Function of Receptor Tyrosine Kinases in $G_1$ deficient cells: preferential suppression of insulin signalling

By

Clement Sun

A thesis submitted in conformity with the requirements
For the degree of Master of Science
Graduate Department of Laboratory Medicine and Pathobiology
University of Toronto

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Function of Receptor Tyrosine Kinases in Gi deficient cells: preferential suppression of insulin signalling

Clement Sun
Master of Science, 2000
Department of Laboratory Medicine and Pathobiology
University of Toronto

Abstract

Heterotrimeric G proteins function as critical links in the signaling pathways of heptahelical receptors, but their role in signaling by receptor tyrosine kinases, such as the insulin receptor is less clear. We have developed stable L6 myocyte lines in which a loss of Gi function can be controlled by tetracycline-regulated expression of a full-length Gi-alpha2 antisense RNA. Antisense clones cultured in the absence of tetracycline exhibit a 50 to 70 % reduction in endogenous Gi-alpha protein. Transcriptional readout assays indicate that Gi depletion causes selective inhibitory effects on signalling by both heptahelical receptors and receptor tyrosine kinase receptors. Insulin activation of the c-fos serum response element and hexokinaseII is significantly reduced in Gi-antisense myoblasts and myotubes. In contrast to insulin signaling, PDGF-dependent activation of target gene promoters is largely independent of Gi protein levels in our antisense L6 cell model. These experiments illustrate a role for Gi in the signaling pathway of specific receptor tyrosine kinases and support a model in which loss of Gi function can contribute to an insulin resistant phenotype.
Acknowledgements

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<tr>
<td>β-gal</td>
<td>β-galactosidase</td>
</tr>
<tr>
<td>AC</td>
<td>adenylyl cyclase</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
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<tr>
<td>AM</td>
<td>activated mutant</td>
</tr>
<tr>
<td>Amp</td>
<td>ampicillin</td>
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<td>adenosine triphosphate</td>
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<td>cyclic guanosine monophosphate</td>
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<td>calf-intestinal phosphatase</td>
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<td>CSF-1R</td>
<td>colony stimulating factor 1 receptor</td>
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<td>DMSO</td>
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<td>DN</td>
<td>dominant negative</td>
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<td>DOK</td>
<td>docking protein</td>
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<td>epidermal growth factor</td>
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<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<td>GTPase activating protein</td>
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<td>guanosine diphosphate</td>
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<td>Growth factor receptor bound protein 2</td>
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<td>glycogen synthase kinase 3</td>
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<td>guanosine triphosphate</td>
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<td>GTP[S]</td>
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<td>Hyg</td>
<td>hygromycin</td>
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<tr>
<td>IDDM</td>
<td>insulin dependent diabetes mellitus</td>
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<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
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<td>IP</td>
<td>immunoprecipitation</td>
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<td>IR</td>
<td>insulin receptor</td>
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<td>IRS</td>
<td>insulin receptor substrate</td>
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<td>Description</td>
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</tr>
<tr>
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<td>kilobase</td>
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<td>kilo-dalton</td>
</tr>
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</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
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<td>lysophosphatidic acid</td>
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<tr>
<td>Luc</td>
<td>luciferase</td>
</tr>
<tr>
<td>M</td>
<td>molarity</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>mitogen-activated protein kinase kinase</td>
</tr>
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<td>MEM</td>
<td>modified eagle’s medium</td>
</tr>
<tr>
<td>mmol</td>
<td>millimole</td>
</tr>
<tr>
<td>Mol</td>
<td>moles</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>NIDDM</td>
<td>noninsulin dependent diabetes mellitus</td>
</tr>
<tr>
<td>p70^6K</td>
<td>p70 ribosomal S6 kinase</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>platelet-derived-growth-factor</td>
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<tr>
<td>PDGFR</td>
<td>platelet-derived-growth-factor-receptor</td>
</tr>
<tr>
<td>PDK</td>
<td>phosphatidylinositol-dependent kinase</td>
</tr>
<tr>
<td>PEPCK</td>
<td>phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin homology</td>
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<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B (also AKT)</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PPARγ</td>
<td>peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>PTB</td>
<td>protein tyrosine binding</td>
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<tr>
<td>PtdIns</td>
<td>phosphatidylinositol</td>
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<tr>
<td>PTP</td>
<td>protein-tyrosine phosphatase</td>
</tr>
<tr>
<td>PTP-LAR</td>
<td>protein tyrosine phosphatase–leukocyte common antigen related molecule</td>
</tr>
<tr>
<td>PTX</td>
<td>Bordetella pertussis toxin</td>
</tr>
<tr>
<td>RKS</td>
<td>receptor kinase substrates</td>
</tr>
<tr>
<td>RIPA</td>
<td>radio immunoprecipitation assay</td>
</tr>
<tr>
<td>Rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulfide</td>
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<td>SH2</td>
<td>src-homology 2</td>
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<td>SH3</td>
<td>src-homology 3</td>
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<tr>
<td>Shc</td>
<td>src-homology-collagen</td>
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<tr>
<td>SIE</td>
<td>sis-inducible element</td>
</tr>
<tr>
<td>SOS</td>
<td>son-of-sevenless</td>
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<tr>
<td>SRE</td>
<td>serum response element</td>
</tr>
<tr>
<td>SRF</td>
<td>serum response factor</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with tween</td>
</tr>
<tr>
<td>TCF</td>
<td>ternary complex factor</td>
</tr>
<tr>
<td>Tet</td>
<td>tetracycline</td>
</tr>
<tr>
<td>TK</td>
<td>thymidine kinase</td>
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<tr>
<td>TNF-α</td>
<td>tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol 13-acetate</td>
</tr>
<tr>
<td>UV</td>
<td>ultra-violet</td>
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</table>
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A. Introduction
A. 1. Diabetes

