this first step, the B-chain is degraded into three additional fragments [193] Insulin degradation on the extracellular surface of hepatocytes has shown a similar pattern of degradation products, but with slightly different end products [80]. Whether extracellular insulin degradation is achieved by the same IP enzyme responsible for intracellular insulin degradation is unknown. It may be that additional or different insulin degrading enzymes exist for intracellular insulin over extracellular insulin.

How these degradation products may be involved in insulin action is not known There is strong evidence supporting the notion that degradation, or at least intracellular routing, is important for insulin-dependent cellular responses [26,84,153,263] For example, Kahn *et al.* have successfully prepared clearly delineated plasmalemma and endosomal subcellular fractions from rat liver which has allowed the comparison insulin receptor kinase activity at the cell surface and in hepatic endosomes as a function of dose and time after injected insulin [151,152]. The tyrosine kinase activity of receptors partially purified by wheat germ agglutinin were measured for their ability to phosporylate poly(Glu:Tyr). It was shown that following the injection of a subsaturating dose of insulin (150 ng/100 g body weight), the plasmalemma had peak activation in its

tyrosine kinase activity with in half a minute, where peak activation in the endosomal occurred only after two minutes. Furthermore, phospoamino acid analyses of activated insulin receptors in the plasamlemma and endosomal fractions showed differences in the degree and type of phosphorylation activity. Insulin receptors located in the plasmalemma fraction, were found to be autophosphorylated at both serine (55%) and tyrosine (45%) residues; where activated insulin receptors located in the endosomal fraction were phosphorylated exclusively on tyrosine resisdues. The insulin receptors in the endosomal fraction also illustrated an autophosphorylation specific activity 3- to 4-fold higher than that observed for the insulin receptors of the plasmalemma. This suggests that there may exist an intracellular site of action in which the insulin receptor kinase can activate *second messengers*. Studies by Eckel and Reinauer [84] have also illustrated that blockage of endosomal internalization by phenylarsine oxide affects the early phase (8 min) of insulin-activated glucose transport, where chloroquine affects the late phase (30 min) of insulin-activated glucose transport. With regards to insulin-dependent cell growth, it is known that most cells require at least a 10 hour exposure of insulin [236]. There have been reports stating that insulin can be trafficked to the nucleus [111,265]. Yet these studies involved either subcellular fractionation or cytochemical techniques. The appearence of insulin in the nucleus as assessed by subcellular fractionation techniques may not be entirely justified, since "leakage" may occur from one subcellular comparment to another (J. Bergeron, per. commun.). In addition, the amount of insulin located with in the nucleus determined by cytochemical technics was less than 2% of the total amount of cellular insulin [265]. Since a high degree of background noise occurs with this technique, more reliable and accurate methods must be pursued. Nonetheless, it has also been demonstrated that insulin has a direct effect on isolated nuclei and that blocking the intracellular processing of insulin results in the loss of amino acid uptake [111,265]. Thus, long term insulin effects such as cell growth and even translocation of GT to the plasma membrane may involve the activation of secondary messengers in specific intracellular compartments either by the receptor's active kinase or by certain insulin breakdown products.

1.6.5 Summary

It is now generally thought that insulin mediates two distinct pathways (a cascade of tyrosine kinase-dependent phosphorylated proteins and the appearance of secondary mediators or modulators) that may act either independently or in unison with each other [244]. The simple conclusion that insulin-receptor complex internalization is important only in regulating the turnover, expression, and degradation of insulin and/or its receptor is naive. Much evidence supports the theory that compartmentalization is an important step in insulin action. Internalization and compartmentalization allow insulin and its receptor to interact with potential intracellular secondary messengers to produce cellular effects. Compartmentalization also allows insulin to migrate to certain sites within the cell, suggesting that insulin or degraded products of insulin have direct effects on cell growth (nuclei) and/or metabolism (intracellular hexose transporter pools for translocation).

1.7 Toxic Insulin Molecules and Insulin Action

1.7.1 Introduction

This section deals with the methods that have been employed for studying insulin action through the use of toxic proteins linked to insulin. Two toxic proteins which have been used widely in this field are ricin and diphtheria toxin. The first part of this section deals with DT. Although there are many types of bacterial and plant toxins that one can link to carrier proteins such as ligands and antibodies (i.e. *Pseudomonas* exotoxin A, ricin, moddencin, gelonin, and poke weed anti-viral protein [211,212,218]), DT was the molecule employed in this project. The later half deals with the construction of toxic-ligand molecules and their uses in studying ligand-mediated cellular action.

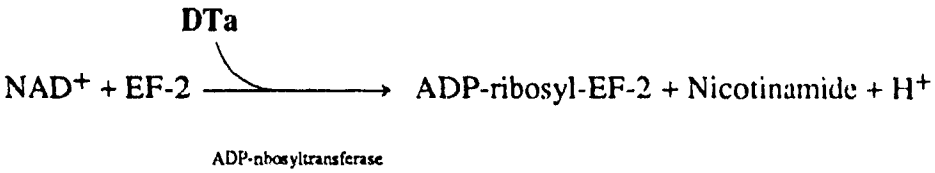
1.7.2 Diphtheria Toxin

Structure and Binding

DT is secreted from the bacterial strain *Corynebacterium diphtheriae* [219]. When purified, it has a molecular weight of 60,782 which can be separated into two fragments upon limited proteolysis and reduction of a single sulfhydryl bond [49,218,219]. The A fragment (MR: 23,145) is the enzymatically active part of the toxin [70], where the B fragment (MR: 39,637) is the carrier portion of the protein [166]. The B-chain binds to a specific cell surface receptor which is a glycoprotein of 153,000 mol. wt. [191,229] and allows for the internalization of the A-chain into the cytoplasm. Though evidence supports the fact that both the A-chain and B-chain of DT get internalized into an endosomal vesicle [201,213], evidence also supports the idea that the B-chain, due to its containing certain hydrophobic lipid associating domains, can insert itself into the lipid bilayer forming a channel to facilitate the entry of the A-chain [195]. Nonetheless, if the A-chain is cleaved from the B-chain before binding, the A-chain is unable to enter the cytoplasm except through random events such as pinocytosis or endocytosis, making the reduced (cleaved) DT non-toxic, except at extremely high concentrations [196].

Mechanism of Action

The A-chain, once it enters the cell, migrates to the area of protein synthesis and shuts down protein synthesis by inactivating elongation factor-2 (EF-2) by ADP-ribosylation [50,106]. As can be seen below the A-chain of diphtheria is an ADP-ribosyltransferase enzyme which facilitates the transfer of the ADP-ribose moiety from NAD^+ to a novel histidine residue (diphthamide-(2,3-[carboxyamido-3-trimethylammonio)propyl]-histidine) found specifically in EF-2 [288].



EF-2 is responsible for the transfer of peptidyl tRNAs from the A site to the P site on the ribosome, a step which is crucial for the amino acid peptide formation of proteins [107,177]. The A-chain catalyzes this reaction only on free EF-2 substrates not bound to the ribosome, making them inactive. This in turn, inhibits protein synthesis [196,218]. Furthermore, because this is a catalytic reaction, as little as one DTa molecule can ADP-ribosylate 2000 EF-2 molecules per minute depending on the cell type (discussed below). Thus, a single DTa molecule at the site of protein synthesis is capable of inactivating all cellular EF-2 and can kill the cell [214,307]. Based on the reaction above, an excess of NAD^+ will allow the reaction to go to completion by using all available EF-2. Since this reaction is highly specific for EF-2, due to its novel histidine residue, the amount of ADP-ribose transferred from NAD^+ to EF-2 will give a direct indication of the amount of EF-2 present in cell extracts [196]. The rate of ADP-ribosylation can also be measured if an excess of both NAD^+ and EF-2 are used. Thus, looking at both the amount and rate of DT-dependent ADP-ribosylation in a variety of cell types has helped in characterizing species differences in sensitivity to the toxin and has aided in further understanding the toxin's mode of action.

It has been observed for a number of cell types that the time period for the inhibition of protein synthesis involves a lag period of at least 30 minutes, where in cell-free extracts, protein synthesis inhibition is observed almost immediately [196]. This lag

is also dependent on DT concentration, indicating that the lag observed is most likely due to the cell's mechanism of uptake plus the time required for the A-chain to enter the cytoplasm. A number of species and cell types are either extremely or partially sensitive to DT (i.e. human diploid fibroblasts [120], primary guinea pig kidney [100], Chinese hamster ovary (CHO) [119,198], and Chinese hamster lung (CHL) fibroblasts [119]) where others are not (i.e. rat [98] and primary mouse kidney and L-cells [197]). One reason is that the number of DT receptors per cell can vary from undetectable levels to 2 x 10^5 [98,118,119,191,198]. Whether DT-resistant cells derived from rat or mouse contain any functional DT receptors is still under discussion [41,191,211]. It has been shown however, that mouse L-cells show similar sensitivities to DT when protein synthesis is monitored in cell-free extracts [41,197]. Thus the resistance could either lie at the level of the receptor (expression or function) or at some step involved in carrying the A-chain to the site of protein synthesis.

Numerous studies have been undertaken to produce mutants resistant to DT in order to better understand its mechanism of action [41,118,196,197]. This has been achieved by exposing cells to varying concentrations of DT and has resulted in the production of mutants which can be separated into two classes. The first class have a defect either at the level of binding, internalization, or vesicular trafficking. The second class of mutants show decreased abilities to ADP-ribosylate EF-2 or decreased sensitivities to DT in a cell-free system which monitors protein synthesis. V-79 (CHL fibroblasts) have shown a mutation frequency of 1.7 x 10^{-6} for spontaneous mutants and 1.6 x 10^{-4} for cells chemically induced with 300 mg/ml ethyl-methyl-sulphonate (EMS) in the presence of 1 lethal fragement/ml of DT [119]. In this particular case, DT-resistant V-79 cells were shown to be of the second class since the specific activity of EF-2 (pmol ^{14}C-ADP-ribose incorporated per milligram of protein) was <0.1 as compared to the value of 80.8 of DT-sensitive V-79 cells [119]. To date, V-79 cells resistant to DT as a class one defect have not been reported.

1.7.3. Using Toxic Insulin Molecules for Studying Insulin Action

There are a number of approaches that have been employed to study the mechanisms involved in insulin action. Some of these have employed the study of cells obtained from diabetic patients or animals [263,277,278]. Others have used chemicals to alter insulin receptor binding and processing [69,87,269], while transfection of receptors which have been mutagenized through site-directed molecular approaches have been employed [162,244,298]. These have been mentioned briefly in the above sections and will not be covered here. Rather this section deals with a review on the particular approach that has been applied to this project, i.e. the use of a toxic insulin molecule.

Two successful approaches have been taken to construct toxic insulin hybrid molecules. The first approach involved the chemical modification of insulin's carboxyl groups with cystamine-dihydrochloride and ethyl-3-(dimethylaminopropyl) carbodiimide-hydrochloride (EDAC) [255,259]. The result was an *activated* insulin molecule (cystaminyl insulin) which could bind to the reduced form of an enzymatically active protein (i.e. DTa or Ricin A) through a sulfhydryl exchange. The disadvantage to using this method lies at the level of producing cystaminylated insulin. This method generates a number of active sites on the insulin molecule, such that once conjugation occurs with the selected toxin, a heterogeneous production of $(toxin)_n$-insulin molecules are formed. This can be avoided by purifying the various species of cystaminylated insulin by DE52 ion-exchange chromatography, yet the actual yield and true purity used in this conjugation process is low [259]. The second approach involves conjugating a disulfide-containing toxin to the free amino groups of the insulin molecule by using the heterobifunctional reagent N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP) [255,259]. Prior to using SPDP on insulin, the amino acid terminal groups of insulin are blocked leaving a single internal amino group free. The end result is the construction of an insulin molecule containing one toxin molecule [259]. This latter approach has been successful in constructing insulin-ricin A chain (insulin-RTa), EGF-ricin A chain (EGF-RTa), and EGF-DTa molecules [39,258,259]. The former approach has been used to construct insulin-RTa and insulin-DTa molecules [255,257,259].

RTa-linked hormono-toxins have been successful in eliciting toxic effects in a number of cells that express the conjugate's receptor [39,256-258]. DTa-linked hormono-toxins however, have not been as successful [39,255,256]. For example, EGF-RTa has

been shown to be toxic to 3T3 cells, while EGF-DTa exposed to the same cell line is non-toxic [39]. In these particular experiments, DTa maintains its ability to ADP-ribosylate EF-2 when conjugated to EGF. EGF-DTa was also shown to interact with the EGF receptor and internalize [39]. The differences in cellular toxicity between EGF-RTa and EGF-DTa are not well understood. It is known that the toxin must be linked to the ligand (growth factor) through a disulfide bond in order to cause cytotoxicity [210], but this may not be the only necessity. RTa contains two hydrophobic regions which are lacking in DTa and may help facilitate its entry into the cytoplasm [97]. In addition RTa, unlike DTa, does not require a low pH for entry into the cytoplasm; it may even prefer a slightly alkaline pH [187]. Thus, it is entirely possible that the endocytic route for EGF conjugated with a toxic molecule in 3T3 cells does not go through an acidification process before the toxin is either degraded or expelled from the cell. The lack of a hydrophobic domain for a ligand-bound DTa molecule may also represent its sole dependence on the ligand for internalization into the cell, where ligand-bound RTa molecules may non-specifically interact with the cell's plasma membrane [259].

The use of insulin-DTa and insulin-RTa molecules in selecting resistant cells has yielded interesting results with respect to insulin dependent/non-dependent growth and metabolism. In the case of mouse fibroblast and 3T3 pre-adipocyte cells, insulin-DTa resistant cells showed both decreased and enhanced abilities to bind insulin suggesting abnormal functioning of the insulin receptor [257]. Insulin-DTa resistant pre-adipocytes also showed various abilities to grow in a serum-free hormonally defined medium [257]. Though no conclusive results were made, these cells were shown to be either dependent on insulin for growth, did not require insulin for growth, or unable to grow at all in a serum-free medium [257]. Furthermore, no correlation was made between the ability for these cells to bind insulin and their ability to grow in a serum-free medium containing insulin. Insulin-DTa resistant H35 rat hepatoma cells also exhibited decreased insulin binding abilities [259]. These cell lines were not able to exhibit any insulin-dependent tyrosine aminotransferase (TAT) activity unlike their insulin-DTa sensitive counter parts. Yet, both cell types were able to produce dexamethasone-induced TAT activity [259]. This would indicate that insulin may have a different mechanism than cortical steroids in stimulating TAT synthesis. Whether insulin-dependent TAT synthesis is directly related to insulin receptor activation is not known. There have been no further studies conducted with these cell variants. Nonetheless, these results illustrate how the construction and

employment of toxic insulin hybrids on cell cultures can be used in selecting cells which are altered at the level of insulin binding and insulin responsiveness.

1.7.4 Summary

Diphtheria toxin is a protein molecule comprised of a receptor binding subunit (B-chain; 40 kD) and enzymatic subunit (A-chain; 21 kD) held together by a single sulfhydryl bridge. Once DT enters the cell, the A-chain, after entering the cytoplasm, will inhibit protein synthesis by ADP-ribosylating unbound cellular EF-2. Nicked DT (DT having undergone mild proteolysis) which has been subjected to sulfhydryl reduction is not toxic to the cell. Only at high concentrations can it show toxicity, since the A-chain does not have a specific route of entry. DT has varying degrees of toxicity in different cells types with virtually no toxic effect on cells derived from rat or mouse. The difference observed between cell types may lie at the receptor level and/or intracellular routing of the toxin, since DT is capable of inhibiting protein synthesis in a cell-free mouse extract.

To conclude, toxic-ligand hybrids are useful tools in producing cell variants which are defective in various parts of the ligand-receptor pathway. Insulin-DTa and insulin-RTa conjugates have allowed the isolation of cell variants which show alterations in insulin binding, growth, and insulin-dependent TAT activity. Little else has been uncovered with regards to insulin action using this approach. Thus, further investigation of cell variants resistant to insulin-DTa and RTa conjugates would be useful for dissecting the mechanisms involved in insulin action.

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1.9 Figures for Chapter 1

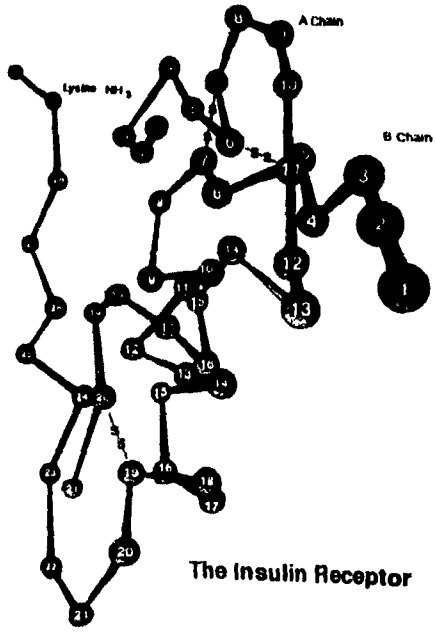

Figure 1. The insulin molecule.

The structure respresents the three dimensional structure of the porcine insulin molecule as determined by X-ray crystallography and molecular model computer generation. Numbers on the backbone molecule correspond to the amino acids numbered from the amino-terminal end of the protein chains. -S-S- donotes disulfide bridges between amino acids. The inserted legend lists the amino acid sequence for both the A chain and the B chain and shows inter- and intra-disulfide bridges for porcine insulin (information obtained from reference 297).

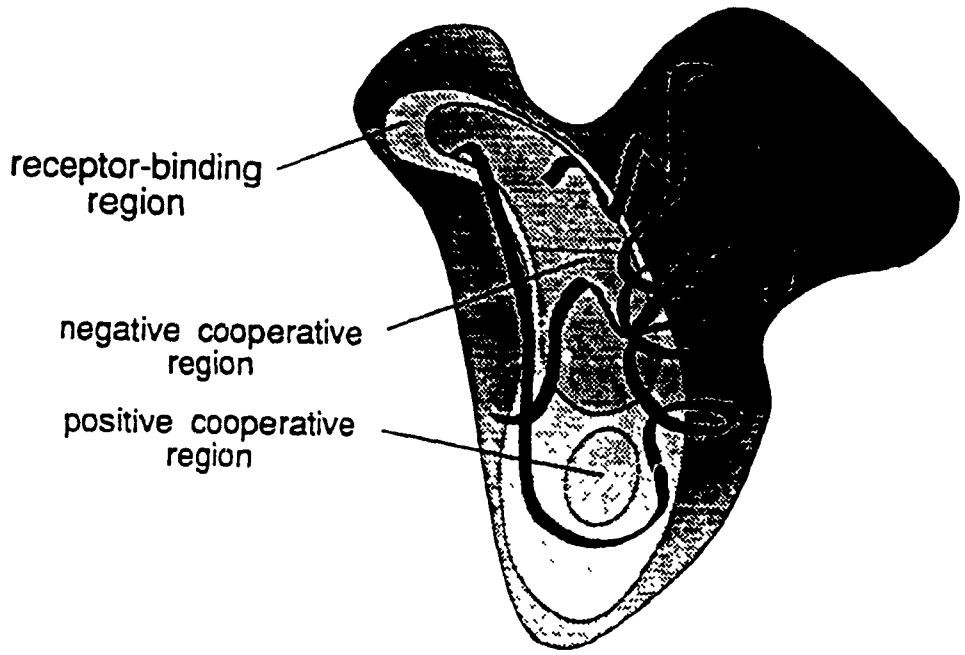

Figure 2. The insulin molecule and its recognition site.

This figure shows a graphical representation of the areas believed to be responsible for receptor binding, positive cooperativity, and negative cooperativity. Regions reflect those portions of the insulin molecule that have been shown to affect its binding to its receptor through alterations in the amino acid sequences (information obtained from references 72,73, and 99).

Figure 3. The insulin receptor.

This figure shows a cartoon of the insulin receptor and its tyrosine kinase domain. The lower left panel illustrates the structure of the insulin receptor inserted into the plasma membrane. Insulin can bind to the α-subunit (α) of the insulin receptor and phosphorylate the β-subunit (β) at specific tyrosine sites (numbers, upper right panel). Phosphorylated tyrosine sites are believed to mediate a number of effects as illustrated. Lysine at postion 1018 (Lys-1018) is the ATP binding region of the receptor. See text for further explanation. Numbers represent the amino acid position from the amino terminal end. Information obtained from references 43, 268, and 298 (Gly, glycine; Lys, lysine; Pro, proline).

Chapter 2

Construction of a Highly Specific Toxic Insulin Molecule: Selection and Partial Characterization of Cells Resistant to its Killing Effects

2.1 Preface

Before presenting the next three chapters which contain original manuscripts, the structural setup of the project is presented here.

Stage One:	Conjugate construction and characterization
Stage Two:	Mutant isolation
Stage Three:	Mutant characterization

The first stage was to construct the DTaI molecule using SPDP as the linker molecule and to characterize this conjugate with respect to its cellular toxicity and insulin receptor specificity. This would indicate whether DTaI could elicit its toxic effects specifically through the insulin-receptor pathway as well as through the interaction of another surface receptor (i.e. the IGF-1 receptor). The second stage was to use DTaI to select and isolate cell variants which were DTaI-resistant yet still DT-sensitive. This would indicate that resistance was due to an alteration in the insulin-receptor pathway and not at the site of DTa action. Cell variants fitting this criteria were then used in stage three. This first involved a preliminary characterization of all DTaI-resistant mutants for their sensitivity to the whole toxin (DT), insulin binding, and growth in serum containing medium. Second, it involved a more extensive characterization of one variant, IV-A1-j.

With regards to the classification of DTaI-resistant mutants the following nomenclature was used:

e.g. IV-A1-j

where,

- IV denotes the experiment that involved application of the DTaI to the culture medium.
- A represents the concentration used (in this case 3 times the LD50)
- 1 represents the plate number at that concentration (i.e. A1 - A5)
- j represents the clone picked from that plate (this clone was then sub-cloned, yet it maintained the nomenclature after the first cloning)

This chapter looks at the construction of the DTaI molecule, for which a general outline of the methodology can be found in the appendix (Figure 1.) This chapter also describes the selection and isolation of DTaI-resistant cells In all cases, the variants described in this chapter were shown to be DT-sensitive at the same concentrations observed for the DTaI-sensitive V-79 cell line. Figure 2 in the appendix illustrates this sensitivity for IV-A1-j cells.

For assessing the binding kinetics of these cell variants, it was necessary to employ steady-state conditions. For this, times courses to establish equilibrium were performed at two different temperatures. This is illustrated in Figure 3 of the appendix for both V-79 and IV-A1-j cells.

This chapter looks at (1) the method used to construct the DTaI molecule, (2) the ability of DTaI to specifically elicit its effects through the insulin receptor and not the IGF-1 receptor, (3) the selection and isolation of the number of DTaI-resistant, DT-sensitive clones, and (4) that characterization of these variants to (a) bind insulin, (b) grow in serum containing medium, and (c) grow in the hormonally-defined medium where insulin is the predominant mitogen.

All work was performed by the candidate.

2.2 Abstract

We have constructed an insulin-diphtheria hormono-toxin by using the heterobifunctional reagent N-succinimidyl-3-(2-pyridyldithio) proprionate (SPDP). The conjugate migrates as a single 29 kd band on 10% SDS polyacrylamide gel electrophoresis which corresponds to a one to one molar ratio of the diphtheria A-chain (23 kDa) and insulin (6 kDa) molecules. The diphtheria A-chain:insulin (DTaI) hormono-toxin demonstrates cytotoxicity in V-79 Chinese hamster cells exhibiting an LD50 of 1.1 x 10^{-8} M, which is 22X more potent than whole diphtheria toxin. Also, DTaI can competitively displace [^{125}I]-insulin with an ED50 of 1.1 x 10^{-8} M, which is similar to the ED50 of insulin (1.1 x 10^{-8} M), and showed limited cross-reactivity with the IGF-1 receptor (12% displacement of [^{125}I]-IGF-1 with a DTaI concentration of 1.1 x 10^{-8} M). Furthermore, DTaI which has been blocked at the amino terminals by citrate groups is not capable of displacing [^{125}I]-insulin nor is it cytotoxic up to a concentration of 1 x 10^{-6} M We have used DTaI to select conjugate-resistant clones from the V-79 Chinese hamster fibroblast parental cell line. Conjugate-resistant variants expressed insulin binding levels ranging from just below normal (8.0 ± 2.0 fmoles/mg P) down to a 60% average decrease (3 6 ± .5 fmoles/mg P) in insulin binding when compared to the V-79 parental cell line (11 2 ± .2 fmole/mg P) Additionally, a number of conjugate resistant clones show a decreased ability to grow in medium containing 5% serum and/or in a serum-free defined medium containing insulin as the predominant mitogen. The altered ability of these clones to grow in a serum or serum-free medium containing insulin did not correlate directly with the changes observed for insulin binding. Based on these observations we suggest that (1) DTaI elicits its cytotoxicological effects through the insulin-receptor trafficking pathway, (2) DTaI can be used to isolate cells altered at the level of insulin binding and/or action, and (3) mechanisms responsible for mediating insulin-dependent cell growth along the insulin receptor trafficking pathway may also be shared by other growth factors.

2.3 Introduction

Insulin is known to bind a specific cell surface receptor which results in its internalization, compartmentalization, and subsequent degradation [1-3]. During this process insulin can mediate its metabolic and mitogenic actions. Yet, the mechanisms that are responsible for coupling the insulin-receptor trafficking pathway to insulin action remain obscure. One approach towards understanding the relationship between the insulin-receptor pathway and insulin action has been to employ methods which alter the internalization and trafficking pathways through the use of acidotrophic agents (i.e. ammonium chloride, chloroquine, and methylamine [4]) and carboxylic ionophores (i.e. monesin, nigercin, and X537A [5,6]). Another approach has involved the use of toxic-insulin molecules which have been used to isolate variants defective at the level of insulin receptor expression, binding, internalization or response [7,8]. A previous report on the construction of such a molecule has yielded heterologous proteins which contained multimeric forms of a Diphtheria A chain bound to a single insulin molecule, decreasing its affinity for binding to the insulin receptor [9,10]. Although this conjugate mixture has proven fruitful in selecting insulin response-altered rat hepatoma cell lines [9,10], there would be significant disadvantages of using a bulky selective agent with cell lines expressing both insulin-like growth factor I (IGF-1) and insulin receptors [11,12]. Using the approach described herein we linked a single molecule of diphtheria A-chain to a single molecule of insulin abolishing the formation of bulky multimeric species and greatly enhancing its specificity of interaction with the insulin receptor with low cross reactivity to the IGF-1 receptor. The data indicate that this molecule makes an excellent selective agent for isolating and studying cells altered in insulin binding and action which contain normal levels of IGF-1 binding. We further present evidence which suggests that the insulin-receptor pathway is independent of and coupled in part to pathways that are involved with other growth factors responsible for promoting growth.

2.4 Materials and Methods

2.4.1 Reagents and Radioactive Materials

Wild type V-79 cell lines (Chu *et al*, 1968) were a gift of Dr. I. Scheffler (Department of Biology, University of California, San Diego). ß-mercaptoethanol was purchased from Aldrich, acrylamide gel reagents from Biorad, N-succinimidyl-3-(2-Pyridyldithio) proprionate (SPDP) from Pierce Chemicals, and Sephadex G-25 and G-75 from Pharmacia. Diphtheria toxin (DT) (lot #D-396) was purchased from Connaught Laboratories, Toronto. IGF-1 from AMGen Biochemicals, Thousand Oaks, California, and both citraconic anhydride and bovine pancreas insulin were from Sigma Monocomponent insulin was a gift from Lilly Research Laboratories, Indianapolis and [^{125}I]NaI was obtained from Amersham Corp. Apo-transferrin (Tf) was graciously provided by Dr. P. Ponka (Lady Davis Institute, Montreal) and EGF was from Biomedical Technologies Inc. (Stoughton, MA).

2.4.2 Cell Culture

V-79 cells were grown in Dulbecco's modified Eagle's medium (DME) containing essential and non-essential amino acids supplemented with 5% (v/v) fetal calf serum (FCS) in an atmosphere of CO_2 plus air (5:95) at 37°C. Cells were harvested at confluence in 75-cm^2 culture vessels after incubation with 0.02% (w/v) EDTA and 0.04% (w/v) trypsin (Difco Labs, Detroit) [13]. Cells were plated in either 35-mm plastic petri plates or 24-multiwell plates (Falcon Co.) at a density of 4 x 10^4 cells/cm^2 except where indicated. After 24 hours, cells were used for growth studies (see below) or serum-deprived for 24 hours in Eagle's serum-free minimal essential medium (0% MEM) containing 4 mg/ml glucose for receptor binding studies.

2.4.3 Purification of Diphtheria Toxin A-chain (DTa)

Purified DT (3000 lethal fragments/ml) is first nicked by reacting the protein (1 ml) with 1 mM trypsin for 30 minutes and terminated by adding 10 mM soybean trypsin inhibitor (Boehringer-Mannheim). Following reduction in 5% ß-mercaptoethanol for 30 min, the mixture is placed into a hot water bath (90°C) for 5 min, then centrifuged at 13,000 rpm in a microfuge (John's Scientific) for 2 min. The supernatant is removed and applied to a Sephadex G-25 column equilibrated with 20 mM TES buffer (pH 8.0)

containing 0.1 M NaCl. The void volume contains DTa which is then added directly to insulin linked with SPDP (see results and discussion).

2.4.4 Synthesis of an Insulin-Toxin Conjugate

Insulin was cross-linked to DTa by using SPDP. The construction of an activated insulin molecule (SDP-insulin) was achieved with slight modifications of the method of Shimizu [7], lyophilized, and directly mixed with a freshly prepared batch of purified DTa at varying molar ratios (see results). The mixture was dialyzed against one liter of 20 mM TES (pH 7.8) containing 0.1 M NaCl for a minimum of 12 hours at 4°C. The dialysate was then analyzed for its degree of conjugation by 10% SDS-PAGE [14] and purified by a 1 cm x 40 cm column containing Sephadex G-75 superfine (SF) gel matrix equilibrated with 20 mM TES (pH 7.4) containing 0.1 M NaCl.

2.4.5 ADP-Ribosylation

The ability for DT, DTa, and DTaI to ADP-ribosylate Elongation Factor-2 (EF-2) was determined by the method of Moehring and Moehring [15]. EF-2 was isolated from rabbit erythrocytes as previously described [16].

2.4.6 Cytotoxicity Assays

Cells were plated at 4 x 10^4 cells/cm^2 and grown to 70 - 80% confluence in either 35mm plastic petri plates or 24 multiwell culture plates and rinsed twice with 0% MEM containing 4 mg/ml glucose. Varying concentrations of either DT or purified DTaI were added to the cultures and incubated at 37°C for two hours. Cultures were then washed once with 0% MEM medium and replaced with DME medium containing 5% FCS. After 24 hours cell viability was determined using the method of trypan blue exclusion or inhibition of protein synthesis which was determined by the incorporation of [^{3}H]-leucine into TCA-precipitable material [17].

2.4.7 Insulin and IGF-1 Binding Assays

Serum-deprived confluent monolayer cultures were washed three times with 3 ml of 20 mM Hank's-Hepes buffer (pH 7.4) with 0.2% BSA at 22°C. Cells were then incubated with 1 ml (or 0.2 ml in 24-multiwell plates) of the same buffer containing various concentrations of either insulin, IGF-1, or DTaI. [^{125}I]-labelled insulin or IGF-1

(specific activity 200 μCi/μg) were added at a concentration of 2 ng/ml and incubation was carried out at 22°C for 2 hours. At this time point equilibrium had been reached and less than 2% of the radio-labelled ligands had been degraded as assessed by the appearance of TCA-soluble radioactivity in the medium (data not shown). Cold insulin and IGF-1 at a concentration of 40 μg/ml and 10 μg/ml, respectively, were used to determine non-specific binding. The ability of insulin, IGF-1, and DTaI to displace [^{125}I]-labelled insulin or IGF-1 from their respective receptors was determined by the percent of radioactivity remaining specifically bound to that receptor. The percent specific binding for insulin and IGF-I was 82 ± 7% and 91 ± 3% where the amount of ligand specifically bound was 11.2 ± 1.2 and 19.8 ± 1.2 fmoles/mg protein, respectively.

2.4.8 Selection of DTaI resistant clones

Clonal selection involved two separate procedures denoted as series IV and VI. The major difference between these two series is that series IV was not subjected to mutagenesis prior to DTaI exposure. Series VI cells were pre-mutagenized by incubating exponentially growing V-79 cells to 5 x 10^{-5} M methyl-nitrosyl-urea for 1 hour. These cells were then transferred into DME + 5% FCS for 3 days to allow the expression of altered phenotypes prior to conjugate exposure.

For both series, 5 x 10^{8} exponentially growing cells were washed 3 times with DME minus serum and treated with purified DTaI at a concentration of 5 x 10^{-7} M for either 2 hours (Series IV) or 16 hours (Series VI). The selection medium was then removed and replaced with DME + 5%FCS for 2 days. The cells were then re-exposed to the selection medium for the same time interval (2 or 16 hours), and then repeated a third time. After the third exposure, cells were grown for 2 - 3 weeks. When individual colonies were formed these were isolated and re-cloned with cloning rings. Clones were then tested for: (1) their sensitivity to DTaI and DT, (2) their ability to grow in serum containing medium, and (3) their ability to bind insulin and IGF-1 (See Results and discussion).

2.4.9 Growth of cells in a serum-free medium containing insulin

Cells were then seeded with 1.0 ml DME+S in 35-mm culture plates at a density of 60,000 cells per plate. After 24 hours the medium was replaced with a serum-free medium containing DME, essential and non-essential amino acids, 0.2% (v/v), dialyzed heat inactivated serum, 60 nM Tf, and 1 nM EGF alone, with 5% (v/v) FCS, or with two different concentrations of insulin (1 and 4 ug/ml). Cells were counted on days 0, 1, 3, and 4, and corrected for viability as determined by trypan blue exclusion.

2.5 Results And Discussion

2.5.1 Synthesis and Purification of DTaI

To ensure that SPDP activation would be localized to a single amino acid located on the B-chain of insulin (B29-lysine), the amino-terminal groups of insulin were blocked with citraconic anhydride which preferentially reacts with free α-amino groups over ε-amino groups [8].

Various molar ratios of insulin to SPDP were used (1:1 to 1:20, respectively), to determine the amount of SPDP required to yield a one to one molar substitution of a 2-pyridyl disulfide (SDP) linkage molecule to insulin. The degree of substitution was determined by the method of Carlson [18]. We then mixed SPDP with insulin (previously capped with citrate groups) at a molar ratio of 1:10, which would allow for the maximal substitution of 2-pyridyl disulfide to the internal B29-lysine. Citrate groups were then removed by slowly lowering the pH to 2.0 by dialyzing against water adjusted to pH 2.0 with HCl for 16 hours. The pH was then gradually brought back to 7.4 (by dialysis) and the mixture was lyophilized.

For the purification of DTa a heat precipitation method was used (see materials and methods). In order to access the purity of DTa in the supernatent, samples (supernatent and precipitate) where applied to a 10% SDS-Poly Acrylamide Gel run under reducing conditions (Figure 1). Either supernates (lane 3) were added directly to a 2X Laemmli sample buffer (1:1 v/v ratio), or precipitates pelleted by centrifugation (lane 2) were first partially reconstituted by adding 0.5 ml of 20 mM TES (pH 7.6) containing 0.1 M NaCl and then added to Laemmli sample buffer. Lane 2 of Figure 1, represents the precipitate and shows a number of different molecular weight species. Lane 3 (which represents the supernatent) depicts purified DTa. DTa retained greater than 80% of its ability to ADP ribosylate Elongation Factor-2 (EF-2) (183 ± 5 mol of ADP-ribose-EF-2 formed/mol DTa/minute *vs* 153 ± 6 mol of ADP-ribose-EF-2 formed/mol DTaI/minute).

Completely reduced DTa was then added in varying amounts to the lyophilized SDP-insulin. The data in figure 2, lane 5, illustrates the presence of three protein bands with mol. wt. of ~23 kDa, ~29 kDa and ~48 kDa which correspond to DTa, DTaI, and a dimeric form of DTa, respectively. At a DTa to SDP-Insulin molar ratio of 1 to 6, the optimal amount of DTaI was formed (45 to 63 percent of total DTa used as determined by scanning densitometry). At lower ratios there was an increase of dimeric DTa, where

at higher ratios, the yield of DTaI remained approximately the same (~50%, data not shown). The 29 kDa mol. wt. band is consistent with a one to one molar ratio of DTa to insulin and as indicated in lane 4 of Figure 2, the conjugated protein revealed two distinct bands and after reduction with ß-mercaptoethanol, one for DTa (23 kDa) and the other for reduced insulin A and B chains (~3kDa), respectively. The crude conjugate mixture was then applied to a Sephadex G-75 SF column (1 x 40 cm, void volume, 72 ml), eluted with 20 mM TES containing 0.1 M NaCl (pH 7.4), and 1 ml fractions were collected 5 ml prior to the exclusion of the void volume eluent. Figure 3, lanes 3 to 7, show the elution profile of DTaI fractionated by size-exclusion chromatography by SDS-PAGE. Lane 4 (fraction 15) gave the greatest yield (as determined by scanning densitometry of the commassie blue stained gel) of DTaI with the lowest degree of DTa and dimeric DTa contamination. The faint band at 31 Kd in lanes 3 and 4 was not present in all preparations. Additional SDS-PAGE analysis (data not shown) indicated the presence of dimeric DTa between fractions 6-21, DTaI between fractions 7-39, DTa between fractions 12-45, where insulin did not appear until fraction 30. Relatively pure preparations of DTaI which contained no insulin (fractions 11-17) were extensively dialyzed in water, aliquoted, lyophilized, and stored at -20°C until further use. Previous work involved the conjugation of DTaI to insulin by linkage to the free carboxyl groups of the insulin molecule [7]. This resulted in the appearance of many high molecular weight protein bands on a polyacrylamide gel, which indicated the presence of heteromeric conjugated $(DTaI)_nI$ species [7,9]. We did not obtain any heteromeric conjugates with our procedure. In fact, our results indicate that we have a monosubstituted insulin molecule (i.e. a 1:1 molar ratio of insulin:DTa). Upon reconstitution in PBS, DTaI retained greater than 60% of its ADP-ribosylation activity when compared to purified DTa (133 ± 9 mol of ADP-ribose-EF-2 formed/mol DTaI/minute *vs* 212 ± 9 mol of ADP-ribose-EF-2 formed/mol DTa/minute, respectively).

2.5.2 DTaI Cytotoxicity and Displacement Profiles

DTaI's biological effects on V-79 cells were tested to determine the degree of cytotoxicity. This cell line is sensitive to DT and exhibits an LD50 (the dose at which the lethal response is 50%) of 2.45×10^{-7} M (data not shown) and is in agreement with other reports [19]. The LD50 for DTaI was found to be 1.1×10^{-8} M, more than 22 times

lower than for the whole toxin itself suggesting that the route of entry differs from that of the whole toxin.

Next we looked at the ability for DTaI to competitively displace [^{125}I]-insulin [Figure 4]. The data indicate that DTaI exhibits the same competition profile as native insulin in displacing [^{125}I]-insulin from its receptor, indicating that the attachment of DTa to the insulin molecule results in no significant alteration in its binding to its receptor. The fact that DTa substitution to the insulin molecule does not interfere with insulin binding is a significant advancement over previous methods to obtain substituted insulin molecules For example, rhod$_7$-α-lactalbumin-insulin lost more than 90% of its binding ability, where α-lactalbumin conjugated solely with the ε-amino acid of insulin lost 10% of its binding potential to the insulin receptor [20]. Furthermore, the biological activity for these two lactalbumin-substituted insulins were greatly diminished (75% and 1% biological activity, respectively) [20]. It is interesting to note however, that the capped conjugate has little ability to competitively displace [^{125}I]-insulin from its receptor (Figure 4) substantiating the importance of the amino terminal group of the insulin A chain for insulin receptor-ligand binding. Capped DTaI also showed no toxic effects at concentrations as high as 10^{-6} M (data not shown).