Diabetes mellitus is a metabolic disorder caused by decreased production of insulin or by decreased ability to use insulin in which chronic hyperglycemia is the essential feature. Insulin-dependent diabetes (IDDM or type 1) usually occurs early in life before the age of 30 and is a direct result of an auto-immune destruction of the β-cells of the islets in the pancreas, which results in the loss of insulin production. Noninsulin-dependent diabetes (NIDDM or type 2) is a polygeneic disorder frequently associated with obesity and age (i.e. older adults) and involves increased insulin resistance. Both type I and type II diabetes can be controlled with the proper medicine, diet and exercise. Defining the actions and role of the hormone insulin and its signalling mechanisms is a key strategy for understanding the basis of this disease.

In addition to the two well known forms of diabetes, a third form is called Maturity Onset Diabetes of the Young or MODY. MODY as the name implies, usually develops in young persons with raised blood glucose but does not have the high ketone levels frequently present in IDDM patients. MODY is usually a result of an inherited defective gene disorder and currently, there have been five MODY genes identified; they are hepatocyte nuclear factor-4 (HNF-4alpha/MODY1), glucokinase/MODY2, HNF-1alpha/MODY3, insulin promoter factor-1 (IPF1/MODY4) and HNF-1beta/MODY5 (Chevre et al 1998). Genetic screens have suggested the existence of additional loci that can cause MODY which have yet to be discovered. Disruption of each gene leads to a form of MODY and shows the complexity and variability of diabetes as a polygeneic disease.
A. 2. Insulin resistance

Insulin resistance can be defined as a state in which normal amounts of insulin produce a subnormal biological response (Kahn 1978). Factors contributing to NIDDM can include insulin resistance, obesity, elevated free fatty acids, tumor necrosis factor-α (TNF-α), and specific defects in the insulin signalling pathway. Reducing insulin resistance is important for managing NIDDM with programs developed for weight control, exercise and moderate alcohol consumption to improve insulin responsiveness.

The molecular mechanisms that can impair insulin signalling leading to hormone resistance are complex and wide ranging. For example, a recent study by Barroso et al. (1999), showed that dominant negative mutation in the transcription factor, peroxisome proliferator-activated receptor gamma (PPARγ), is associated with severe insulin resistance. PPARγ is a nuclear receptor, which when activated by an endogenous ligand or agonist drugs (e.g. thiazolidinediones) regulates transcription of target genes. The dominant negative mutations resulted in extreme insulin resistance, early onset diabetes and hypertension due to interference of wild-type PPARγ. Alternatively to this mechanism, involving a specific transcription factor, MODY-2 is caused by a mutation in the glucokinase gene (review Velho and Froguel 1998). Glucokinase, also called hexokinase type IV, is found in the liver and controls glucose uptake. It serves as a glucose sensor to regulate insulin release from the pancreatic β-cells. Defects in glucokinase function impair glucose tolerance, and elevate fasting glucose levels. The genetic lesions in glucokinase can include missense or nonsense mutations, nucleotide deletions or frameshift mutations at RNA splice sites (Burke et al. 1999). These
examples illustrate that specific gene defects in either insulin target cells or insulin secretory cells can lead to insulin resistance.