Next we compared DTaI's toxicity profile to its displacement profile of [^{125}I]-labeled insulin or IGF-1. As illustrated in Figure 5, the concentration range for DTaI to produce 100% killing of V-79 cells occurs between 10^{-8} and 10^{-7} M. Similar results were obtained for the ability of DTaI to inhibit protein synthesis as determined by the incorporation of [^{3}H]-leucine into TCA-precipitable material (data not shown). In this same concentration range, DTaI could competitively displace 80% of [^{125}I]-insulin from its receptor but was unable to significantly displace [^{125}I]-IGF-1 from its receptor. Thus, the evidence presented herein indicates that DTaI causes cell death by gaining entry into the cell solely via its interaction with the insulin receptor. This is supported by the following evidence. first, the decrease in the LD50 for DTaI versus DT suggests a different route for DTaI than for DT. It has previously been reported that this observation could be partly attributed to the differences in affinity for insulin and DT for their respective receptors [7]. Second, the increase in DTaI's cytotoxic effects correlate with its ability to displace insulin from its receptor. If the toxic effects of the conjugate were mediated through the IGF-1 receptor the data presented here would indicate that only a one to five percent occupancy of the IGF-1 receptor sites are needed to effectively translocate DTa to its site of action and kill the cells. This seems unlikely since there are

other factors which play important roles in determining the effectiveness of DTa once it enters an endocytic [20-22] vesicular apparatus.

2.5.3 Selection of DTal resistant clones

At this point, we employed this DTal toxic conjugate in a selection procedure allowing us to isolate a number of clones resistant to DTal but still sensitive to DT (data not shown). Our studies show that these DTal-resistant clones expressed variation in their ability to bind [^{125}I]-Insulin (Table 1). Levels of insulin binding observed ranged from a 29% decrease (e.g. VI-A4-d) to a 68% decrease (VI-A5-d and VI-A2-C). In addition to insulin binding, [^{125}I]-IGF-1 binding was determined for a number of these clones. In all cases examined there was no significant alteration in the amount of [^{125}I] IGF-1 specifically bound suggesting that resistance to DTal is probably related to alterations in the insulin-receptor trafficking pathway and not at the level of IGF-1-receptor interactions (Table 1).

2.5.4 Mitogenic action

DTal resistant clones were also studied for their ability to grow in DMF containing 5% FCS. Out of all the clones tested, two appeared to have normal doubling times when compared to the parental cell line (IV-A1-j and VI-A5-d). Thus it would appear that even though there was a greater than 50% decrease in [^{125}I]-insulin binding for these two cell lines (55% and 60%, respectively), growth in 5% serum was unaffected (Table 1). This is not unusual since insulin is not a major growth factor in fetal calf serum. Yet it is interesting to note that some of the mutants isolated did express growth abnormalities in fetal calf serum (IV-A3-g, VI-A3-b, VI-A4-d). Cell variants with a defect in this insulin-receptor trafficking pathway, which also show a decreased ability to grow in serum indicate that parts of the insulin-receptor signalling pathway may be shared with other growth factors in generating growth promoting effects [23].

We have also studied the ability of these variants to grow in a serum-free medium where insulin at two different concentrations was used as the predominant mitogen (Figure 6). Both VI-A3-b and VI-A5-d cells exhibited insulin-dependent cell growth, where IV-A1-j cells did not. It is interesting to note that even though VI A3-b cells could exhibit insulin-dependent cell growth, its doubling times in serum or in serum-free medium with insulin are nearly two times as long as compared to V-79 cells.

In summary, the construction of a monomeric DTaI molecule has allowed us to isolate DTaI-resistant, DT-sensitive cell variants expressing defects along the insulin-receptor trafficking pathway. Alterations have been observed at the level of insulin binding, insulin-dependent cell growth and also serum-dependent growth. This would indicate that parts of the insulin-receptor trafficking pathway are important not only for insulin but for other growth factors in generating cell growth. Further examination is underway to understand how these alterations affect the cell's insulin-dependent growth and metabolic response characteristics.

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2.7 Figures and Tables for Chapter 2

Figure 1. Purification of DTa by heat precipitation.

1 ml samples of DT were treated with trypsin, reduced with 5% ß-mercaptoethanol for 30 min, placed into a hot water bath (90°C) for 5 min to enhance precipitation of DT, and centrifuged (described under materials and methods). Supernates (lane 3) of samples were added directly to sample buffer. Precipitates pelleted by centrifugation (lane 2) were first partially reconstituted by adding 0.5 ml of 20 mM TES (pH 7.6) containing 0.1 M NaCl and then added to Laemmli sample buffer. Low molecular weight standards (Bio-Rad) are in lane 1. Samples were then applied to a 10% SDS-Polyacrylamide Gel according to the method of Laemmli [15]. The gel was electrophorised at constant current (18 mA) for 1 hour, stained with 0.1% Coomassic Blue (made fresh) for 16 hours, and then destained using a 1:4:5 solution of acetic acid:methanol:water with an absorbent sponge for three hours.

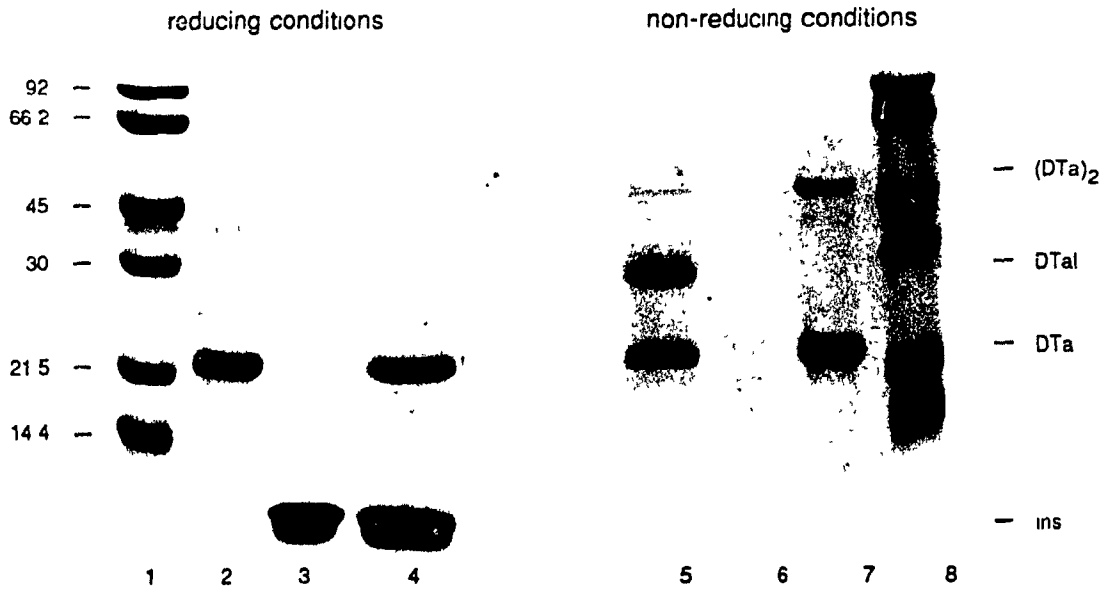

Figure 2. Formation of the insulin-diphtheria A hormono-toxin.

Lanes 1 to 4 contain samples which use Laemmli sample buffer with 1% ß-mercaptoethanol as a reducing agent. Laemmli sample buffer used for samples in lanes 5 to 8 did not contain a reducing agent. Procedures for electrophoresis and staining are the same as in Figure 1. Lanes 1 and 8 contain low mol. wt. protein standards ($x10^{-3}$), 2 and 7 contain DTa, 3 and 6 contain SDP-insulin (ins), and lanes 4 and 5 contain the crude DTaI mixture (see text for explanation). Note: non-reduced SDP-Insulin (lanes 5 and 6) does not absorb coomassie blue dye effectively and is mostly removed after 5 to 6 hours of destaining.

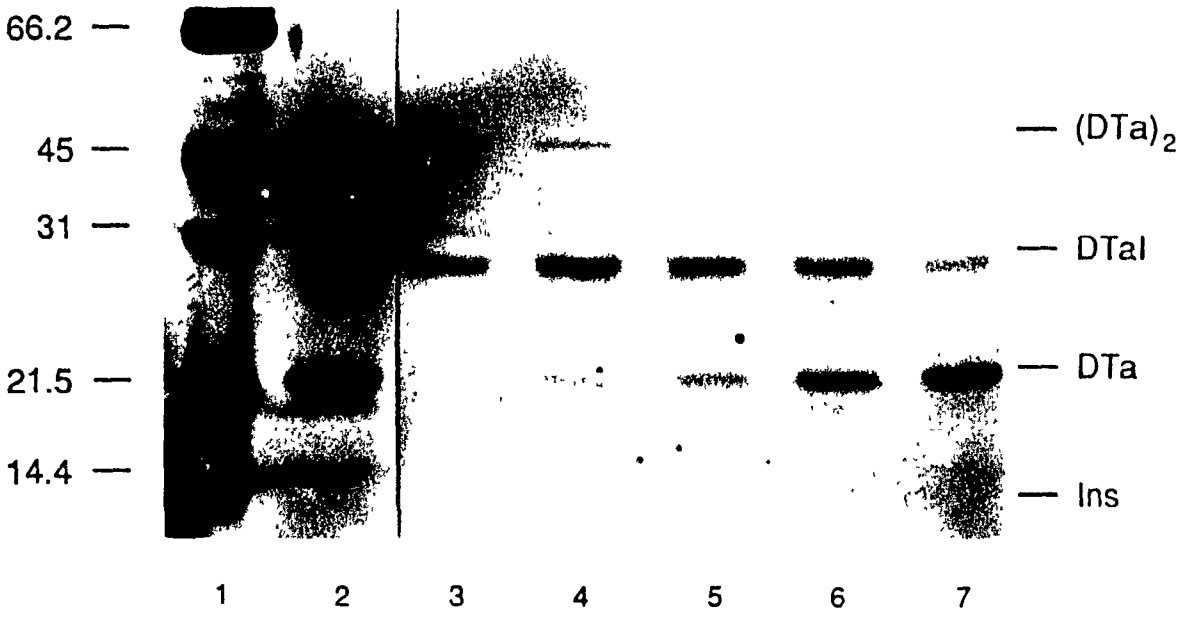

Figure 3. Elution profile of DTaI from Sephadex G-75 superfine column.

The crude conjugate mixture was applied to a Sephadex G-75 SF column (1 x 40 cm, void volume; 72 ml), and eluted with 20 mM TES containing 0.1 M NaCl (pH 7.4). 1 ml fractions were collected from which 10 µl samples were added to non-reducing Laemmli sample buffer, applied to a 10% SDS polyacrylamide gel, and electrophorised under the same conditions employed in Figure 1. Lanes 3 to 7 correspond to fractions 9,15,21,27, and 33 collected after the void volume Lane 1 included low molecular weight protein standards ($x10^{-3}$), lane 2 contained the crude conjugate mixture which was applied to the column. $(DTa)_2$; dimeric form diphtheria A fragment, DTaI, insulin-diphtheria A conjugated toxin: DTa, diphtheria A fragment and Ins, insulin.

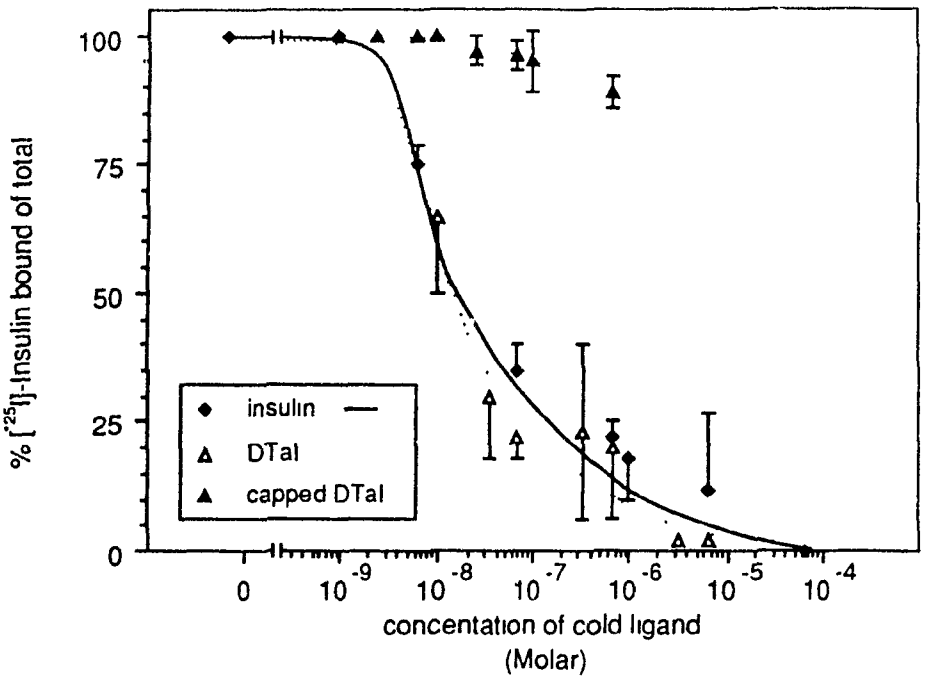

Figure 4. Ability of insulin, DTaI or capped-DTaI to competitively displace [^{125}I]-insulin from its receptor.

V79 cells were incubated with trace amounts of [^{125}I]-insulin and increasing concentrations of either insulin, DTaI, or capped-DTaI for 2 h. Cell-associated [^{125}I]-counts where then calculated as described under Material and Methods. Values represent the % of [^{125}I]-insulin specifically bound of total in the presence of increasing molar concentrations of unlabeled insulin (◆), DTaI (◇), or capped-DTaI (▲). Each data point represents the mean ± SEM of 3 separate experiments, with triplicate plates in each experiment.

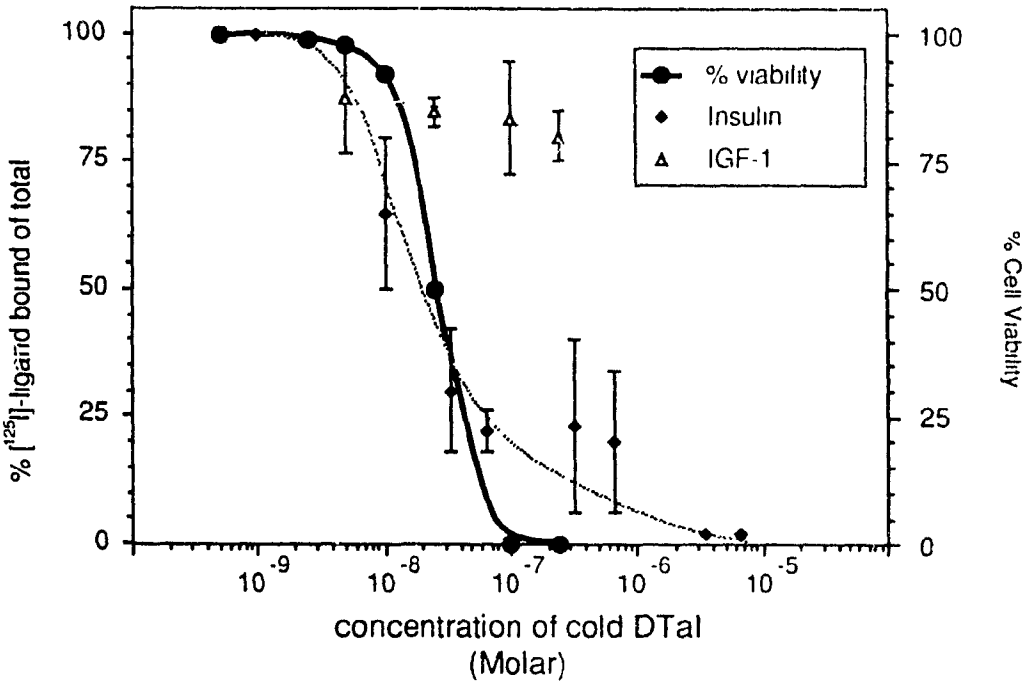

Figure 5. The ability of DTaI to competitively displace [^{125}I]-insulin or [^{125}I]-IGF-1 from their respective receptors and to kill V79 cells.

DTaI was added at increasing molar concentrations with tracer amounts of either [^{125}I]-insulin (◆) or [^{125}I]-IGF-1 (△) to V79 cells for 2 h. Values represent the amount of [^{125}I]-labeled ligand (insulin or IGF-1) specifically bound of the total For the DTaI toxicity profile, V79 cells were incubated either in the absence or the presence of an increasing molar concentration of DTaI for 2 h and then counted for cell viability after 24 h DTaI killing of V79 cells (●) was measured as the percent of viable cells remaining from cells grown in the absence of DTaI as determined by trypan blue exclusion (see methods for further details) In all cases, values represent the mean ± SEM of 3-5 separate experiments.

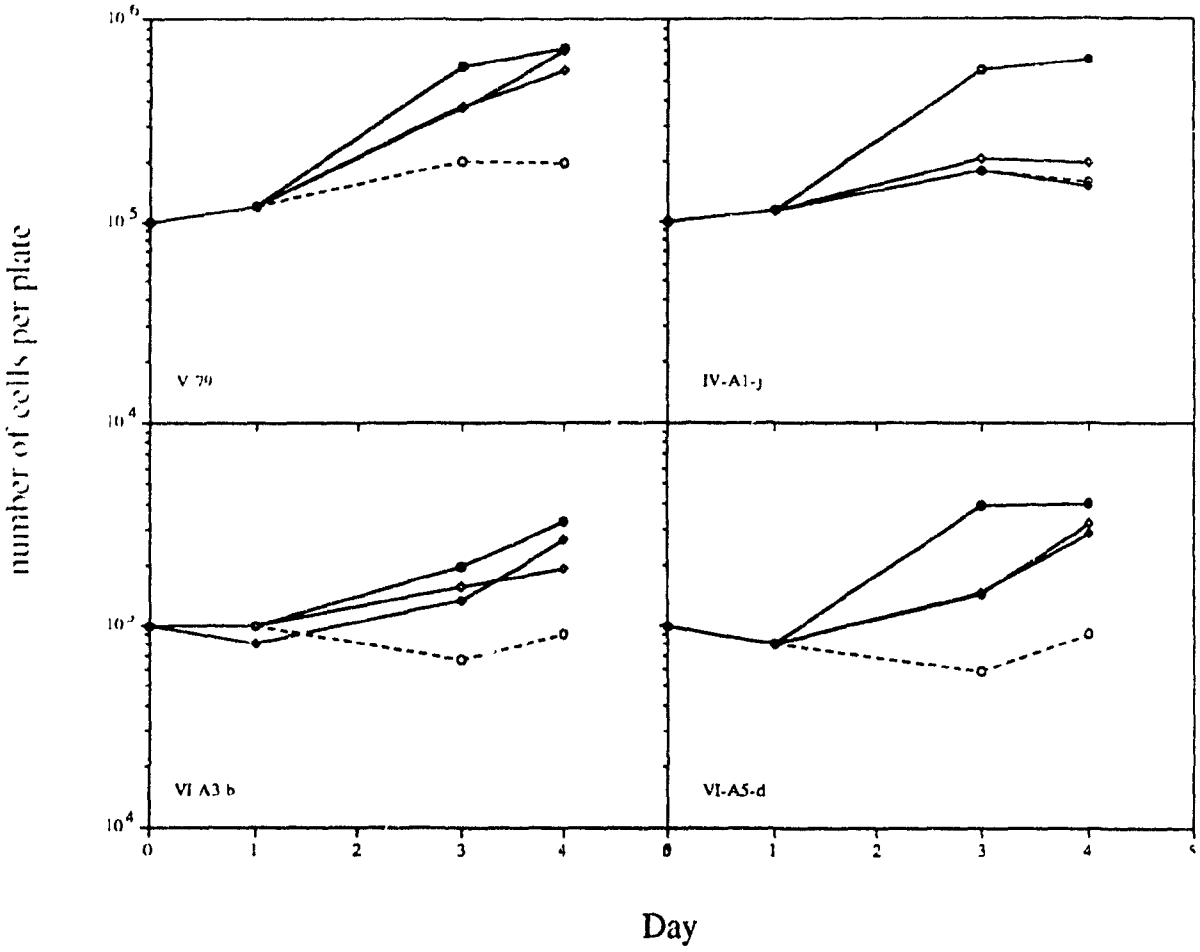

Figure 6. The ability of insulin, IGF-I and serum to stimulate growth of V-79 cells and DTaI resistant mutants.

Cells were treated with either 670 nM insulin (◆), 167 nM insulin (◇), 5% (v/v) fetal calf serum (●) or no additions in a basal medium (○) containing 0.2% (v/v) dialyzed heat-inactivated serum, 60 nM Tf and 1 nM EGF. Cell counts were done on various days and the values represent the number of viable cells per plate in 2 to 7 separate experiments.

Table 1.

Insulin and IGF-1 binding and cell doubling times for isolated clones resistant to an insulin-diphtheria A chain toxic conjugate

Cell (clone) line	[^{125}I]-ligand specifically bound (fmoles/mg protein)		Doubling Times in 5% FCS (hours)
	Insulin	IGF-1	
V-79 (parental)	11.2 ± 1.2	19.8 ± 1.21	13.0 ± 0.2
IV-A1-j	4.0 ± 0.4	18.7 ± 2.2	12.6 ± 0.4
IV-A3-g	6.4 ± 0.7	18.6 ± 1.6	16.1 ± 0.4
VI-A2-c	3.6 ± 0.5	21.0 ± 2.2	15.2 ± 0.2
VI-A3-b	6.3 ± 0.7	ND	20.8 ± 0.4
VI-A4-d	8.0 ± 2.0	ND	18.2 ± 0.6
VI-A5-d	3.6 ± 0.5	19.2 ± 1.6	13.4 ± 0.3

Binding is representative of the total amounts of [^{125}I]-labelled ligand specifically bound (see Materials and Methods) The binding data represent the average of 3 to 5 experiments ± SEM. Doubling times were calculated by counting the number of cells on days 1 to 6 The exponential part of the curve was used to determine the time required for the cells to double in number.
ND = Not Done

Chapter 3
The Growth Promoting Effects of Insulin and IGF-1 Occur at a Step Post-Ligand Binding and are Independent of Other Growth Factors in a Chinese Hamster Lung Fibroblast Cell Line

3.1 Preface

As was shown in chapter 2, a number of cell variants were initially tested at the level of insulin and IGF-1 binding as well as cell growth in serum or insulin. In this chapter, we further characterize IV-A1-j cells for their ability to respond to insulin. Since IV-A1-j cells had a decreased ability to bind insulin, were unable to grow in a hormonally-defined medium containing insulin as the predominant mitogen, but showed normal growth in serum, we extended our studies on this mutant's behavior to growth with other growth factors. This chapter describes the growth properties of both V-79 and IV-A1-j cells with different growth factors in a hormonally-defined medium.

All work in this chapter was performed by the candidate.

3.2 Abstract

Recently, we have isolated a Chinese hamster cell variant (IV-A1-j) resistant to an insulin-diphtheria-A chain toxic conjugate. This cell line exhibits a decreased level of insulin binding, but normal growth in serum-containing medium when compared to the parental cell line (V-79). In this paper we further characterized this variant for its ability to grow in a hormonally-defined medium using insulin or insulin-like growth factor type 1 (IGF-1) as mitogens. We report that although IV-A1-j cells are capable of growing in serum-containing medium, they are insensitive to the mitogenic actions of either insulin or IGF-1. In contrast, epidermal growth factor (EGF) and α-thrombin (THR) are capable of producing a mitogenic effect in IV-A1-j cells comparable to V-79 cells. The combination of EGF and/or THR with either insulin or IGF-1 results in an increase in V-79 cell growth above EGF and/or THR alone. On the other hand, insulin or IGF-I in the presence of other mitogens did not stimulate further growth in IV-A1-j cells.

From these observations we conclude that insulin and IGF-1 have a mitogenic signalling pathway which is distinct from other growth factors and that this pathway is defective in the IV-A1-j cell line.

3.3 Introduction

The ability of insulin to mediate growth through its own receptor has been a topic of much debate [1-6]. It is thought that insulin mediates its effects through the insulin-like growth factor type 1 (IGF-1) receptor, and that both the insulin receptor and IGF-1 receptor share a common post-receptor pathway necessary for mitogenic signalling [7-10]. Chinese hamster lung (CHL) fibroblasts and murine 3T3 fibroblasts have been well documented and characterized for their ability to demonstrate both insulin and IGF-1 dependent DNA synthesis [11-16]. Since these cell lines contain both insulin and IGF-1 growth factor receptors, interpreting the importance and independence of one pathway to the other has proven difficult. One way to overcome this problem is to study CHL cells that have an altered expression of insulin receptors but normal expression of IGF-1 receptors (IV-A1-3 cells) [17]. This report examines the ability of insulin and IGF-1 to stimulate cell growth in IV-A1-3 cells compared to parental CHL cells (V-79). Other growth factors were also used with insulin or IGF-1 to help understand the interrelationship that may exist between different classes of growth factors. The data suggest that both insulin and IGF-1 share a common mitogenic pathway which is distinct from stimulation with other growth factors since, (1) unlike V-79 cells, IV-A1-3 cells are unable to grow in hormonally-defined medium containing insulin or IGF-1 as predominant mitogens, (2) exposure of V-79 cells to a combination of insulin and IGF-1 gave no significant increase over incubation with either insulin or IGF-1 alone, and (3) epidermal growth factor (EGF) and/or α-thrombin (THR) are capable of producing growth responses in both cell lines. The characterization of IV-A1-3's growth response in hormonally-defined media and its inability to use insulin and IGF-1 as mitogens may provide a convenient system for studying the mitogenic cascade of insulin and IGF-1 and for understanding their interrelationship with other growth factor receptor signalling pathways.

3.4 Materials and Methods

3.4.1 Materials

Wild type CHL cell lines (V-79) [18] were a gift of Dr. I. Scheffler (Department of Biology, University of California, San Diego) and IV-A1-j cells were selected from the parental V-79 for their resistance to an insulin-diphtheria-A chain toxic conjugate as previously described [17]. Dulbecco's modified Eagle's Medium containing 4.0 mg glucose/ml (DME), modified Eagle's Medium containing 4.0 mg glucose/ml (MEM), 100X essential amino acids (EAA), 50X non-essential amino acids (NEAA), and ethylenediaminetetraacetic acid (EDTA) were purchased from Flow Laboratories. Culture flasks and 35 mm culture petri plates were from Falcon Co. and trypsin was from Difco Labs, Detroit.

Bovine pancreas insulin was purchased from Sigma, IGF-1 from AmGem Biological (Thousand Oaks, CA), and both EGF and THR were purchased from Biomedical Technologies Inc. (Stroughton, MA). Apo-transferrin (Tf) was graciously provided by Dr. P. Ponka (McGill University, Montreal) and fetal calf serum (FCS) was purchased from Flow Laboratories.

3.4.2 Methods

V-79 and IV-A1-j cells were grown in T-75 culture flasks using DME supplemented with EAA, NEAA, and 5% (v/v) FCS (DME+S) in an atmosphere of CO_2 plus air (5:95) at 37°C. For experiments, cells were harvested at confluence after incubation with 0.02% (w/v) EDTA and 0.04% (w/v) trypsin [19]. Cells were then seeded with 1.0 ml DME+S in 35 mm culture plates at a density of 60,000 cells per plate. After 24 hours the medium was replaced with a hormonally-defined medium (DME, EAA, NEAA, 0.2% (v/v) diaylized heat-inactivated serum, and 60 nM Tf; basal medium) containing different concentrations of insulin, IGF-1, EGF, and THR. For studies involving insulin, IGF-1, and THR, the medium was supplemented with 1 nM EGF. After various time intervals (1-4 days), cells were harvested using 0.02% (w/v) EDTA and counted. Except for growth curves, values are expressed as the fold stimulation from basal (average ± SEM), which is representative of the number of cells grown in the presence of various mitogens on specific days and divided by the number of cells present in the basal medium for that same day. Statistical significance was determined by the

student's t-test. In all cases, the number of cells plated in basal medium for both V-79 and IV-A1-j were not significantly different (8.4 ± 0.5 and 8.2 ± 0.6 x 10^5 cells/plate, respectively), where cell viability was greater than 95% as determined by trypan blue exclusion.

3.5 Results

We recently reported that IV-A1-j cells exhibited a greater than 50% decrease in insulin binding (11.2 ± 1.2 vs 4 ± 0.4 fmoles insulin/mg protein, n=3), yet showed no differences in IGF-1 binding when compared to V-79 parental cells [17]. For example, specific IGF-1 binding in the V-79 cell line was 19.8 ± 1.2 fmoles/mg protein while in the IV-A1-j cells IGF-1 binding was 18.7 ± 2.2 fmoles/mg protein, (n=3). Furthermore, both cell lines demonstrated the same ability to grow in DME supplemented with either 5% or 10% FCS [17]. Since overall growth of this cell line in serum appeared to be normal, we created a growth culture system where we could study IV-A1-j's ability to grow in a hormonally-defined medium where insulin could be used as a predominant mitogen. Our results indicate that while V-79 cells were capable of insulin-dependent proliferation in a medium containing insulin at a concentration of 170 nM, IV-A1-j cells showed no significant differences in growth between the basal and insulin-containing medium over a 4 day period (Figure 1). Further, a 4-fold higher concentration of insulin (i.e. 670 nM) could not stimulate any insulin-dependent cell growth on IV-A1-j cells (Table 1). IV-A1-j cells were monitored for up to 5 days with no significant increase in growth above basal (data not shown).

The data in Table 1 also show that IGF-1 at a concentration of 30 nM was capable of producing a growth response in V-79 cells nearly 2 times the basal, while IV- A1-j cells showed no apparent response to IGF-1. This latter result was surprising, since both cell lines expressed similar levels of IGF-1 binding. We next established dose-dependent profiles by incubating each cell line with different concentrations of insulin (Figure 2) or IGF-1 (Figure 3) and performing cell counts on day 3. Even at the lowest insulin concentration employed (i.e. 33 nM), V-79 cells gave a significant increase in cell growth (2.7 ± 0.3 fold above the basal). IV-A1-j cells, on the other hand, showed no increase in growth at all insulin concentrations tested (1.02 ± 0.12 maximum fold stimulation for 170 nM insulin). A similar growth pattern was observed using IGF-I, such that the optimal IGF-I concentration for V-79 cells was between 30 and 70 nM (2.1 ± 0.2 fold above the basal) where IV-A1-j cells were unresponsive. Also, we examined cell growth changes for insulin and IGF-I in combination with each other at maximal stimulating concentrations. V-79 cells showed no significant increase in cell growth when compared to either insulin or IGF-1 alone (1.68 ± 0.21, 1.76 ± 0.29, 1.70 ± 0.05 fold stimulation over basal for insulin, IGF-1, and insulin + IGF-1, respectively). IV-

A1-j cells showed no significant increase over the basal medium (1.03 ± 0.06 fold stimulation over basal for insulin + IGF-1).

Since IV-A1-j cells showed normal growth in 5% FCS yet lacked insulin or IGF-1 stimulated cell growth, we determined whether this deficit was specific for insulin and IGF-1 or a general characteristic seen for other mitogens. Therefore, EGF and THR were used since it is thought that they act through regulatory pathways which differ from insulin and IGF-1 [21]. The data in Table 2 show the dose-dependent response for V-79 and IV-A1-j cells, with EGF. Both cell lines were equally responsive to EGF, where a maximal effect was reached at a concentration of 2 nM (1.62 ± 0.05 and 1.68 ± 0.09 fold stimulation over basal for V-79 and IV-A1-j cells, respectively). As illustrated in Table 3, THR was also shown to be a good inducer of cell growth, exhibiting its maximal effect at 30 nM (1.56 ± 0.09 and 1.61 ± 0.14 fold stimulation over basal of V-79 and IV-A1-j cells, respectively).

We also examined insulin and IGF-1 in combination with other growth factors to determine whether these two growth factors had any effects on functional mitogenic pathways in IV-A1-j cells. The data in Table 4 show that with no additional growth factors added, the effect of insulin in V-79 cells produced half (53 ± 3%) the mitogenic response of serum, where the effect of IGF-1 was lower (41 ± 1% for IGF-1, data not shown). IV-A1-j cells failed to exhibit such a growth response (e.g. < 2%, for insulin). Both EGF and THR significantly stimulated growth in the absence of insulin in both cell lines (from 32-37% for THR and 40-47% for EGF), yet lacked any significant additive effect when combined together. It is interesting to note however, that the combination of EGF, THR and insulin allowed V-79 cells to proliferate to the same extent as in serum alone (101 ± 3%), but the loss of any one of these mitogens resulted in a small but noticeable decrease from serum (82 ± 4 and 90 ± 4 for EGF+insulin and THR+insulin, respectively). With regards to IV-A1-j cells, insulin failed to stimulate growth even in the presence of other growth factors. Additionally, when all three growth factors were added together, IV-A1-j gave only a 42 ± 7% increase in cell growth as compared to serum; this can be attributed to the addition of EGF and THR alone (45 ± 9%). When IGF-1 was substituted for insulin in these studies a similar pattern was observed (data not shown).

3.6 Discussion

In this report, we investigated the growth of V-79 and IV-A1-j cells in the presence of insulin, IGF-1, EGF, and THR. It has previously been shown that IV-A1-j cells, a variant derived from the parental V-79 cell line for its resistance to an insulin-diphtheria-A chain toxic conjugate, exhibited a decreased ability to bind insulin, yet showed no alteration in its level of IGF-1 binding or for its growth in serum containing medium [17]. Since insulin is known to be an important factor in regulating cell growth [1,3,5,7-9,22], we examined the ability of V-79 and IV-A1-j cells to grow in a hormonally-defined medium where insulin was the predominant mitogen. A previous report which employed a similar system to monitor cell growth of CC139 Chinese hamster fibroblasts suggested that the mitogenic ability of insulin is solely mediated through the IGF-1 receptor [11]. Since the level of IGF-1 binding was unaltered in IV-A1-j cells, we expected little change in this cell's ability to mediate an insulin-dependent mitogenic response. Our results clearly demonstrate that insulin could not stimulate growth in IV-A1-j cells. Moreover, using IGF-1 as a mitogen failed to cause any significant increase in cell growth for IV-A1-j cells. It was possible that IV-A1-j cells could exhibit an alternate threshold from V-79 cells to produce a response, therefore we expanded the range of concentrations employed for both insulin and IGF-1. Yet at all concentrations tested, IV-A1-j cells failed to show any insulin or IGF-1 dependent cell proliferation. Since, the level of IGF-1 binding was unaffected in IV-A1-j cells, the lack of both an insulin or IGF-1 response supports the idea that these two mitogens might act through a related post-receptor system, which is in agreement with previously related studies [23,24]. This is further substantiated by the idea that the combination of both growth factors shows no significant increase in V-79 cell growth when compared to their abilities to work individually, and that the selection system used to obtain the IV-A1-j variant involved a toxic protein which interacted specifically through the insulin, and not IGF-1, receptor pathway [17]. Nonetheless, the lack of both an insulin and IGF-1 mitogenic response does not rule out the fact that other receptor systems may be operative at the level of mitogenesis, especially since both cell lines showed similar rates of growth in serum-containing medium. For example, it had been reported by others that the addition of insulin and THR to a serum-free medium were required in order to produce a mitogenic response [11]. However in the absence of insulin, a 10 fold increase in the concentration of THR alone could mediate an equivalent growth response [11].

Also, cultured human fibroblasts have been known to possess multiple independent mitogenic pathways for cell growth [21]. In order to study the relationship between insulin and other growth factor pathways, EGF and THR were used. The dose-dependent growth profiles for both EGF and THR were identical in both cell lines indicating that these two mitogens work through similar post-receptor pathways and that these pathways differ from the insulin and IGF-1 pathway.

Since our results suggested that insulin and IGF-1 work through a distinct mitogenic pathway separate from EGF or THR, we next examined the relationship between insulin and IGF-1 in combination with EGF and/or THR. In V-79 cells, insulin caused an additive increase in growth above that observed for EGF, THR, or EGF and THR.

In summary, the variant IV-A1-j cell line which is resistant to an insulin-diphtheria A chain toxin, unlike the parental V-79 cell line, is not capable of growing in a hormonally-defined medium where either insulin or IGF-1 is used as the predominant mitogen. Whether this lack of responsiveness is due to a decrease in receptor binding or expression, an increased degradation of receptor complexes at a post-receptor level, and/or due to altered receptor and/or substrate phosphorylation mechanisms, has yet to be examined. Nonetheless, both cell lines are capable of growing in a hormonally-defined medium when either EGF and/or THR are employed. These results suggest that insulin and IGF-1 share a common pathway that is independent of other growth factors. Furthermore, insulin and IGF-1 elicit their mitogenic actions at a site post-ligand binding since IGF-1 binding in IV-A1-j cells is unaffected. In conclusion, these two cell lines provide us with a system for studying the interrelationship of insulin and IGF-1 with other mitogenic pathways. Further investigation is underway to clarify these pathways and to better understand the mechanisms involved in the unresponsive behavior of IV-A1-j cells.

3.7 References

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3.8 Figures and Tables for Chapter 3

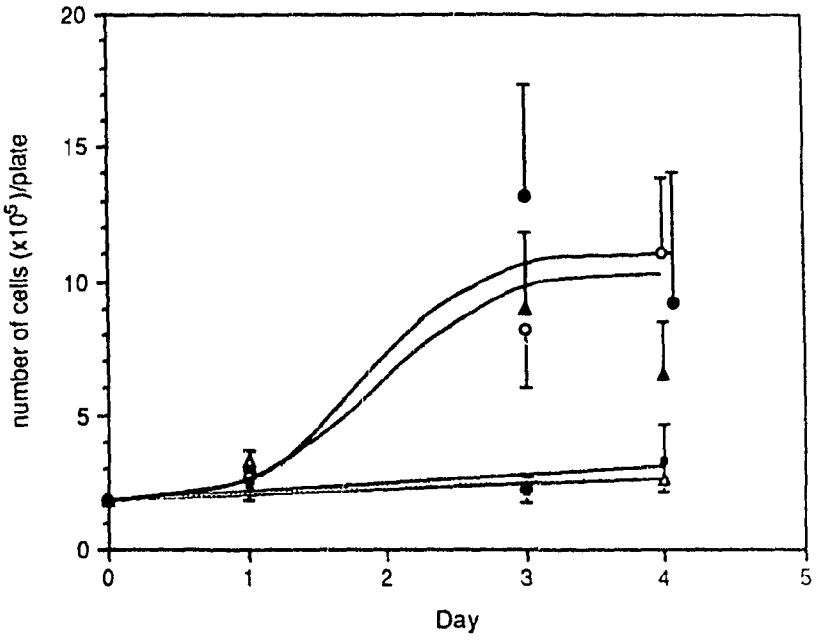

Figure 1. Growth of V-79 and IV-A1-j cells using insulin as the predominant mitogen in hormonally-defined medium.

Values represent the mean (± SEM) for the number of cells per plate grown for various days with serum (● :V-79; ○ :IV- A1-j), 170 nM insulin (▲ :V-79; △ :IV-A1-j) or basal medium (■ :V-79; □ :IV- A1-j) from 4 - 5 individual experiments.