A. 3. Insulin Action

The hormone insulin was first isolated as a pancreatic extract in 1921 by Sir Frederick G. Banting and Charles H. Best, and since that time, understanding its mechanisms of action has been the focus of many laboratories. Insulin is composed of two polypeptide chains called the A chain and B chain joined by disulfide linkages. The normal fasting levels of insulin in the plasma are about $10^{-10}$ to $10^{-9}$ M, which is elevated 10-fold after a detectable increase in blood glucose levels after a meal (basal glucose concentrations are approximately 5 mmol/L rising to 9-10 mmol/L after a meal). The main target tissues for insulin are skeletal muscle, fat and liver. In muscle, it promotes the uptake of glucose and amino acids for protein synthesis. In adipose tissue, insulin facilitates the storage of glucose and its conversion to fatty acids. In liver, insulin helps convert glucose into glycogen and decreases gluconeogenesis. Insulin action is opposed by the presence of glucagon, another islet hormone and by epinephrine. Epinephrine stimulates glycogen breakdown in muscle and to a lesser extent, in liver. Activation by both glucagon and epinephrine involves binding to the G-protein coupled receptors, and stimulates adenylyl cyclase and cyclic AMP levels (review Siess 1989). Insulin alters many functions at the plasma membrane of target tissues including amino acid and cation transport and membrane potential (review Avruch 1998). Many intracellular processes are activated as well, for example, protein synthesis and regulation of specific enzymes such as glycogen synthase, pyruvate dehydrogenase or hormone sensitive lipase. As
described below, insulin like many growth factors, initiates signalling at a specific receptor tyrosine kinase (for general outline see Fig 1).
Fig 1: The insulin signaling system affects numerous intracellular processes.
A. 4. Receptor Tyrosine Kinases (RTKs)

Receptor tyrosine kinases are involved in both cell proliferation and differentiation (reviewed by Schlessinger and Ullrich 1992). Growth factors binding to the extracellular domain of RTKs cause a conformational change that induces receptor oligomerization and activation of the intracellular protein tyrosine kinase domain (reviewed by Hubbard et al. 1998). Substrates for each kinase can be either the RTK itself, or specific docking proteins that facilitate protein interactions. In response to tyrosine phosphorylation of the RTKs, different signalling molecules containing protein tyrosine binding (PTB) domains, src-homology 2 (SH2) domains and src-homology 3 (SH3) domains (discussed below) can dock at specific RTK sequences and activate distinct downstream signalling cascades. Most RTKs can stimulate similar downstream targets even though they are each activated by unique ligands.

A. 4.1. Insulin Receptor

Studies by Cuatrecasas et al. (1969) first showed that insulin acts on target cells without entering the cells, which subsequently led to the identification of a specific, high affinity cell surface receptor. The expression of the insulin receptor is almost ubiquitous throughout mammalian tissues. Although the number of receptors per cell varies among tissues, they are most abundant in the major insulin target tissues, fat, muscle and liver (reviewed by Kahn et al. 1981). The size of the insulin holoreceptor is approximately 350 000 kDa and consists of two α-subunits and two β-subunits linked through disulfide bonds in the form of a heterotetramer on the plasma membrane (Fig 2) (Massague et al. 1981). The two α-subunits are positioned on the extracellular side of the membrane and
contain the high affinity insulin-binding domain. This function has been mapped to the N-terminal portions of the α-subunits (Rafaeloff et al. 1989, Yip 1992.). The α-subunit also plays an allosteric inhibitory role as truncation experiments that removed the α-subunit from the receptor resulted in constitutive activation of the kinase activity of the β-subunit (Shoelson et al. 1988). In contrast to the α-subunits, which are entirely extracellular, each β-subunit is composed of a short extracellular domain, a 23 amino acid transmembrane domain and an intracellular domain which contains the ATP binding site and the tyrosine kinase catalytic domain (Chou et al. 1987, Ebina et al. 1987, McClain et al 1987, Stumpo and Blackshear 1991). Insulin binding leads to autophosphorylation of the insulin receptor at seven possible tyrosine residues (Kahn et al. 1993, Feener et al. 1993, Goren et al. 1987). Three of these residues, Tyr^{1158}, Tyr^{1162} and Tyr^{1163}, are located within the kinase regulatory domain (see Fig 2). Phosphorylation of these residues is necessary for further autophosphorylation and kinase activation, which is required for both metabolic and growth responses, and for regulating insulin sensitivity (Wilden et al 1992). The two C-terminal phosphotyrosine sites, Tyr^{1328} and Tyr^{1334}, may be important for binding or docking for several src homology 2 (SH2) domain containing proteins (review Kahn 1994). SH2 domains are protein modules of approximately 100 amino acids, which bind to phosphorylated tyrosines in specific contexts. For example, two sequences known to be recognized by SH2 domains are pYMXM and pYXXM. Functional mapping studies suggest the two residues (Tyr^{1328} and Tyr^{1334}) can negatively regulate insulin-stimulated cell growth (Thies et al. 1989). Defining a role for the phosphorylation of these two tyrosines in this region has been controversial. One study showed that a mutated form of the insulin receptor with a 43
amino acid deletion at the C-terminus behaves in a manner identical to the wild-type receptor with respect to both glycogen synthesis and DNA synthesis (Myers et al. 1991). However, a second study showed that in Chinese Hamster Ovary cells overexpressing the insulin receptor with the same deletion, a significantly greater level of receptor autophosphorylation and tyrosine kinase activity was achieved compared to the wild-type receptor (Bernier et al. 1994). This suggested that the C-terminal of the insulin receptor has an inhibitory role in insulin stimulation. Although, the autophosphorylation sites in the insulin receptor C-terminal domain appears to regulate insulin signalling, their exact function remains unclear. In addition to tyrosine phosphorylation sites, the insulin receptor β-subunits also contain many serine and threonine residues, phosphorylation of which can inhibit tyrosine kinase activity in vivo and in vitro (Takayama et al. 1988).

Therefore, the consequence of tyrosine phosphorylation on the insulin receptor is to create new binding sites for the recruitment of docking proteins. These proteins in turn bind to specific adaptors and enzymes (Koch et al. 1991, Kavanaugh and Williams 1994) that allow divergence of the insulin signal to various receptor kinase substrates (described in section A. 5).

A. 4. 2. Platelet derived growth factor receptor

Platelet derived growth factor (PDGF) was first identified as a mitogenic activity from human platelets that could stimulate growth of several cell types including fibroblasts, smooth muscle cells and glial cells (Kohler and Lipton 1974, Ross et al. 1974, Westermark and Wasteson 1976). Similarly to insulin, PDGF binds to high affinity RTKs. Unlike the insulin receptor, PDGF receptors form homodimers upon ligand binding (review Claesson-Welsh 1994). The dimerized PDGFR activates the kinase
activity leading to autophosphorylation at multiple tyrosine residues in the juxtamembrane domain, kinase insert, tyrosine kinase domain 2 and c-terminal tail (see Fig 3) increasing the catalytic activity of the receptor (Panayotou and Waterfield 1993). The juxtamembrane domain attracts members of the Src family of kinases and as a consequence, increases the kinase activity of the Src proteins two to four fold. The kinase insert binds several adaptor proteins, Grb2, PI3-kinase, Nck, GTPase-activating protein of Ras (GAP), Shc, and Shb proteins. The C-terminal tail phosphorylation leads to binding of Protein Tyrosine Phosphatase 1D (PTP-1D) and Phospholipase Cγ (PLC-γ).

Upon interaction with the activated receptor, the catalytic activities become up-regulated through tyrosine phosphorylation or other mechanism. The signal generated may be unique for each type of signal transduction molecule but eventually results in a biological response such as immediate early gene activation and/or cell proliferation.

In response to PDGF stimulation, the receptor is autophosphorylated as well as polyubiquitinated. The ubiquitination leads to degradation of the receptor, which internalization and degradation of PDGF receptors may be a mechanism for regulation. The PDGF receptors are also expressed in a broad spectrum of cells (review Raines and Ross R 1993) and a number of growth-modulating factors have been shown to regulate expression. The PDGF receptor is clearly involved in a wide variety of cellular responses and signal transduction pathways, which is useful for comparing and contrasting different receptor tyrosine kinase activities.
FIG 2
Insulin RTK

α subunit

β subunit

O — Tyr Residues

965 Juxtamembrane Region
1158 1162 1163 Kinase Regulatory Domain
1328 1334 C-terminal Domain

FIG 3
PDGFR

579 581 Juxtamembrane domain

Tyrosine Kinase domain 1

716 740 751 771 Kinase Insert

857 Tyrosine Kinase domain 2

1009 C-terminal Domain

1021
A. 5. Receptor kinase substrates

Receptor kinase substrates interact directly with receptors and serve as docking proteins for downstream effectors in the signalling pathway. Several well-studied docking proteins are IRS-1, IRS-2, IRS-3, Shc, Gab-1, and p62DOK. By recruiting additional proteins to the activated receptor, they effectively propagate the downstream signals following autophosphorylation of the receptor.