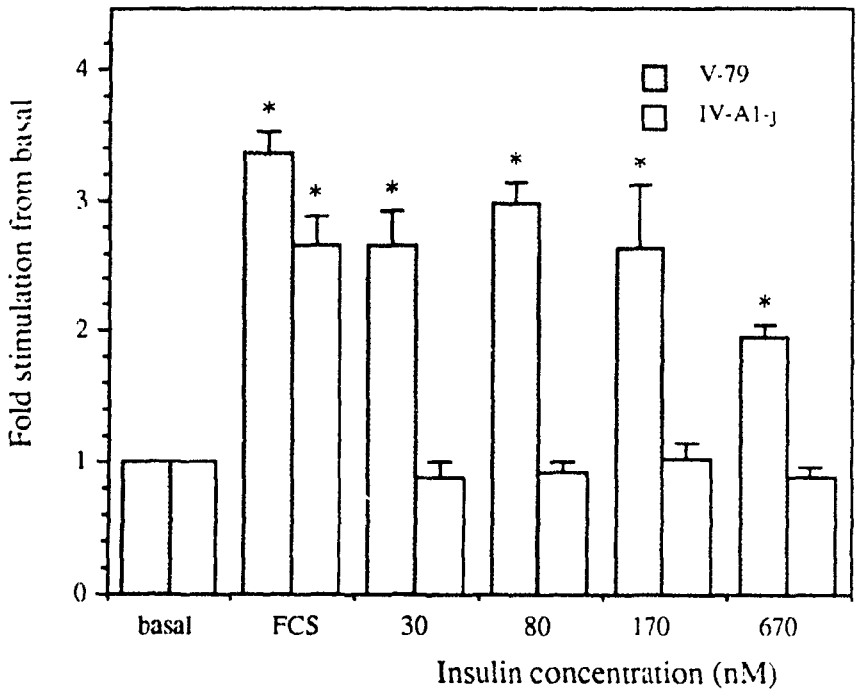

Figure 2. The effect of insulin concentration on the growth of V-79 and IV-A1-j cells in hormonally-defined medium.

Cells were treated with the indicated concentrations of insulin in basal medium for 3 days, then counted. The values represent the ratio of cells grown with different concentrations of insulin or 5% (v/v) fetal calf serum (FCS) over the number of cells grown in basal medium for 5 separate experiments. * indicates values are significantly greater than the values for cells maintained in basal medium ($P < .05$).

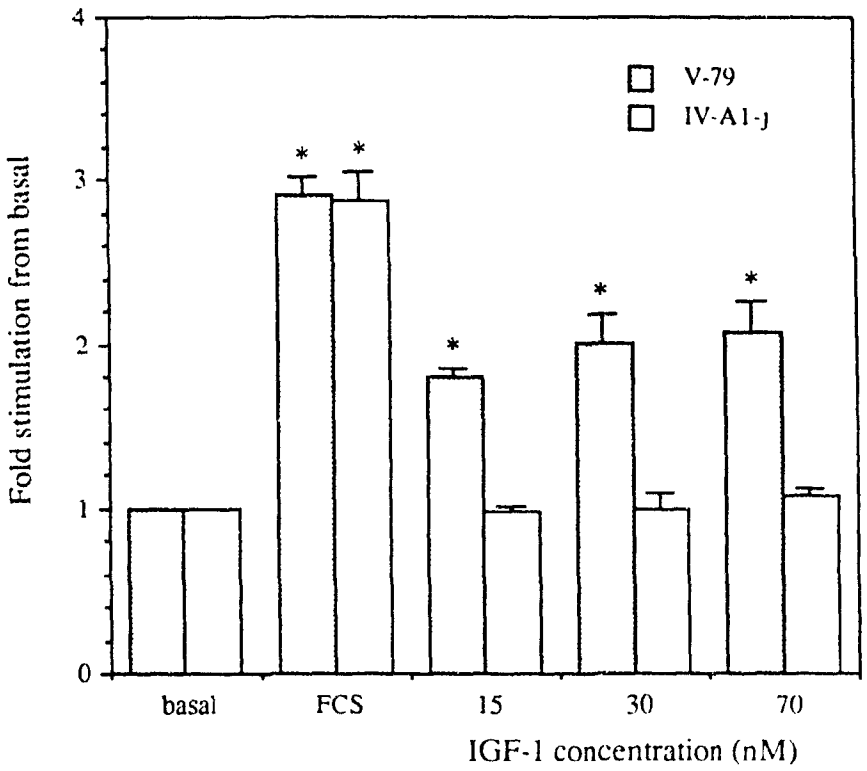

Figure 3. The effect of IGF-1 concentration on the growth of V-79 and IV-A1-j cells in hormonally-defined medium.

Cells were treated with the indicated concentrations of IGF-1 in basal medium for 3 days then counted. The values represent fold stimulation of cell growth over basal medium of 6 (V-79) and 7 (IV-A1-j) experiments * indicates values are significantly greater than the values for cells maintained in basal medium ($P < .05$), (FCS; 5% (v/v) fetal calf serum)

Table 1.

The effect of serum, insulin, or IGF-1 on the growth of V-79 cells and IV-A1-j cells in a hormonally-defined medium

	number of cells ($x10^{-5}$)/plate	
Treatment	V-79	IV-A1-j
basal	2.41 ± 0.29	2.14 ± 0.35
serum	13.2 ± 4.2*	8.2 ± 2.2*
170 nM insulin	9.0 ± 2.8*	2.40 ± 0.24
670 nM insulin	10.0 ± 3.9*	2.48 ± 0.39
30 nM IGF-1	3.98 ± 0.26*	2.39 ± 0.83

The values represent the mean ± SEM for the number of cells per plate after 3 days of growth in basal medium alone or containing either 5% (v/v) fetal calf serum (serum), 170 nM insulin, 670 nM insulin, or 30 nM insulin-like growth factor type one (IGF-1).

* Significant difference from control ($P<.05$), student t-test.

Table 2.

The effect of serum and different concentrations of epidermal growth factor (EGF) on the growth of V-79 cells and IV-A1-j cells in a hormonally-defined growth medium

EGF	fold stimulation of growth	
(nM)	V-79	IV-A1-j
basal	1	1
0.2	1.12 ± 0.05	1.12 ± 0.03
1.0	1.45 ± 0.02*	1.40 ± 0.06*
2.0	1.62 ± 0.05*	1.68 ± 0.09*
5% serum	3.49 ± 0.26*	3.37 ± 0 24*

Fold stimulation of growth represent the ratio of cell number per plate in the treated to basal state after 3 days in culture. The values represent the mean ± SEM for fold stimulation above the basal from 3 separate experiments.

* Significant difference from control (P<.05), student t-test.

Table 3.

The effect of serum and different concentrations of α-thrombin (THR) on the growth of V-79 cells and IV-A1-j cells in a hormonally-defined growth medium

THR	fold stimulation of growth	
(nM)	V-79	IV-A1-j
basal	1	1
15	1.49 ± 0.01*	1.54 ± 0.11*
30	1.56 ± 0.09*	1.61 ± 0.14*
150	1.31 ± 0.17*	1.42 ± 0.08*
5% serum	3.05 ± 0.13*	3.00 ± 0.05*

Fold stimulation of growth represent the ratio of cell number per plate in the treated to basal state after 3 days in culture. The values represent the mean ± SEM for fold stimulation above the basal from 3 separate experiments.

* Significant difference from control ($P<.05$), student t-test.

Table 4.
The effect of insulin in combination with other growth factors on cell growth.

Cell line	Insulin	% of serum-stimulated growth			
		-EGF	+EGF	+THR	EGF+THR
V-79	-	0	48 ± 3	37 ± 3	48 ± 5
	+	53 ± 3	82 ±4 *	90 ± 4*	101 ± 3*
IV-A1-j	-	0	40 ± 5	32 ± 3	45 ± 9
	+	<2	39 ± 5	34 ± 2	42 ± 7

Insulin (170 nM) was added alone or in combination with EGF (2 nM) or α-thrombin (30 nM; THR) in serum-free medium to V-79 and IV-A1-j cells for three days. At this time cells were counted. The data are from three separate experiments (duplicate plates in each experiment) and growth represents the increase in cell number above the number obtained in the absence of insulin as a % of the serum-stimulated growth.

For example,

[(cell number+insulin) - (basal cell number)]/[(cell number+serum) - (basal cell number)]x 100 = % of serum-stimulated growth

Values represent the mean ± SEM and were analyzed by the student's t-test. * Significant difference with the addition of insulin ($P<.05$).

Chapter 4

Tyrosine Phosphorylated Substrate pp175 and Insulin Degradation May Be Involved in Insulin and IGF-1 Dependent Cell Growth in Chinese Hamster Lung Fibroblasts

4.1 Preface

Since the mitogenic pathway for insulin action was affected in IV-A1-j cells we then looked at the metabolic pathway to see if it was similarly affected or if the mutation generated in IV-A1-j cells was working at a level which only affects the mitogenic response. In addition, we examined insulin-receptor internalization and routing to determine which parts of this trafficking pathway might be important for generating an insulin response. This was achieved by looking at insulin binding, internalization, and degradation. This chapter also uncovered the possible involvement of putative second messengers phosphorylated at tyrosine specific sites.

All work in this chapter was performed by the candidate.

4.2 Abstract

We have recently isolated a cell line (IV-A-j) resistant to an insulin-diphtheria A-chain molecule (DTaI) that shows decreased insulin binding and an inability to grow in a hormonally-defined medium where insulin is the predominant mitogen (Leckett, B. and Germinario, R.J. (1991) *Exp. Cell Res., in submission*). In this report we have further characterized IV-A1-j cells. Our results indicate that the decreased ability of IV-A1-j cells to bind insulin is not due to an alteration in insulin receptor affinity, but rather due to a decrease in the number of insulin receptors capable of binding insulin at the cell surface. Time course studies using [^{125}I]-insulin showed no differences in the internalization rates for both cell lines, yet there was a significant difference in [^{125}I]-insulin degradation, such that the variant cell line exhibited a 3-fold increase in insulin degradation compared to V-79 cells at 15 minutes post-insulin binding. Hexose transport studies showed no differences in insulin-stimulated hexose transport between IV-A1-j cells and V-79 cells. Lastly, protein tyrosine phosphorylation was studied in both cell lines using an antiphosphotyrosine antibody which detected an insulin-inducible 175,000 dalton protein band (pp175) in V-79 cells but not in IV-A1-j cells.

The evidence presented here and the fact that IV-A1-j cells, unlike V-79s cells, are unable to grow in a hormonally-defined medium containing insulin, suggests that enhanced insulin degradation and pp175 are not related to insulin-dependent glucose uptake, but may be involved in insulin-dependent cell growth in Chinese hamster cells.

4.3 Introduction

The insulin receptor is a glycoprotein which consists of two extracellular α-subunits capable of binding insulin and two transmembrane β-subunits which contain an intracellular tyrosine-specific protein kinase [1]. Upon binding of insulin to its receptor, the β-subunit becomes phosphorylated at tyrosine-specific sites and the receptor/ligand complex is internalized into endosomes [2-4]. Eventually insulin can be degraded, sequestered within certain parts of the cell, and/or recycled back to the surface [5]. It is somewhere during this process of insulin binding, internalization, intracellular routing, and degradation that insulin can mediate its cellular effects. Studies which employ antibodies directed toward the tyrosine kinase (TK) domain of the insulin receptor [6,7] and look at receptors mutated in this region (Lys-1018/1030) [8,9], indicate that insulin receptor tyrosine kinase activity is crucial for mediating both the metabolic effects and the mitogenic effects of insulin action. Interestingly, truncated insulin receptors [10] or mutated receptors lacking tyrosine-specific phosphorylation sites on the β-subunit between the TK domain and the carboxyl-terminal (i.e. positions 1146,1150,1151 [11]) can not mediate insulin-dependent hexose uptake, yet do show an ability to produce a mitogenic response. A more recent study showed that the mutation of a potential tyrosine-specific phosphorylation site upstream from the TK domain (Tyr-960) diminishes the incorporation of thymidine into DNA and shows a decreased ability to phosphorylate a 185,000-dalton protein (pp185) in Chinese hamster ovary (CHO) cells [12]. While these studies do not indicate that pp185 may be directly involved in the mitogenic signalling pathway, they do reflect the importance of the TK domain for mediating insulin action, and that there exist at least two different pathways for the activation of the insulin-dependent metabolic and mitogenic cascades. The fact that most cells require at least a 10 hour exposure of insulin to induce cell growth [13] and that the insulin receptor-ligand complex maintains its TK activity even after being internalized [14], strongly suggests that cellular routing and/or degradation of the insulin-receptor complex may be important for insulin's mitogenic action, possibly through the tyrosine-specific activation of key substrates located in different parts of the cell.

In order to test this hypothesis, we had at our disposal a cell line which shows resistance to a diphtheria A-chain-insulin toxic conjugate (DTaI) [15]. This cell line (IV-A1-j) showed a decreased ability to bind insulin when compared to wild type cells (V-79), yet had no alteration in its insulin-like growth factor type one (IGF-1) binding

ability. In addition, while IV-A1-j cells showed no alteration in their ability to grow in serum, they were unable to grow in a hormonally-defined medium in which insulin or IGF-1 was used as a predominant mitogen. Thus, we examined, (1) the insulin binding characteristics of IV-A1-j cells, (2) the ability of the mutant cell line to mediate insulin-dependent receptor internalization and insulin-stimulated hexose transport, and (3) its ability to phosphorylate endogenous substrates at tyrosine-specific sites. Our results show that IV-A1-j cells, in comparison to V-79 cells, have fewer insulin receptors which are capable of binding insulin and that these receptors are capable of mediating insulin-dependent internalization. Furthermore, IV-A1-j cells, when compared to V-79 cells, are unaltered in their ability to demonstrate insulin-stimulated hexose transport.

In contrast, IV-A1-j cells show an augmented ability to degrade insulin and are unable to phoshorylate a 175,000 molecular weight protein at tyrosine-specific sites when exposed to insulin. In the present study, we provide further proof that both the metabolic pathway and the mitogenic pathway for insulin action are mediated through different mechanisms. We also present evidence that enhanced insulin degradation and absence of pp175 do not affect insulin-stimulated hexose transport in Chinese hamster lung fibroblasts, but may be involved in insulin-dependent and IGF-1-dependent cell growth.

4.4 Experimental Procedures

4.4.1 Materials

Wild type CHL cells (V-79) [16] were a gift of Dr. I. Scheffler (Department of Biology, University of California, San Diego) and IV-A1-j cells were selected from the parental V-79 for their resistance to an insulin-diphtheria A-chain toxic conjugate (DTaI) as previously described [15]. Dulbecco's modified Eagle's Medium containing 4.0 mg glucose/ml (DME), modified Eagle's Medium containing 4.0 mg glucose/ml (MEM), 100X essential amino acids (EAA), 50X non-essential amino acids (NEAA), and ethylenediaminetetraacetic acid (EDTA) were purchased from Flow Laboratories. Culture flasks and 35-mm culture petri plates were from Falcon Co. and trypsin was purchased from Difco Labs, Detroit.

Bovine pancreas insulin was purchased from Sigma, monocomponent insulin was a gift from Lilly Research Laboratories, Indianapolis and [^{125}I]NaI was obtained from Amersham Corp. [^{125}I]-insulin (180-210 uCi/ug) was made by the chloramine-T method [17], [^{3}H]-2-deoxy-D-glucose was from New England Nuclear Corp., Boston, mouse monoclonal anti-phosphotyrosine antibodies (clone PT-66) were purchased from Sigma, and [^{125}I]-sheep anti-mouse antibody was graciously provided by Dr. Mark Wainberg (McGill University, Montreal, Canada).

4.4.2 Cell Culture

DTaI-resistant IV-A1-j cells [15] and V-79 cells were grown in Dulbecco's modified Eagle's Medium (DME) supplemented with 5% (v/v) fetal calf serum (FCS) in an atmosphere of CO_2 plus air (5:95) at 37°C. To carry cultures, cells were harvested at confluence in 75-cm^2 culture vessels after incubation with 0.02% (w/v) EDTA and 0.04% (w/v) trypsin (Difco Labs, Detroit) [18]. For insulin binding and hexose transport assays cells were plated in 35-mm plastic petri plates, where substrate phosphorylation assays involving cell extraction employed 100-mm plastic dishes (Falcon Co.) seeded at a density of 4 x 10^4 cells/cm^2. After 24 hours, cells were serum-deprived for 24 hours in 0% MEM medium containing 4 mg/ml glucose for receptor binding, hexose transport, and substrate phosphorylation studies.

4.4.3 *Insulin Binding Studies*

Serum-deprived confluent monolayer cultures were washed three times with 3 ml of 20 mM Hank's-Hepes buffer (pH 7.4) with 0.2% BSA at 22°C. Cells were then incubated with 1 ml of the same buffer containing different concentrations of insulin and [^{125}I]-insulin at a concentration of 0.17 nM and incubations were carried out at 22°C for 2 hours. At this time point equilibrium had been reached for both cell lines and less than 2% of the radio-labelled ligands had been degraded as assessed by the appearance of TCA-soluble radioactivity in the medium (data not shown). Non-specific binding was calculated using 6.7 μM of cold insulin and subtracted from total binding. The amount of radioactivity was assessed by solubilizing the cells with 1.2 ml of 1N NaOH and counting 1 ml samples as previously described [19]. Identical plates not treated with [^{125}I]-insulin were solubilized directly with 1 ml of 1N NaOH and 0.2 ml aliquots were taken for protein determination [20].

4.4.4 *Insulin Internalization Studies*

Binding experiments were performed as above with slight modifications. Cells were pre-incubated with [^{125}I]-insulin at 4°C for 4 hours to allow steady-state equilibrium. [^{125}I]-insulin was then removed by washing the cells 4 times with 20 mM Hank's-Hepes buffer (pH 7.4) containing 0.2% BSA at 4°C. At zero time the medium was replaced with 1 ml of 37°C buffer. To stop [^{125}I]-insulin internalization, cells were washed rapidly 4 times with 4°C buffer and immediately placed at 4°C. Internalized [^{125}I]-insulin was assessed by incubating the cells with 0.2 M acetic acid (pH 2.7) containing 0.5 M NaCl for 6 minutes at 4°C. Under these conditions greater than 95% of all surface-bound insulin was removed. Cells were then solubilized with NaOH to determine the amount of [^{125}I]-insulin incorporated. The amount of surface bound [^{125}I]-insulin was determined by subtracting the amount of internalized insulin from the amount of total insulin bound (plates not treated with acetic acid) at each time frame.

4.4.5 *Measurement of Insulin Degradation*

In order to determine the amount of [^{125}I]-insulin that was degraded both intracellularly and extracellularly, the above procedure was performed with two additional steps. First, after the indicated time points for incubation at 37°C, the media

for each plate was stored in microcentrifuge tubes pre-chilled at 4°C. Cells were then rinsed and placed at 4°C as above. Second, cells which were stripped of their surface bound insulin were exposed to 1 ml of Triton X-100 (Trix) for one hour. No detectable amounts of degradation were observed on the surface of either cell line during the entire 37°C incubation time frame, nor was there any detectable increase in the amount of [^{125}I]-degraded insulin in the medium during the one hour exposure to Trix (unpublished observation). 0.4 ml aliquots of the media were taken directly for [^{125}I] counting, where an additional 0.4 ml was incubated with 0.4 ml of 20% TCA for one hour in an microcentrifuge stored at 4°C. The contents were centrifuged at 14,000 RPM for five minutes and protein pellets were counted. The amount of TCA soluble material was determined by subtraction of TCA precipitable material and the total amount counted in 0.4 ml. In all cases, the amount of TCA soluble material did not exceed 10% of the total amount of [^{125}I]-insulin initially bound for all time frames. To determine the amount of TCA soluble material in the medium, 0.4 ml media aliquots were collected and subjected to the same process as for Trix soluble aliquots.

4.4.6 Measurement of [^{3}H]-2-Deoxy-D-Glucose

Serum-deprived cells were washed 3 times with glucose-free phosphate buffered saline, pH 7.4 (PBS) at 37°C. 0.8 ml of 0.05 mM [^{3}H]-2-DG (specific activity 50 μCi/μmole) was then added for 2 minutes. Hexose transport has been found to be linear up to 10 minutes in both cell types for this concentration. After the required incubation time, cells were washed 4 times with 2.0 ml of PBS (pH 7.4) containing 5 μM cytochalasin B (Aldrich Chem. Co.) at 4°C. Cells were then dissolved in 1N NaOH and aliquots were taken for liquid scintillation counting and protein determination [20].