A. 5.1. IRS proteins

Members of the IRS family bind to the insulin receptor via an NH2-terminal Pleckstrin Homology (PH) domain (discussed below) and a tyrosine binding domain. Currently, four different IRS proteins have been identified and characterized in their relation to insulin signalling (review Cheatham 1995, review White 1997). The IRS-1 protein is approximately 185 kDa in size when fully phosphorylated and associates with the insulin receptor through protein tyrosine binding (PTB) domains. The PTB domains of IRS-1 recognize autophosphorylation sites in RTKs with the consensus sequence NPXpY (van der Geer et ai. 1999). IRS-1 binds to the phosphorylated tyrosines on the juxtamembrane region of the insulin receptor (Eck et al. 1996), particularly, at Tyr\textsuperscript{960} (White et al. 1988) (see Fig 2). Mutations of the Tyr\textsuperscript{960} residue can prevent phosphorylation of the IRS-1 without affecting insulin binding, kinase activation or receptor autophosphorylation (White et al. 1988). IRS-2 is structurally similar to the IRS-1 and like IRS-1, it is phosphorylated and activated by the insulin receptor (review White 1997). Overexpression of IRS-1 and -2 has been examined in translocation experiments using a co-transfected epitope-tagged GLUT4 glucose transporter (GLUT4-
HA) (Zhou et al. 1997). In these studies, IRS-2 (like IRS-1) is capable of recruiting GLUT4 to the plasma membrane in response to insulin. A second domain found on the amino terminus of the IRS proteins is called the pleckstrin homology (PH) domain (Yenush et al. 1996). The pleckstrin homology domain, named for the protein in which it was first identified, a conserved sequence of approximately 100-amino-acids found in numerous proteins of diverse functions. It forms a discrete structural module (Mayer et al. 1993) and has been speculated to play a role in targeting proteins (like IRS) to the cell membrane (Lemmon et al. 1996). The ligands for the PH domains in IRS proteins have not yet been defined, nor has a direct interaction between the insulin receptor and the PH domain of IRS-1 been detected (Burks et al. 1998, Gustafson et al. 1995). However, the IRS-1 PH domain appears to be essential for signalling as deletion of the PH domain significantly reduces insulin stimulated IRS-1 tyrosine phosphorylation (Yenush et al. 1996). IRS-1/2 proteins contain more than 20 well-conserved tyrosine residues that can be phosphorylated and serve as potential docking sites for src homology 2 (SH2) domain-containing proteins. There are six pYMXM and three pYXXM motifs found on the IRS-1 protein (review Myers and White 1996), that serve as potential docking sites for effectors, in the insulin signalling cascade.

In addition to tyrosine phosphorylation, IRS proteins can be phosphorylated at multiple serine or threonine residues. In the absence of insulin, IRS-1 is known to be phosphorylated on many of its approximately 40 potential serine and threonine phosphorylation sites (Sun et al. 1991). After stimulation with insulin, there is a total increase in both tyrosine and serine phosphorylation (Sun et al. 1992). The elevated serine/threonine phosphorylation of IRS-1 has been shown to inhibit its binding to the
juxtamembrane region of the insulin receptor and prevents insulin induced tyrosine phosphorylation of IRS-1 (Paz et al 1997). Several potential kinases for the serine and threonine phosphorylation sites are casein kinase II, protein kinase C, protein kinase A, cGMP-dependent protein kinases, MAP kinases, and cdc2 kinase (Sun et al. 1991, Tanasijevic et al. 1993). MAP kinase was found to co-precipitate with IRS-1, implicating this kinase as a potential regulator of IRS-1 tyrosine phosphorylation (Fea and Roth 1997). Regulation of IRS proteins may therefore be dependent on the balance between such serine/threonine phosphorylation and tyrosine phosphorylation by the insulin receptor.

IRS-1 and IRS-2 have very similar structures and accordingly, in mice deficient in IRS-1, IRS-2 is capable of transducing insulin signals and compensating the loss of IRS-1 without an increase in protein expression. However, although IRS-1 deficient mice failed to develop type 2 diabetes, they still showed some insulin resistance and significant growth retardation (Patti et al. 1995). By contrast, mice deficient in IRS-2 developed marked insulin resistance followed by type 2 diabetes and impairment of pancreatic β-cell function (Withers et al. 1998). These in vivo models show the important functional distinction between IRS-1 and IRS-2.

Recently, the roles of IRS-3 and IRS-4 in insulin signalling have been characterized. IRS-3 is located mainly in the plasma membrane fraction and contributes to PI3-kinase activation (Anai et al 1998). IRS-3 and IRS-4 are suggested to mediate PI3-kinase dependent metabolic actions of insulin in adipose cells and play a physiological role in mediating translocation of GLUT4 (Zhou et al 1999).
A. 5.2. Phosphoinositide 3-kinases