4.4.7 Detection of Tyrosine-phosphorylated Endogenous Substrates

24 hour serum-deprived cells grown in P-100 petri plates (~15x10^6 cells per plate) were incubated with or without insulin (17 nM) for 10 min at 37°C. Cells were washed quickly 4 times with 5 ml of PBS (pH 7.4), then scraped from the plates using a rubber policeman and 3 ml of PBS containing 200 mM NaF and 5 mM $NaVO_3$ (PBS-V). Samples were centrifuged (500g) and cell pellets were resuspended in 0.35 ml (total volume) of PBS-V. 10 μl aliquots were removed to determine protein, where 0.3 ml aliquots were added to 0.1 ml of a 4X solution of Laemmli-sample buffer containing 3%

SDS and 1% β-mercaptoethanol [21]. The mixture was vortexed vigorously and sonicated at 4°C for 5 min. Lysates were applied at equal concentrations to an SDS-polyacrylamide gel (7.5%) and fractionated. The proteins were then transferred onto nitrocellulose [22] and the blots were exposed to a mouse monoclonal antibody specific for phoshotyrosyl residues [23] followed by [^{125}I]-labeled sheep anti-mouse antibody. Protein bands were scanned using a LKB Ultrascan XL densitometer interfaced with an IBM personal computer and GelScan XL software (LKB).

4.5 Results

4.5.1 Insulin Binding and Growth

In initial studies we looked at the ability for DTaI-resistant IV-A1-j cells and parental V-79 cells to bind both [^{125}I]-labeled insulin and IGF-1 [24]. We also examined their ability to grow in a hormonally-defined medium where either growth factor was the predominant mitogen. The data in Table 1 show that IV-A1-j cells bind 4.0 ± 0.4 fmoles of [^{125}I]-insulin/mg protein where V-79 cells bind 11.2 ± 1.2 fmoles/mg protein. This results in a greater then 50% decrease in insulin binding for IV-A1-j cells, where IGF-1 binding was essentially unchanged in both cell types (18.7 ± 2.2 and 19.8 ± 1.21 fmoles of [^{125}I]-IGF-1 bound per mg protein for IV-A1-j cells and V-79 cells, respectively). In addition, insulin failed to mediate a significant growth response for IV-A1-j cells in a hormonally-defined medium unlike V-79 cells (1.02 ± 0.12 vs 3.0 ± 0.2 fold stimulation in cell growth over the basal for IV-A1-j cells and V-79 cells, respectively). IGF-1 also failed to stimulate growth in IV-A1-j cells, even though IV-A1-j cells showed no alteration in their ability to bind IGF-1.

4.5.2 Insulin Receptor Characterization

To determine whether the decrease in binding observed for IV-A1-j cells was due to an altered receptor affinity or a decreased number of functional receptors, [^{125}I]-insulin binding was measured in the presence of different competing concentrations of unlabeled insulin. Figure 1 shows the competitive displacement profile for IV-A1-j cells and V-79 cells with insulin. In both cell lines the amount of unlabeled insulin required to cause a 50% displacement in the total amount of [^{125}I]-insulin bound was 0.5nM, suggesting that the affinity for the insulin receptor to bind insulin is similar in both cell lines. Furthermore, 167 nM insulin was required to completely displace all [^{125}I]-insulin in V-79 cells, where only 17 nM insulin was required to displace all [^{125}I]-insulin in IV-A1-j cells, indicating that the difference in binding between the two cell lines may be due to a change in the number of functional receptors. This was further confirmed by Scatchard analysis of the data [25], where the amount of insulin bound to high affinity sites was found to be 2.5 ± 0.7 x 10^{-2} and 5.8 ± 1.2 x 10^{-2} pmoles per mg protein for IV-A1-j and V-79 cells, respectively.

4.5.3 *Internalization and Degradation of [^{125}I]-Insulin*

Although IV-A1-j cells were shown to express a decreased number of functional insulin receptors on the cell surface, this alone could not explain the cell's resistance to DTaI, as only a small number of receptors need to be occupied to cause cytotoxicity [15]. Thus, in order to determine whether resistance to DTaI could be attributed to a defective mechanism involving internalization, we next assessed the rate of insulin receptor/ligand internalization in both cell types by pre-incubating the cells with [^{125}I]-insulin at 4°C for 4 hours and monitoring ligand uptake for various times at 37°C (Figure 2). The results indicate that initially, V-79 cells bound 13.0 ± 1.1 fmoles of [^{125}I]-insulin/mg protein, where IV-A1-j cells only bound 4.1 ± 0.4 fmoles/mg protein. These results are consistent with the results obtain in Table 1 and Figure 1. Within three minutes 50% of the total amount of ligand bound for both cell lines is taken up into the cells. By normalizing the amount of [^{125}I]-insulin bound between cells lines as an expression of the percent internalized from the total amount bound (upper panel Figure 2) it can be seen that the rates of internalization are nearly identical in both cell lines.

Since internalization appeared unaffected in IV-A1-j cells, it could be assumed that DTaI resistance was occurring at a site distal to insulin receptor/ligand internalization. One possibility for this resistance was that DTaI could be degraded before reaching its site of action. We therefore looked at [^{125}I]-insulin degradation for various times (Figure 3). The percent of total insulin initially bound which was degraded at 16 minutes in IV-A1-j cells was 3-fold higher than for V-79 cells (28% ± 3% versus 7% ± 2% for IV-A1-j cells and V-79 cells, respectively). It also appeared that the time required to reach 10% degradation was slightly longer for V-79 cells than for IV-A1-j cells (~16 and 5 min, respectively) which could suggest differences in cellular routing for insulin degradation between the two cell lines.

4.5.4 *Stimulation of Hexose Transport by Insulin*

In a further attempt to characterize insulin-mediated cellular effects in V-79 cells and IV-A1-j cells we exposed the cells to varying concentrations of insulin for 2 hours and monitored hexose transport (Table 2). The results show that insulin can stimulate hexose transport in a dose-dependent manner for both cell lines and that the degree of stimulation observed for each concentration of insulin was not significantly different between each cell line. This suggests that the effector system for hexose transport in IV-

A1-j cells is completely functional unlike the effector system to produce insulin-dependent cell growth.

4.5.5 Insulin-mediated Endogenous Protein Tyrosine Phosphorylation

The fact that IV-A1-j cells were not able show a mitogenic response involving cell growth, yet showed normal coupling between insulin binding and hexose-uptake, led us to test the possibility that certain key substrates for mitogenic action may have altered degrees of insulin-dependent tyrosine phosphorylation. Although the physiological relevance of tyrosine phosphorylated proteins in insulin action has not been established, a number of these proteins have been shown to be insulin-senisitive [26]. Western analysis using a mouse monoclonal antibody that recognizes the phosphorylated tyrosine residues of proteins was used with cell extracts treated with and without insulin (Figure 4) Immunoblotting with this antibody reacted with a single protein band of 175,000 daltons (pp175) which was insulin-sensitive in V-79 cells (lane B, Figure 4A) and not IV-A1-j cells (lane D, Figure 4A). In addition, the quantitative binding of anti-phosphotyrosine antibodies to an insulin-sensitive protein band in the 95 to 100 kDa range was different between V-79 and IV-A1-j cell extracts (lanes B and D of Figure 4A, respectively). Densitometric scanning of pp95/100 from three separate autoradiograms confirmed that under basal conditions, V-79 cells had a nearly two-fold increase over IV-A1-j cells (Figure 4B). Under insulin-stimulated conditions, this pp95/100 increased in intensity 25.6 ± 3.2 percent and 23.4 ± 4.0 percent over the basal for V-79 and IV-A1-j cells, respectively. Even in insulin-treated IV-A1-j cell extracts, anti-phosphotyrosine antibodies failed to react with a 95 to 100 kDa band at an intensity equal or greater than that observed for V-79 cell extracts not treated with insulin (i.e 4.1 ± 0.1 and 3.1 ± 0.1 relative units for V-79 and IV-A1-j cells, respectively). Between the 45 and 80 kDa range at least three additional bands were found to be insulin-sensitive for both cell lines An additional band (~116 kDa) was observed in non-insulin treated V-79 cells which does not correspond to pp175 and has not been reproduced from 4 additional experiments. Whether this band reflects an artifact of the system is unknown.

4.6 Discussion

4.6.1 The Insulin Receptor Pathway

Our data suggest that the decreased ability of IV-A1-j cells to bind [^{125}I]-insulin was not due to a change in receptor affinity. Rather, the data support the idea that the difference in binding between the two cell lines lies in the number of receptors located on the surface capable of binding insulin. Our data also illustrate that both cell lines, despite the decreased number of surface receptors in IV-A1-j cells, are capable of internalizing insulin at the same initial rate. Nonetheless, the resistance to DTaI could not be attributed to the difference in the number of insulin receptors alone. The possibility that the insulin receptor in IV-A1-j cells has been structurally altered so that the binding affinity towards DTaI was changed seemed unlikely, since (1) DTaI has been shown to be highly specific in displacing native insulin from its receptor, indicating little (if any) structural alterations in the binding site of the toxin-attached insulin molecule [24] and (2) the affinity for insulin itself, towards the insulin receptor in the DTaI-resistant cell line is nearly identical to the DTaI-sensitive parental cell line. In addition, both cell lines are sensitive to diphtheria toxin cell killing [15], indicating that DTaI resistance in IV-A1-j cells occurs somewhere along the insulin-receptor pathway post-internalization and not the site of action for DTa.

Further support of a post-internalization defect is the observation that IV-A1-j cells showed no alteration in their ability to bind IGF-1, but did lose their ability to exhibit IGF-1-dependent cell growth. This would suggest a growth mechanism post-receptor binding common to both insulin and IGF-1, possibly through a shared intracellular routing process [32]. Based on these speculations, DTaI-resistance of IV-A1-j cells most likely resides at a site(s) post-receptor internalization.

One approach to test this theory was to look at insulin degradation in parental and mutant cells. IV-A1-j cells at 15 minutes post-insulin binding, showed nearly a 3-fold increase in their ability to degrade insulin over V-79 cells. It is currently not known whether the insulin receptor undergoes the same degree of degradation, or that insulin degradation occurs at the surface of the cell and/or inside endocytic compartments. Furthermore, the degree of degradation may be much higher than indicated, since the method to monitor degradation involved TCA precipitation [33]. Thus, the mechanism of resistance could involve enhanced degradation, which would interfere with DTa's ability

to migrate to its site of action and inhibit protein synthesis [34]. Therefore the fact that the rate of insulin-receptor internalization is identical in both cell lines unlike the rate of insulin degradation, suggests that IV-A1-j cells are more efficient at presenting the internalized insulin to the cell's degradative enzymes, possibly through different intracellular trafficking mechanisms [28,29]

4.6.2 Insulin Action

It was originally proposed that the primary purpose for insulin internalization and degradation was to regulate the number of insulin receptors on the cell surface [35]. Yet the fact that endothelial cells show no detectable amounts of intracellular degradation suggested otherwise [36,37]. From this a number of reports were generated to show that internalization and degradation of insulin were unrelated to insulin's ability to produce metabolic effects (i.e. anti-receptor antibodies which mimic insulin action [38] and the use of chemical reagents which inhibit internalization and intracellular degradation [39]), yet no direct evidence supported the idea that these two stages of the insulin-receptor pathway might be important in generating a mitogenic signal. Since our variant cell line showed an enhanced ability to degrade insulin, but was lacking the ability to produce an insulin-related mitogenic stimulus, we next assessed IV-A1-j cells to produce a metabolic response by monitoring insulin-dependent hexose uptake. Interestingly, this cell line showed an identical threshold to V-79 cells for insulin-stimulated hexose transport. This indicates that IV-A1-j cells had no noticeable change in its effector system for glucose uptake using insulin as a stimulus. Therefore the enhanced degradation observed in IV-A1-j cells had no effect on insulin-mediated hexose uptake. This does not rule out the possibility, however, that the mechanism(s) involved in the metabolic effects of insulin action can occur at a step prior to the degradation step that has been enhanced in the IV-A1-j cell line. Nonetheless, the fact that IV-A1-j cells show an unaltered response in their ability to promote an insulin-dependent metabolic response, but not a mitogenic response, suggests that at least two separate mechanisms are involved in these effects

The fact that IV-A1-j cells could exhibit differences between these two types of responses led us to investigate the possibility that these cells may be defective in their ability to promote insulin-induced tyrosine phosphorylation of endogenous substrates. The rational for this approach is two fold; first, CHO cells which express insulin receptors that are mutated at the ATP-binding site are unable to phosphorylate

endogenous substrates and have a decreased ability to produce a number of insulin responses such as the stimulation of hexose uptake, glycogen synthesis, and thymidine incorporation into DNA [8,9,23], and second, White *et al.* [12] showed that a mutation in the β-subunit of the insulin receptor at Tyr-960 had no effect on the receptor's ability to autophosphorylate or to produce tyrosine kinase activity. Yet, when cells are transfected with this mutated receptor, there ability to phosphorylate pp185 and to elicit insulin-activated glycogen synthesis and DNA synthesis was strongly lacking [12]. The possibility for tyrosine-phosphorylated endogenous substrates playing key roles in insulin action is enticing, yet their functions still remain largely unknown. V-79 cells contain a number of insulin-sensitive protein bands which are reactive to anti-phosphotyrosine antibodies, one of them being a band in the 175,000 molecular range (pp175). The fact that we do not see pp175 in either insulin-treated or untreated IV-A1-j cells, suggests that activation of this particular protein is not necessary for the insulin-dependent mechanism(s) involved in stimulating hexose uptake, but may be involved in a mitogenic response. Increased auto-radiographic exposure and protein application of cell extracts to polyacrylamide gels have also failed to produce a noticeable pp175 band in IV-A1-j cells.

Our findings reflect those of McClain *et al.* [40] who studied growth and metabolic signalling by insulin and IGF-1 in Rat-1-fibroblasts that have an enhanced expression of normal or mutated insulin receptors (at the ATP-binding domain). They observed two insulin-stimulated phospho-proteins (pp220 and pp170) which are not present in the cell line expressing mutated insulin receptors. In addition, cells lacking pp220 and pp170 have lost their ability to produce a mitogenic response to either insulin or IGF-1, but maintain their ability to promote hexose uptake. Whether pp175 in our system is similar to the insulin-stimulated tyrosine phosphorylated proteins of McClain's group (pp170) or White's group (pp185) has yet to be established. Furthermore, the lack in ability of the mutant cell line to produce a 175 kDa band that is reactive with the antiphosphotyrosine antibody employed herein, suggests that this band is not involved in promoting hexose uptake, but may be involved in promoting a mitogenic response. This is further substantiated by the fact that neither insulin nor IGF-1 can promote cell growth in IV-A1-j cells, nor can they promote the appearance of pp175. Nonetheless, it was conceivable, though less likely, that pp175 is necessary for insulin-stimulated hexose uptake, and that the amount of pp175 to promote this response was below the resolution of our detection system. We also employed a different detection system which included a

secondary antibody to anti-phosphotyrosine antibodies and linked this complex to [^{125}I]-labeled protein A. This system, thought to enhance the detection of anti-phosphotyrosine antibody-reactive proteins, also failed to detect a 175 kDa protein band in IV-A1-j cells.

A number of other bands were found to be differentially phosphorylated between V-79 and IV-A1-j cells with or without the addition of insulin. Of interest is a protein band between 95 and 100 kDa. Reports suggest that this band is reflective of the β-subunit phosphorylation of the insulin and IGF-1 receptor [40,41]. Both cell lines are capable of stimulating this band (pp95/100, Figure 4), however, IV-A1-j cells exhibited less expression of this protein than V-79 cells. Insulin-induced IV-A1-j cells also failed to express this band above the level observed in non-insulin-stimulated V-79 cells. Whether this band is indeed the insulin or IGF-1 receptor β-subunit or whether differences observed are due to a quantitative difference in insulin receptor number or simply a decreased ability for IV-A1-j cells to cause this phosphorylation has yet to be confirmed. Furthermore, it can not be ruled out that IV-A1-j cells may have alterations in their level of phosphotyrosine phosphatase activity. Enhanced activity could explain the differences seen in a number of the protein bands presented in this report, yet this can not explain the disappearance of pp175 in IV-A1-j cells.

4.6.3 Significance

It is apparent that IV-A1-j cells have a differential degree of insulin degradation as compared to V-79 cells and that this could be reflective of a difference in intracellular routing. The fact that IV-A1-j cells show differences in their ability to stimulate the tyrosine-specific phosphorylation of an endogenous substrate, could reflect one of three possibilities. First, for the cell to become resistent to DTaI, an alteration in the insulin-receptor trafficking pathway may have occurred. Second, an alteration in the receptor may have caused an alteration in the insulin-receptor trafficking pathway. Or third, there may be an alteration in the insulin receptor itself which would account for its inability to activate pp175. An alteration in the insulin-receptor trafficking pathway could account for the increased degree of insulin degradation. In turn, this would not allow the phosphorylated insulin-receptor complex to enter an endosomal compartment which may have associated with it, messenger molecules that in turn, need to be activated by either insulin, partially degraded insulin, and or the tyrosine-kinase activated receptor.

Studies using adipocytes have shown that vanadate can selectively phosphorylate the insulin receptor and shunt insulin from a retroendocytic pathway to a degradative pathway [39,40]. Thus, it is entirely possible that the phosphorylation of the insulin receptor may be important for its intracellular routing. It would be interesting to see if the insulin receptor in IV-A1-j cells has undergone any alterations which affect autophosphorylation, since a protein band in the vicinity of the insulin β-subunit shows different intensities between V-79 and IV-A1-j cells. This could support some of the present concepts of receptor phosphorylation and cellular trafficking [14,39,40].

In summary, this approach to studying the insulin-receptor pathway and its relationship to insulin action has allowed us to separate parts of the mitogenic and metabolic signalling pathways. The study of this cell line should allow us to better define the pathways involved in insulin action and intracellular trafficking.

4.7 References

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4.8 Figures and Tables for Chapter 4

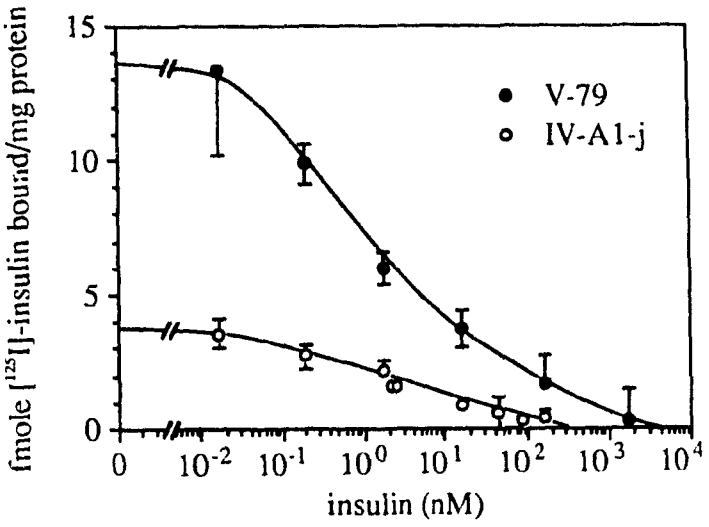

Figure 1. Competitive diplacement profile of insulin binding for V-79 cells and IV-A1-j cells.

The values represent the amount of [^{125}I]-insulin bound (fmoles per mg of protein) to V-79 (●) and IV-A1-j (○) cells in the presence of increasing concentrations of unlabeled insulin. Error bars for points with error less than 0.3 fmoles/mg protein have been omitted. Mean ± SEM of 4 to 7 experiments, triplicate plates per experiment.

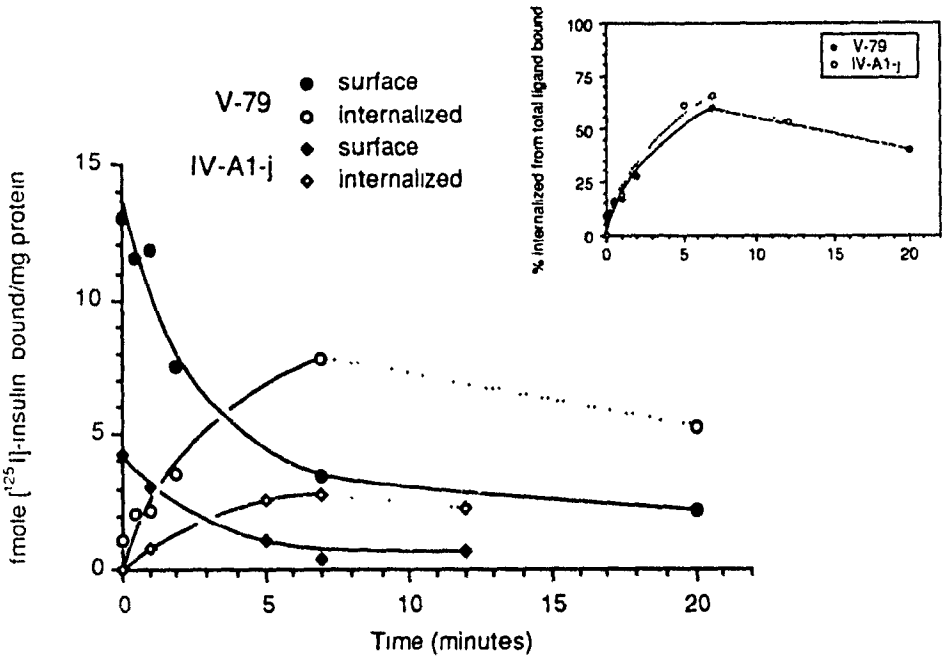

Figure 2. Internalization of [^{125}I]-insulin into V-79 and IV-A1-j cells.

Cells were preincubated in the presence of tracer amounts of insulin (0.16nM) for 4 hours at 4°C. Unbound [^{125}I]-insulin was then removed by washing and bound [^{125}I]-insulin was monitored at 37°C for the times indicated. Surface bound insulin (filled symbols) was calculated by subtracting the amount of acid wash-insensitive [^{125}I]-insulin (open symbols) from total binding for each time point (see text for explanation). The values represent the average taken from three separate experiments for V-79 cells (circles) and IV-A1-j cells (diamonds) (triplicate plates in each experiment). In all cases, the error was found to be less than 10% for each point. The upper right panel represents the percent of [^{125}I]-insulin internalized as a percent of the total bound at time zero.

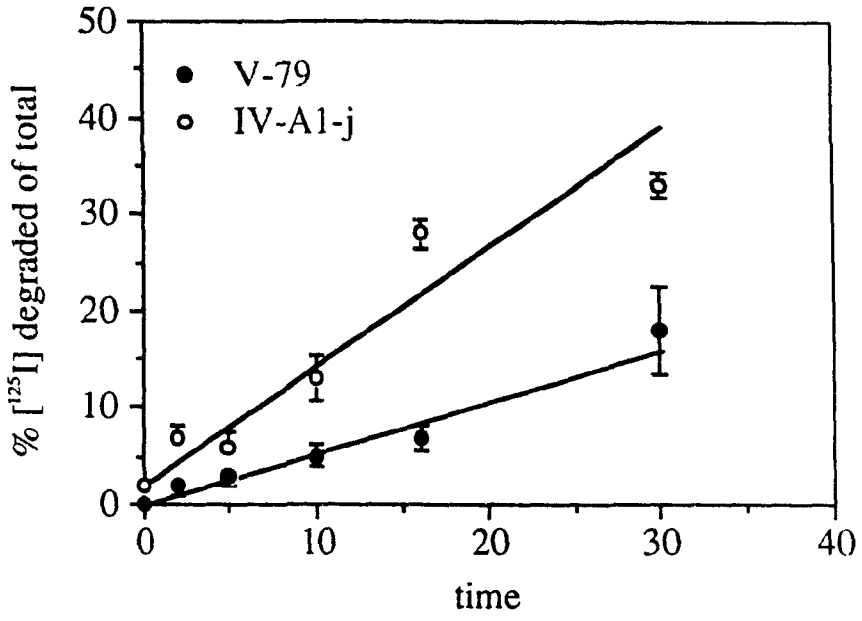

Figure 3. [^{125}I]-insulin degradation profiles for V-79 and IV-A1-j cells.

Cells preincubated with [^{125}I]-insulin were washed and monitored at 37°C for the times indicated. At the end of each time point, the media was removed and assessed for [^{125}I]-insulin degradation by calculation of the amount of TCA-soluble material. Cells were also assessed for degradation (see text). The values represent the amount of TCA-soluble [^{125}I] counts both in the media and in the cells as a percentage of the total amount bound for V-79 cells (●) and IV-A1-j cells (o). Mean ± SEM of 2 to 4 separate experiments, triplicate plates per experiment.

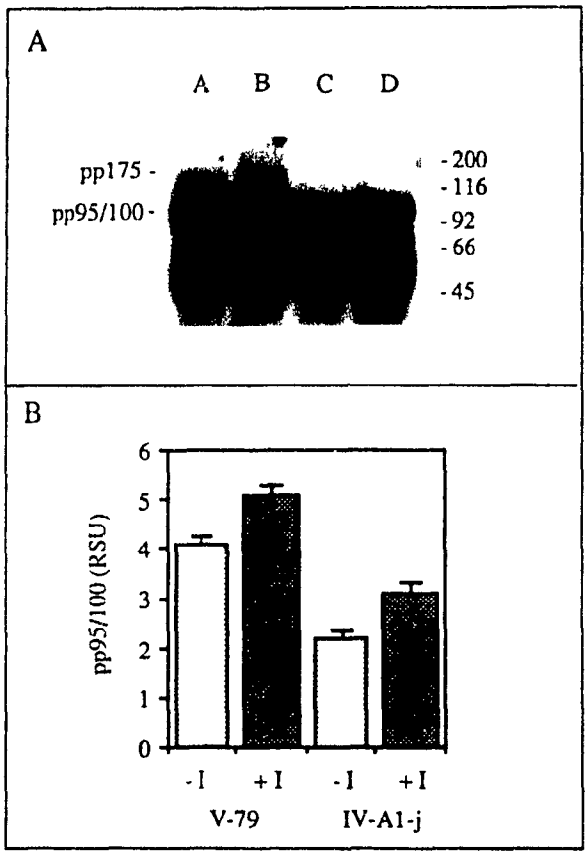

Figure 4. Insulin-stimulated tyrosine phosphorylation of endogenous substrates.

24-hour serum-deprived V-79 cells (right) or IV-A1-j cells (left) were incubated with (+) or without (-) insulin (17 nM) for 10 min at 37°C, lysed, and fractionated on a 7.5% SDS-gel. Protein concentrations were equivalent for each sample applied (120 µg/lane) and immunoblotted using a mouse monoclonal antibody directed towards the phosphotyrosyl residues of proteins. Values (right) represent the position of molecular weight markers in kilodaltons. Two phosphoprotein bands (left) are indicated (pp175 and pp 95/100). Insert B shows the densitometric readings for pp95/100 from 3 separate experiments (RSU; relative scanning units).

Table 1.

[^{125}I]-labeled ligand binding and growth for V-79 and IV-A1-j cells with insulin and IGF-1.

hormone	[^{125}I] binding [a]		growth [b]	
	V-79	IV-A1-j	V-79	IV-A1-j
insulin	11.2 ± 1.2	4.0 ± 0.4*	3.0 ± 0.2 [o]	1.0 ± 0.1
IGF-1	19.8 ± 2.2	18.7 ± 2.2	2.1 ± 0.2 [o]	1.1 ± 0.1

a. values represent the amount of [^{125}I]-labeled ligand bound (mean ± SEM) to cells at steady state (fmoles/mg protein) from 3 to 5 separate experiments.

b. values represent the fold stimulation in growth for the number of cells grown for 3 days in a hormonally-defined medium containing either growth factor as the predominant mitogen over the number of cells in defined medium without the respective mitogen for that same day (mean ± SEM of 3 to 4 experiments) [15,24].

* indicates a significant difference from V-79 cell binding
[o] indicates a significant stimulation in cell growth
$P < .05$, Student t-test.

Table 2.

The effect of insulin concentration and serum on the hexose transport in V-79 and IV-A1-j cells.

Insulin (nM)	2-DG Uptake (fold stimulation from basal)	
	V-79	IV-A1-j
0	1.0	1.0
6.7	1.25 ± 0.04	1.35 ± 0.01
66.7	1.49 ± 0.02	1.49 ± 0.02
667	1.53 ± 0.25	1.65 ± 0.20
5% FCS	1.50 ± 0.19	1.73 ± 0.01

The values represent the fold stimulation in hexose uptake over the basal (mean ± SEM) for 8 individual experiments. The average basal uptake values were 2145 ± 204 and 2066 ± 256 for V-79 cells and IV-A1-j cells, respectively (picomoles 2-DG/mg protein/5 min). All values show a significant increase in hexose uptake above basal ($P < 0.05$), where no significant differences in hexose uptake were observed between V-79 cells and IV-A1-j cells ($P > .05$), as determined by Student t-test.

Chapter 5
General Discussion

5.1 Discussion

5.1.1 Advantages, Significant Findings, and Speculations

The first stage of this project was to construct a toxic DTaI molecule that would specifically interact with the insulin receptor and not the IGF-1 receptor. The purpose of this approach was to generate a number of cell variants defective in a part of the pathway responsible for insulin binding, internalization, cellular compartmentalization, and degradation. We could then examine, as based on our hypothesis, how a defect in the insulin-receptor pathway can alter insulin's ability to produce a cellular response.

There are many advantages as to why we chose this approach. First, our system does not employ the use of drugs to alter the insulin-receptor pathway. Acidotropic agents and carboxylic ionophores have a diverse number of side-effects [1,2] which complicate the interpretations of results in studying the insulin-receptor pathway and insulin action. Second, though transfection studies using truncated [3] or single-site-altered [4,5] insulin receptors have aided in understanding what roles the β-subunit of the insulin receptor has in producing cellular effects, they do have serious drawbacks, one being the introduction of "foreign" cDNA to express mutated insulin receptors [3-5]. In order to study the effect of mutated receptors in the cell system, a control involving the transfection of unaltered receptors must be used for comparison. Furthermore, comparisons should be performed with cell-types that are expressing the same number of receptors. In addition, when the transfected receptors are studied for their ability to produce insulin-dependent responses, the degree of the response does not necessarily correlate with the number of receptors expressed. Whether this is due to expressed receptors having an inefficient coupling mechanism to the response pathway or due to limiting amounts of response-producing substrates is left open to speculation. Defects that occur in our system are not chemically induced or forcibly expressed by molecular technics. We merely provide the cells a selection pressure in an environment where these defects can be expressed. Third, the study of cells obtained from diabetic individuals has helped in studying the cellular mechanisms associated with non-insulin dependent diabetes mellitus [6,7]. However, (1) it is not always possible to obtain enough of these cells for study, (2) sometimes it is difficult assess the problems associated with these cells due to the lack of a proper control, and (3) there may be some extreme cases of insulin resistance that are highly lethal and are not expressed in the normal population

(i.e. only heterozygous mutations involving the TK region of the insulin receptor have been reported in individuals [7]). Defects of this nature could be expressed in individual cell lines maintained in culture. Our approach isolates spontaneous, naturally occurring mutants (i.e. IV-A1-j cells) that may arise as defects associated with diabetes. Defects which normally occur at low frequency in the gene pool. A fourth advantage is that we can exercise some control in our system by looking specifically at the insulin-receptor pathway. Any defects seen in other growth factor pathways or their abilities to generate a cellular response are a consequence of an alteration created in the insulin-receptor pathway. Thus, the inter-relationship between insulin and other growth factors can be studied in our system.

With this long list of advantages we set out to construct a DTaI molecule that would specifically interact with the insulin receptor. Previous reports to produce this toxin involved modifying the insulin molecule at cystamine sites [8,9]. This would produce a heterogeneous population of $(DTa)_nI$ molecules which could have a low degree of specificity toward the insulin receptor. While this toxin proved fruitful in studying H35 rat hepatoma cells [10], we were interested in studying a cell line which also contained the IGF-1 receptor. Another procedure involved the construction of a one to one molar ratio of RTa to insulin through a single amino group (B27) on the insulin molecule. RTa, however, did not suit our purpose. First, RTa contains two hydrophobic regions which may aid in its ability to nonspecifically bind a cell's plasma membrane [10]. DTa does not contain this region, which suggests that the sole method of entry for DTaI would be dependent on binding to the insulin receptor. Second, the Ricin pathway involved in cell killing does not require an acidification stage, unlike the mechanism employed for DT [11]. Since part of the insulin-receptor pathway involves an acidification process, it was in our best interest to employ the use of DTa. The fact that both the insulin-receptor pathway and DT pathway require an acidification step may indicate a similarity in their processing pathways. The result was that we constructed and purified a DTaI molecule which contains a one to one molar ratio of DTa to insulin. We also modified the purification step of DTa. Rather than use a conventional size-exclusion chromatography technic to purify DTa (a technic that usually involves two days of work), our method using heat precipitation, generates a >95% pure DTa fragment with in 3 hours which maintains its ADP-ribosylation activity.

Characterization of the DTaI molecule showed that it specifically interacted with the insulin receptor and not the IGF-1 receptor to cause cytotoxicity. There have been no

previous reports on this kind of specificity of a substituted insulin to the insulin receptor. Nonetheless, this data suggests that DTaI-resistant cell variants will have defects which are directed toward the insulin-receptor pathway.

We isolated and cloned a number of DTaI-resistant cell variants. Some had a large decrease in their ability to bind insulin (>50%)[7] where others had only slight alterations, or none at all. A number of these cell variants also showed no change in their ability to bind IGF-1, further suggesting the specificity of the DTaI molecule to interact specifically to the insulin receptor. Additionally, our results showed that there was no correlation between a variant's ability to bind insulin and its ability to grow in a medium containing 5% or 10% serum. For example, IV-A1-j cells show a >50% decrease in their ability to bind insulin, yet showed no change in their growth properties. VI-A3-b cells however, showed no alteration in their binding ability, but were greatly affected in their ability to proliferate. The former observation was not surprising since it is thought that other growth factors can take the place of insulin in producing a growth response [12,13]. The second observation is startling, however. Since DTaI interacts specifically along the insulin-receptor pathway, this mutant must have a defect post-insulin-receptor binding. The fact that this cell line does not grow properly in a 5% serum containing medium, suggests that the insulin-receptor pathway is somehow coupled to mechanisms involved with other growth factors to induce proliferation. Cell variants of this nature definitely warrant future study.

We next developed a hormonally-defined medium where various growth factors could be studied for their effects on cell growth in CHL cells. Studies on V-79 cells showed that insulin or IGF could work in conjunction with EGF or THR to stimulate cell proliferation. Insulin and IGF-1 stimulated growth separately, yet when added together, failed to show any additive increase. This suggests that insulin and IGF-1 may be working through similar mechanisms. In addition to this information, IV-A1-j cells generated the most compelling evidence that insulin and IGF-1 share a common pathway for cell proliferation. IV-A1-j cells lost their ability to proliferate in hormonally-defined medium containing either insulin or IGF-1. Yet, they showed no changes in their ability to grow with EGF or THR. This cell line provides us with an important tool for studying

[7] Note: In all instances, when talking about various effects (i.e. decreased binding, an alteration in growth, unaffected metabolic responses, etc..), these responses are in reference to the responses observed in the parental DTaI-sensitive cell line (V-79).

the inter-relationship of various growth factors with each other. The fact that this cell line is unresponsive to IGF-1 further suggests that whatever alterations have occurred along the insulin-receptor pathway are also related to the IGF-1 pathway.

Another discovery was that IV-A1-j cells could produce normal insulin-stimulated hexose uptake. This indicated two distinct pathways which are responsible for growth and maintenance. Further characterization of IV-A1-j cells showed that their decreased binding was due to a decrease in the number of insulin receptors capable of binding insulin and not due to an affinity change towards insulin. With regard to DTal resistance, the decrease in receptor number alone could not explain why IV-A1-j cells were able to survive, since in V-79 cells only a small amount of internalized DTal is needed to kill the cell. One could speculate that while the insulin receptors of IV-A1-j cells showed no alteration in their affinity towards insulin, there could still be an affinity change towards DTal. However, since DTal can competitively displace insulin at roughly the same concentration as insulin itself, this would indicate that there is little, if any, structural change in the conjugated insulin molecule's binding site. Thus, any affinity changes towards DTal would also affect insulin.

To determine further what defects may have occurred in the insulin-receptor pathway, insulin-receptor internalization and insulin degradation were studied. IV-A1-j cells showed no loss in their ability to internalize insulin, yet did show enhanced insulin degradation. Thus, if DTal enters the same degradative pathway, then DTa could be destroyed before going to its site of action. The fact that DT could cause cytotoxicity (Appendix, Figure 2) indicated that the mechanisms involved in insulin degradation are indigenous to the insulin-receptor pathway. The evidence presented here strongly suggests that whatever mutation is causing enhanced degradation, it does not affect insulin-stimulated hexose uptake. It does reflect, however, that whatever mechanisms are involved in producing insulin- or IGF-1- dependent mitogenic responses, they may be interrupted by the enhanced insulin-degradation pathway. It would be interesting to examine IGF-1 degradation in these cells (see below)

The characterization of IV-A1-j cells also involved studying their ability to phosphorylate endogenous substrates at tyrosine-specific sites. pp175, an insulin sensitive band found in V-79 cells, was not present in IV-A1-j cells. Based on our results, and the results of others [14,15] pp175 may be important in regulating the cell's insulin and IGF-1 mitogenic effects. The fact that insulin and IGF-1 can stimulate hexose uptake further suggests that this protein band may not be involved in this latter response.

Interestingly, we have recently shown that pp175 can be stimulated with IGF-1 in V-79 cells but not in IV-A1-j cells. This could further support the above speculations.

We have shown that a cell line resistant to DTaI will have a defect somewhere along its insulin-receptor pathway. We have also shown that a defect in this pathway could lead to an alteration in the cell's ability to respond to insulin. In the case of DTaI-resistant IV-A1-j cells, these cells show an alteration in their ability to bind insulin and have an enhanced insulin degradative pathway. As a consequence, they are unable to respond to insulin in a hormonally-defined medium, yet exhibit a normal insulin-dependent hexose uptake profile. The exciting part is that, though the selection system employed was aimed specifically at the insulin-receptor pathway, IGF-1 also failed to produce a growth response in these cells. Thus, this and the fact that IV-A1-j cells exhibit normal IGF-1 binding, gives us a clear indication that the two pathways for insulin and IGF-1 overlap at a site post-ligand binding.

From the information cited above, a theoretical scheme on the relationship between the insulin-receptor, IGF-1/receptor, and response pathways can be presented (Figure 1). First insulin will bind to its receptor, induce autophosphorylation of its β-subunit, and get internalized into an endosomal compartment (Figure 1.a,b). At this point the phosphorylated β-subunit is facing into the cytoplasm and may trigger certain substrates which regulate the intracellular trafficking of the insulin-receptor complex (c). The endosomal pathway for the IGF-1 receptor may also follow a similar or identical route (d). The complex (or part of) then goes to other parts of the cell either by trafficking (e) and/or endosomal fusion with other vesicles (f,g). Eventually, insulin will enter a pathway which causes degradation (h). Based on our evidence, it would seem logical to conclude that the mechanisms necessary to mediate insulin-dependent hexose uptake occur at steps prior to the mechanisms that are involved in cell growth. For initiating a metabolic response, this could result from the activation of messengers available to both insulin and IGF-1 receptors located in the plasma membrane (i) or at sites within the cell shortly after internalization (j). For insulin and IGF-1 dependent cell proliferation, certain proteins need to be activated in order to cause cell growth (pp175?), for example, substrates which are only located in certain parts of the cell and are bypassed in IV-A1-j cells (k). Similarly, it may be that this pathway is also responsible for delivering insulin to the nucleus in order to aid in producing cell growth (l). The fact that differential phosphorylation is seen between V-79 cells and IV-A1-j cells could also suggest that the receptor plays a key role in insulin action. Based on the degree of β-

phosphorylation, it may be that the insulin receptors of V-79 cells have a greater chance to activate certain trafficking proteins (via its tyrosine kinase or tyrosine phosphorylated regions of the receptor?) over IV-A1-j cells, which in turn, may be required to push the insulin-receptor complex into a growth promoting pathway. As for EGF and THR their mechanisms are different and involve pathways that are not related to either insulin or IGF-1. It should be pointed out, however, that this is a simplistic interpretation of how the insulin-receptor pathway may be involved in insulin action based on the above results. A number of approaches can be taken to assess the exact mechanisms that may be involved in the differential regulation of insulin degradation, mitogenic, and metabolic responses between V-79 cells and IV-A1-j cells (see next section). Nonetheless, the information presented here gives new and exciting insights on the relationship between the insulin-receptor pathway, IGF-1, and cellular responsiveness.