Phosphoinositide 3-kinases (PI3-kinase, PI3K) are important effectors of RTK signalling (review Vanhaesebroeck et al. 1997). They are composed of a 110 kDa catalytic subunit (p110) and a 85 kDa SH2-containing regulatory subunit (p85) that binds to phosphorylated tyrosines in RTKs and receptor kinase substrates, such as IRS (review White 1998). PI3-kinases fall into three classes based on their lipid substrate specificity in vitro, their structure and likely their mode of regulation. Class I PI3-kinases phosphorylate phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P2) and PtdIns(4)P. The main substrate target for class I PI3-kinase is the D-3 position of the inositol ring of PtdIns(4,5)bis-phosphate to give PtdIns(3,4,5)phosphate (PIP3) (Shepherd et al. 1996, Vanhaesebroeck et al. 1997). The class I heterodimeric p85/p110 PI3-kinase is known to be activated by receptor tyrosine kinase such as the insulin RTK or PDGFR (Ruderman et al. 1990, Wiese et al. 1995). Class II PI3-kinases phosphorylate PtdIns and PtdIns(4)P in vitro, but not PtdIns(4,5)P2. According to Vanhaesebroeck et al. 1997 it is still unclear whether the class II enzymes are regulated by extracellular stimuli. Class III PI3Ks phosphorylate PtdIns selectively; the current hypothesis is that they perform housekeeping role in constitutive membrane trafficking and vesicle morphogenesis (review De Camilli et al. 1996). The regulatory PI3-kinases p85 subunits contain SH2 domains, which bind phosphorylated tyrosine residues, specifically pYXXM motifs (Gout et al 1992). This allows the class I PI3-kinase catalytic subunits to bind to docking proteins like IRS-1, or directly to growth factor receptors in the case of PDGFR (Giorgetti et al 1993).
Binding of PI3-kinase to phosphorylated pYXXM motifs of IRS-1 activates the catalytic function of the p110 subunit, and leads to translocation of the complex to cellular membranes where it interacts with specific substrates (Shepherd et al. 1996, Stephens et al. 1993). PI3-kinases have been implicated in the insulin stimulated activation of S6 kinase (p70	extsuperscript{S6k}), protein kinase B (PKB), glycogen synthase kinase-3 (GSK3), and also in glucose transport and recruitment of GLUT4 to the plasma membrane (Clarke et al. 1994, Okada et al. 1994). The activities of phosphatidylinositol 3-kinase (PI3-kinase) are inhibited by wortmannin, a fungal metabolite that acts as a potent, selective, cell-permeable and irreversible inhibitor (Yano et al. 1993). Through inhibitor experiments with wortmannin, PI3-kinase has been implicated in a wide range of growth factor responses, both metabolic and mitogenic, involving the activation of MAPK pathway (Cross et al. 1994, Yamauchi et al. 1993).

A. 5. 3. Activation of the MAP Kinase Pathway

The serine kinase cascade centered around a family of enzymes called mitogen-activated-protein kinases (MAPK), plays a key role in regulating cell growth and differentiation in organisms ranging from yeast to humans. MAP kinases can be regulated by many different cell surface receptors. In the case of the insulin receptor, activation can be mediated by interaction of the receptor with the Src-homology-collagen like protein (Shc). Shc, an adaptor molecule without enzymatic activity, is capable of binding to the insulin receptor via its PTB domain, then forms a complex with a second adaptor protein Grb2, which in turn is bound to the GDP exchange factor son-of-sevenless (SOS) through SH2 and SH3 domains (Baltensperger et al. 1993). Alternatively, the Grb2-SOS complex can be recruited to the plasma membrane by
interacting directly with insulin receptor-bound IRS-1 through SH2 domains. The guanine nucleotide exchange activity of the SOS protein catalyses GTP-loading of the small G protein p21-Ras (Medema et al. 1993, Valencia et al. 1991). GTP bound Ras, interacts with and activates the serine/threonine kinase Raf-1 by recruiting it to the plasma membrane. Activation of Raf-1 phosphorylates and activates the mitogen-activated protein kinase/extracellular signal regulated kinase kinases (MAPKK or MEK1 and MEK2) (Cobb and Goldsmith 1995). The MEK dual specificity kinases phosphorylate both tyrosine and threonine residues and thereby directly activate their substrate mitogen activated protein kinase or MAPKs (also known as extracellular signal-regulated kinase 1 and 2 (ERK1/2)). The inhibitor PD 98059 is a specific inhibitor of MAPK kinase (MEK) and acts in a noncompetitive inhibition with respect to ATP binding (Blenis, 1991). As PD 98059 can block the insulin stimulation of the MAP kinase pathway it should prevent insulin-stimulation of c-fos and other early genes involved in the mitogenic response (Hill and Treisman 1995). Activation of the c-fos SRE is generally believed to involve the MAPK-dependent phosphorylation of the TCF/Elk-1 and SRF transcription factors (Gille, 1992).

A. 5. 4. Activation of the 70 kDa ribosomal protein S6-kinase

Stimulation of PI3-kinase is an essential step for activation of glucose transport and metabolism by insulin. The PI3-kinase inhibitor wortmannin blocks insulin stimulation of glucose transport and the recruitment of GLUT4 to the plasma membrane (Clarke et al. 1994). Wortmannin blocks the activation of ribosomal S6 protein kinase (p70^60k) by insulin or insulin-like growth factor-1 (IGF-1) and it is presumed that p70^60k
requires PI3-kinase for activation (Cross 1994, Chung et al. 1992, Cheatham et al. 1994). This activation of p70^{65k} is selectively blocked by rapamycin by inhibiting the phosphorylation and activation of p70 S6 kinase (p70^{65k}) without affecting PI 3-kinase activity (Chung et al. 1992). Rapamycin inhibits p70^{65k} activation through the binding and inactivation of the Mammalian Target of Rapamycin (mTOR), a kinase that has been shown to directly phosphorylate and activate p70^{65k} in vitro. Insulin is hypothesized to activate the p70^{65k} through the PI3-kinase-dependent phosphorylation of PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3. This lipid phosphorylation signal activates PtdIns-dependent kinase (PDK) and protein kinase B (PKB), the latter of which has been shown to regulate activation of mTOR (Scott et al. 1998) probably by direct phosphorylation (Nave et al 1999). Using a three-step kinase assay, Isotani et al (1999) showed that PDK and mTOR synergistically activate p70^{65k}. Whether this mechanism is functional in vivo is still unclear.

A. 6. Transcriptional targets of insulin

Insulin regulates the transcription of several target genes in insulin responsive tissues that play key roles in cell growth and metabolism. For example insulin induces expression of the c-fos and hexokinase II genes. C-fos is needed for cell proliferation and differentiation, while hexokinase II is important for facilitating glucose uptake by catalyzing its phosphorylation to glucose-6 phosphate. Insulin responsive regions have been mapped in both target genes.
A. 6.1. C-fos gene promoter

Insulin regulation of immediate early genes like c-fos, c-jun, are needed for expression of immediate-early genes, for the regulation of cell differentiation, proliferation and apoptosis (review Cheatham and Kahn 1995). Rapid, transient induction of c-fos follows treatment of cells with insulin and other growth factors (Stumpo et al, 1988). The induced c-fos proteins can heterodimerize with members of the c-jun family to form AP-1, a transcription factor, which regulates many secondary genes, including metabolic genes (Gurney et al. 1992). Transcription of c-fos is controlled by several DNA regulatory sequences in the proximal promoter region. These include the serum response element (SRE) (review Treisman 1990) and a sis-inducible element (SIE) (Hayes et al. 1987, Wagner et al. 1990). The SRE serves as an assembly point for a number of transcription factors including the serum response factor (SRF), which bind to a specific portion of the SRE element (Norman et al. 1988). Adjacent to the SRF binding site is an Ets site, which is targeted by ternary complex factor (TCF) proteins. These can bind to SRE following binding of the SRF (Shaw et al. 1989, Hipskind et al. 1991, Dalton and Treisman 1992). Several Ets related proteins have been shown to exhibit SRF-dependent binding to the SRE; these are Elk-1, Sap-1 and Sap-2. Binding of the TCF-Elk-1 proteins to the SRE Ets site and SRE-dependent transactivation is regulated by MAP kinase phosphorylation in response to a wide range of extracellular signals (Gille et al. 1992, Hill et al. 1993, review Treisman 1994), which besides insulin, can include lysophosphatidic acid (LPA) (Perkins et al. 1994), 12-O-tetradecanoylphorbol-13-acetate (TPA), epidermal growth factor (EGF), and PDGF (Hill and Treisman 1995).
A. 6.2. Hexokinase II gene promoter

Transport of glucose across the plasma membrane by GLUT4 and phosphorylation of glucose by hexokinase II constitute the first two steps of glucose utilization in skeletal muscle, heart and adipose tissue. By catalyzing the conversion of glucose to glucose 6-phosphate, hexokinase II maintains the gradient necessary for glucose entry into cells through GLUT4 transporters (Bell et al. 1990). Hexokinase II mRNA is decreased in adipose tissue from diabetic rats, but restored by insulin treatment to the levels found in nondiabetic control rats. Insulin has been shown to induce hexokinase II mRNA in two adipose cell lines (3T3-F442A and BFC-1B) and two skeletal muscle cell lines (C2C12 and L6) (Printz et al. 1993). In L6 myotubes, insulin was shown to induce hexokinase II primarily by an increase in gene transcription and is dependent on the activation by p70\(^{66K}\) (Osawa et al. 1996). As reviewed earlier the activation of p70\(^{66K}\) was activated by mTOR in the PI3-kinase pathway \textit{in vitro} in response to insulin (Isotani et al. 1999).

A. 7. Heterotrimeric G proteins

Although not generally associated with signalling by receptor tyrosine kinases, experimental evidence has suggested the potential involvement of the "G\(_i\)-type" G proteins in insulin dependent responses (Heyworth 1986). In order to understand this involvement, a review of the effects and functions of heterotrimeric G proteins is presented to assist in explaining potential regulatory interactions.

Heterotrimeric G proteins are an integral part of the signal transduction pathways for seven transmembrane receptors and can link to an array of intracellular effector molecules. In general, classification of G proteins is by reference to the G\(_{\alpha}\)-subunit (see
Table 1). Currently, some twenty $G_\alpha$-subunits, at least five $G_\beta$ and twelve $G_\gamma$-subunits have been identified (Sondek et al. 1996, Clapham 1996). Heterotrimeric G proteins can regulate a diverse group of effector proteins such as adenylyl cyclases (AC), phospholipases (PLCs), and various ion channels. The G-proteins are hydrophilic proteins that are modified by the addition of fatty acid chains to become anchored to the plasma membrane. The $G_\alpha$ subunits are modified by combinations of palmitoylation and/or myristoylation, while the $G_\gamma$-subunits are isoprenylated (review Milligan and Grassie 1997).