5.1.2 Future Prospects

The selection system presented in this thesis has shown to be a useful tool for isolating cellular mutants which have defects along their insulin-receptor pathway. The generation of one particular mutant (IV-A1-j) has helped in locating some of the important steps that are involved in the mitogenic action of insulin. With this unique mutant, many other approaches can be used to generate pertinent information which will be crucial in understanding mechanism(s) in insulin response. This section briefly describes some the approaches that can be taken in studying IV-A1-j cells.

One approach would be to look at insulin degrading enzymes, more particularly, IP and multi-catalytic IP. It may be the simple fact that the enzymatic level or the accessibility for these enzymes to act on insulin (and IGF-1) may be elevated in (or on the surface of) IV-A1-j cells. If this were the case, then mechanisms involving the regulation of these enzymes can be studied. In addition, it would be important to determine whether insulin degradation occurs at the surface of the cell and/or inside endocytic compartments. The insulin degradation profiles could also be examined for the various compartments, where the amount and type of degradation can be assessed by using Sephadex G-50 chromatography [16]. More importantly is to look at the degradation profile for IGF-1. If IGF-1 follows the same route as insulin then their profiles should be the same. This could explain why both hormones are unable to

produce a growth response. If the profiles are different, such that IGF-1 shows a normal degradation pattern, this would suggest a great dependence on insulin, its receptor, and/or its pathways for IGF-1-dependent cell growth. Such an observation could lead to some startling new discoveries. Lastly, it would also be interesting to look at IGF-II and its receptor, since it is thought that this receptor may function as a regulator for protein degradation [17]

Another approach would be to study insulin receptor phosphorylation. As stated above, β-subunit phosphorylation may be essential in signalling not only second messengers for insulin action, but also key substrates required for trafficking vesicles containing insulin-receptor (and IGF-1/receptor) complexes (i.e. trafficking to a location which contains inactivated proteins required in cell growth). Similarly, a Tyr-960 mutation is know to suppress the formation of a phosphotyrosine band of 185 kDa. This mutation also inhibits insulin-stimulated DNA synthesis [5,14]. It would be interesting to see if IV-A1-j cells were lacking the ability to phosphorylate at this site.

Conventional techniques could look at the trafficking of insulin-receptor and IGF-1 receptor complexes to see if they are indeed altered in any significant way from control cells. Studies could also involve looking at the turnover rate of the insulin and IGF-1 receptors themselves. This could help explain why IV-A1-j cells have fewer receptors than V-79 cells on the cell surface. Another approach would be to look at insulin receptor message. Perhaps the altered pathway in IV-A1-j cells has a unique affect on insulin receptor mRNA expression.

Lastly, it would be important to study glycosylation and processing of the insulin receptor. One possibility that should be studied is that the insulin receptor may contain a mutation in an important part of its structure. If this affected glycosylation, it could generate another explanation for the decreased expression of receptors capable of binding insulin. Antibodies directed towards the insulin receptor could also be used to substantiate the idea that IV-A1-j cells have fewer receptors expressed on the external face of the plasmamembrane. A receptor mutation could affect its ability to autophosphorylate (i.e. β-subunit), phosphorylate endogenous substrates (i.e. pp175), and traffic the insulin-receptor complex to proper vesicles before degradation. With relation to the IGF-1 receptor, it has been shown that this receptor and the insulin receptor can cross-phosphorylate each others β-subunits [18]. If we could speculate that the IGF-1-receptor pathway is unaffected, yet IGF-1 can not stimulate cell proliferation, then perhaps transactive phosphorylation with the insulin receptor is necessary to generate a

perhaps transactive phosphorylation with the insulin receptor is necessary to generate a signal strong enough to initiate the mechanisms required for cell growth. These are all speculations, none of which have been proven. Then again, we never had a cell system to prove them in.

5.1.3 Summary

As a quick summary, this work has involved the construction of a DTaI molecule which effects its toxicity through the insulin receptor. A number of DTaI-resistant cell variants have been isolated, of which one has been studied in detail. This cell variant (IV-A1-j) has exhibited the following properties different from its parent V-79 cell line: (1) fewer receptors capable of binding insulin, (2) an inability to use either insulin or IGF-1 as proliferation factors, (3) an enhanced ability to degrade insulin, (4), a suppressed ability to phosphorylate endogenous substrates at tyrosine specific sites, and (5) an inability to produce a phosphotyrosine pp175 protein. Alternatively, IV-A1-j cells showed no change in their ability to (1) bind IGF-1, (2) use EGF or THR as proliferation factors, and (3) exhibit insulin-stimulated hexose uptake.

With these observations, relationships between the insulin-receptor pathway and insulin/IGF-1 action have been proposed (Figure 1). Nonetheless, there are many questions left unanswered. How is degradation related to a decreased ability for IV-A1-j cells to bind insulin? What is the mechanism for decreased degradation? Is this degradation the result of an increase in insulin degrading enzymes and/or is it due to differential compartmentalization of the ligand/receptor complex? What is the role of pp175? What are the mechanisms involved between an enhanced degradation pathway, expression of pp175, and the ability for cells to respond to insulin? A number of conclusions have been drawn from the studies performed in this project. Now many more questions are raised. Fortunately we have uncovered a system which can address these questions, and more.

5.2 Concluding Remarks

A concise understanding of how insulin can mediate diversified effects though its receptor is far from being achieved. From the work constituted in this project we have at our disposal a method which will help us understand some of the relationships between the insulin-receptor pathway and insulin action. We have also generated a number of cellular mutants, one of which provides us with information leading towards our global understanding of insulin action. Just this cell line alone can be characterized at a number of different levels, some of which have been mentioned above and would further contribute to our knowledge of insulin action. Remember, other mutants have been isolated which can be characterized. This project has opened a new door in studying insulin action and has helped us step inside. Unfortunately, much more needs to be done before we can close the door behind us.

5.3 References

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5.4 Figure for Chapter 5

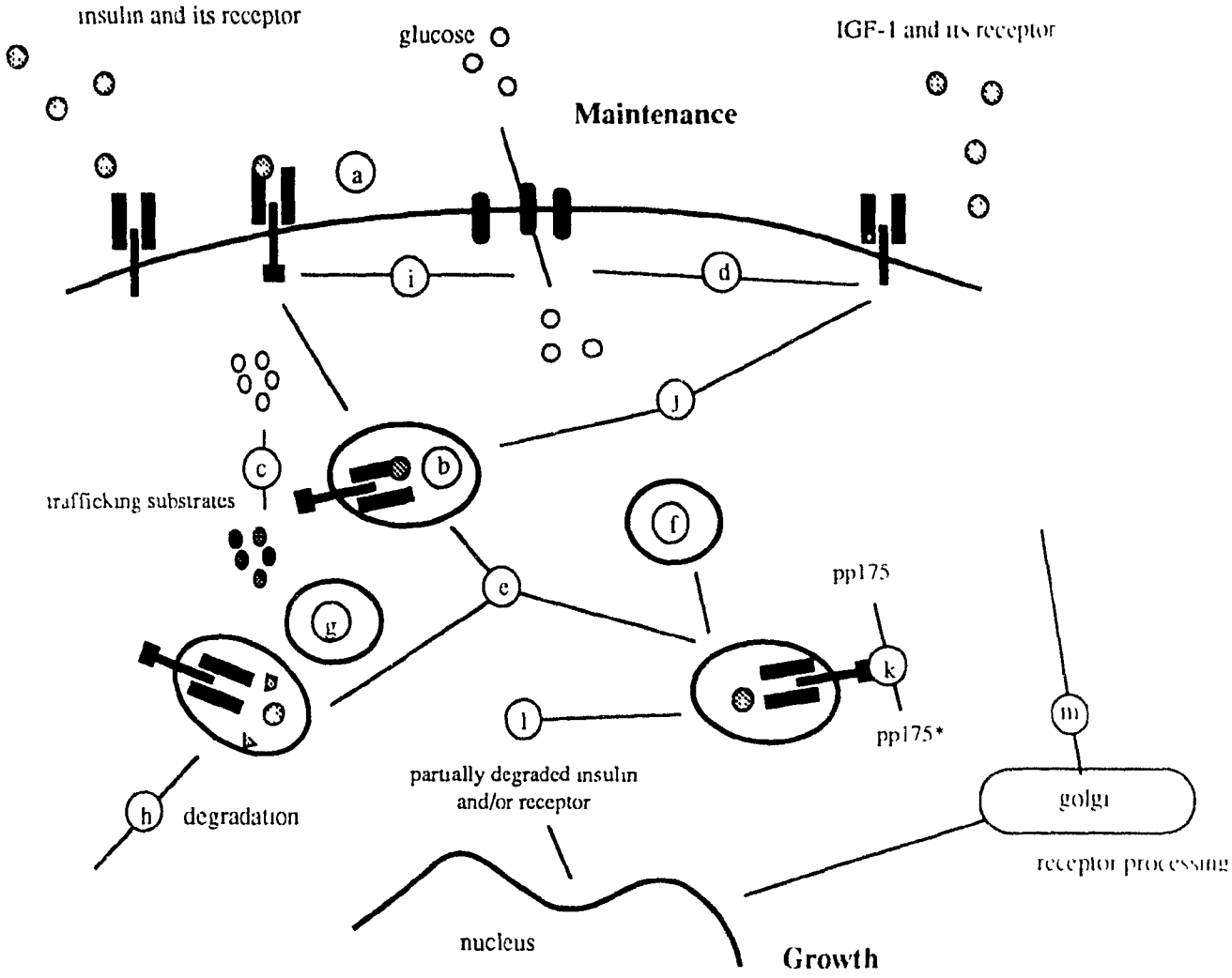

Figure 1. A diagramatic representation of the insulin-receptor pathway.

The above diagram illustrates some of the possible routes that insulin and/or its receptor may take in order to activate intracellular subtrates necessary to elicit ligand-dependent cellular effect. See section 5.1.3 for explanation.

Appendix

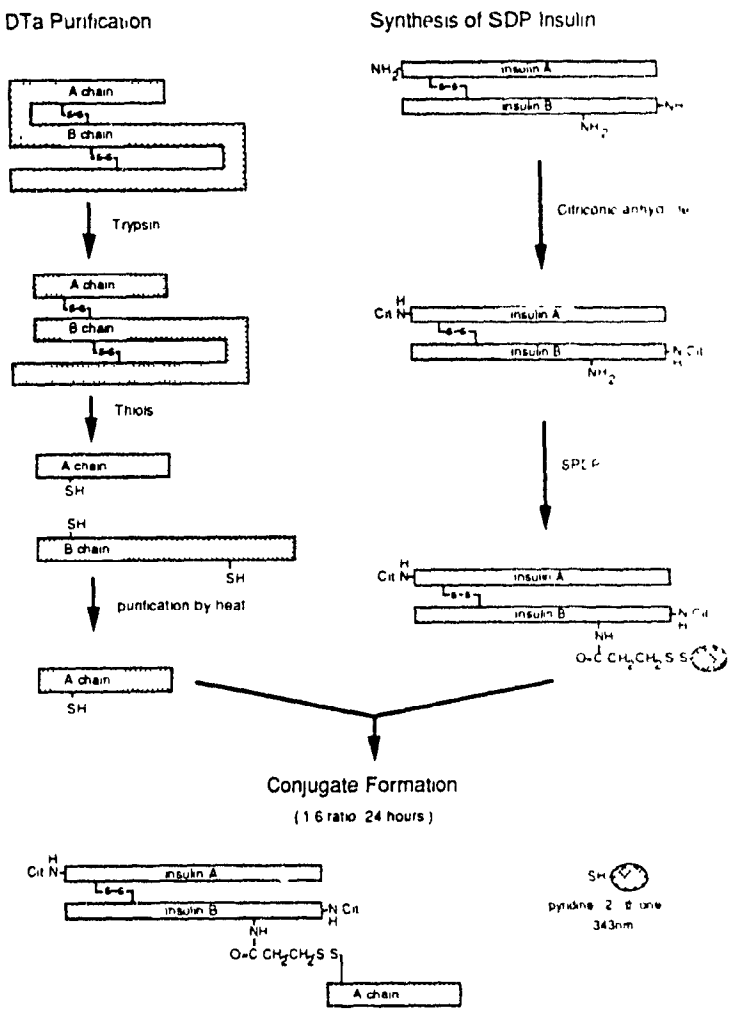

Figure 1. Construction of an insulin-diphtheria A-chain toxic conjugate using SPDP as the heterobifunctional reagent.

Diphtheria toxin was cleaved using β-mercaptoethanol The A-chain of diphtheria (DTa) was then separated from the B-chain (DTb) by heat, which resulted in the precipitation of the B-chain. The insulin molecule was first treated with citraconic anhydride to allow the blocking of amino terminal groups (NH_2) with citrate (Cit). SPDP was then added to the capped insulin molecule to form a linkage molecule. Insulin was then added to reduced DTa. After 24 hours, this resulted in the formation of a 1:1 molar ratio of DTa to insulin with the release of pyridine-2-thione which has a unique absorbance at 343 nM. (-SH. exposed sulfhydryl group)

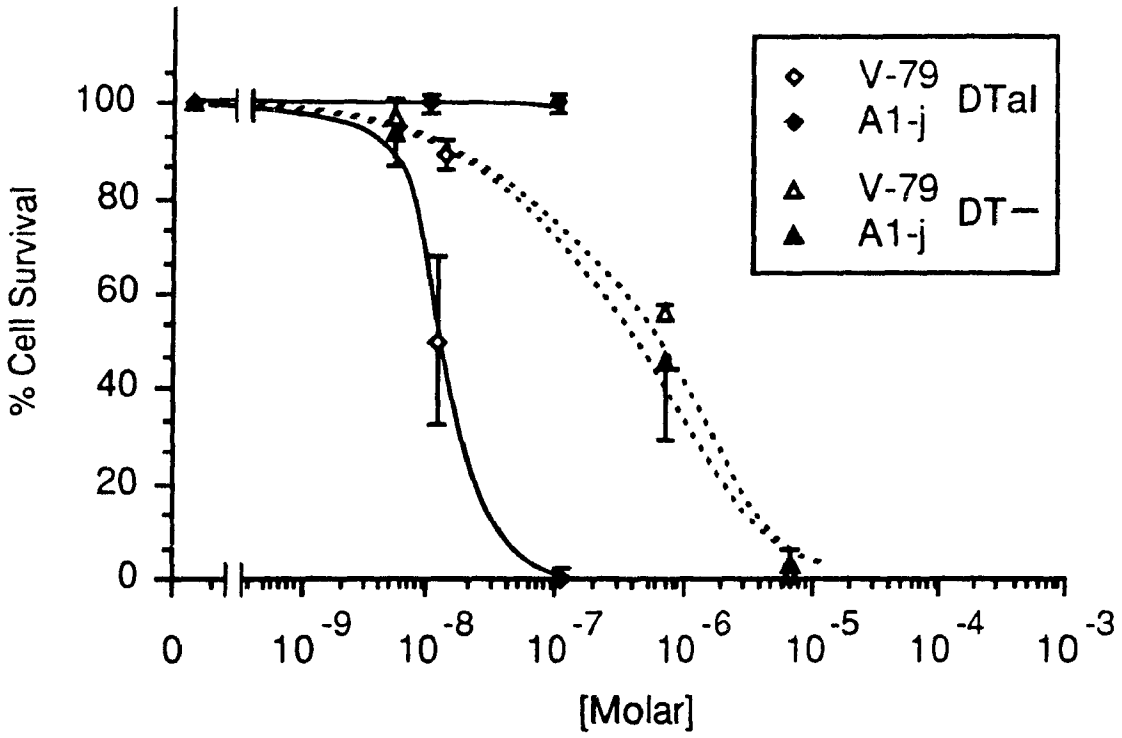

Figure 2. The toxicity profiles for DT and DTaI killing in V-79 and IV-A1-j cells.

DT or DTaI was added at increasing molar concentrations to serum-deprived V-79 and IV-A1-j cells for 2 hours. Cell counts and viability were measured after 24 hours using trypan blue. DT (triangles) and DTaI (diamonds) cell killing was measured as a percent of viable cells remaining from cells grown in the absence of either protein. Values represent the mean ± SEM of three separate experiments.

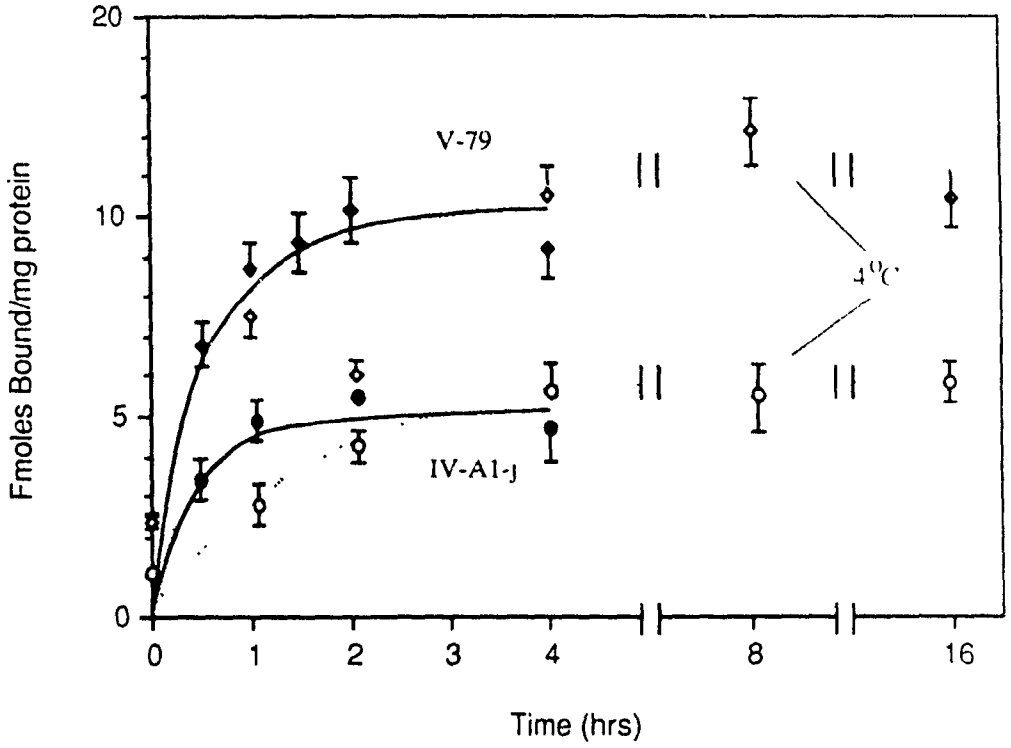

Figure 3. Equilibrium binding studies using [^{125}I]-insulin for V-79 and IV-A1-j cells.

Serum-deprived V-79 (triangles) and IV-A1-j (diamonds) cells were incubated with trace amounts of [^{125}I]-insulin for the indicated periods of time either at 4°C (.....) or 22°C (—). Values (fmoles of [^{125}I]-insulin bound/mg protein) were corrected for non-specific binding and respresent the mean ± SEM of 3 to 7 separate experiments (triplicate plates in each experiment).