All G protein linked receptors (GPCRs) share a heptahelical arrangement of hydrophobic domains that span the lipid bilayer in a serpentine manner (review Ji et al. 1998, review Dohlman et al. 1991). The seven transmembrane domains maintain an intervening arrangement of intracellular and extracellular “loops” of variable length, as well as a cytoplasmic COOH terminal tail and an extracellular NH$_2$ terminus. The extracellular domain acts in signal discrimination and ligand binding, which propagates the signal to the inactive G protein trimer. One or more intracellular loops of the GPCR and sometimes the N-terminal region of the cytoplasmic C-terminal tail are essential for coupling to G proteins (Sano et al. 1997).

In general, ligand binding generates a signal, which is transduced through the GPCR, likely through reorganization of the seven transmembrane helices. The conformation change induces the GDP release from the $G_\alpha$ subunit and binding of GTP. Nucleotide exchange for GTP allows dissociation of the $G_\alpha$ subunit from the $G_{\beta\gamma}$ dimer. Now activated, both G protein components can interact with and activate various effectors within the cell. Activation occurs until the intrinsic GTPase activity of the $G_\alpha$
subunit hydrolyses the GTP to GDP, allowing the inactive $G_\alpha$ to reassociate with the $\beta\gamma$ dimer in the inactive state (See Fig 4) (Bourne et al. 1989). The $G_\alpha$ and $G_{\beta\gamma}$ subunits can regulate the $G$ protein-coupled effectors in a selective manner that can be either independent, synergistic or antagonistic (Neer and Clapham 1988).

Several specific toxins have been found to selectively modify subgroups of $G_\alpha$ subunits. The $G_{3\alpha}$ subunits activate adenylyl cyclase and are selectively ADP-ribosylated between amino acids 100-119 by cholera toxin (Krieger-Brauer et al 1999). The ribosylation catalyzed by the cholera toxin inhibits the intrinsic GTPase activity allowing the $G_{3\alpha}$ subunit to be constantly active (Ui and Katada 1990, Gilman 1989). The $G_{1\alpha}$ class subunits, more specifically $G_{1\alpha2}$, is capable of mediating the inhibitory control of adenylyl cyclase (review Gilman 1987, Moxham et al.1993A, Moxham et al. 1993B, McKenzie and Milligan 1990) and is sensitive to Bordetella pertussis toxin (Ui and Katada 1990). Pertussis toxin targets the cysteine residue at the $G_i$ COOH-terminal resulting in ADP-ribosylation. Unlike cholera toxin, pertussis toxin effectively blocks the ability of $G_{1\alpha}$ proteins to transduce the signals to effectors by stabilizing the $G_i\alpha$-subunit in the heterotrimeric, inactive state.
### Table 1 Classes of Gα subunits

<table>
<thead>
<tr>
<th>Class</th>
<th>Members</th>
<th>Modifying Toxin</th>
<th>Some Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>αₙ</td>
<td>αₙ, αᵣₒᵢ</td>
<td>Cholera</td>
<td>Stimulate adenylyl cyclase,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>regulate Ca²⁺ channels</td>
</tr>
<tr>
<td>α₁</td>
<td>α₁₁, α₁₂, α₁₃, α₁₅, α₁₆, α₁₇</td>
<td>Pertussis (except α₁)</td>
<td>Inhibit adenylyl cyclase,</td>
</tr>
<tr>
<td></td>
<td>α₁₈, α₂₀, α₂₁</td>
<td></td>
<td>regulate K⁺ and Ca²⁺ channels,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>activate cGMP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>phosphodiesterase</td>
</tr>
<tr>
<td>α₉</td>
<td>α₉, α₁₁, α₁₄, α₁₅, α₁₆</td>
<td>-</td>
<td>Activate PLC</td>
</tr>
<tr>
<td>α₁₂</td>
<td>α₁₂, α₁₃</td>
<td>-</td>
<td>Regulates Na⁺/K⁺ exchange</td>
</tr>
</tbody>
</table>
**Fig 4**

1 - Synthesis and targeting of components

2 - Receptor activation by agonist

3 - Receptor activation of G protein

4 - G protein-effector interaction

5 - GTPase

*Fig 4: The G protein GTPase Cycle*

1 - the inactive G protein is a GDP-bound heterotrimer. 2 - Agonist binding causes receptor activation. 3 - Receptor activation catalyzes the exchange of GDP for GTP leading to dissociation of the Gᵦ-subunit from the Gᵦᵣ-dimer. 4 - Dissociated G protein subunits interact with various effectors (see text). 5 - Hydrolysis of GTP by the intrinsic GTPase activity of the Gᵦ-subunit leads to reassociation of the inactive heterotrimer. (Adapted and modified from Spiegel A 1996)*
A. 8. G\textsubscript{ia} protein involvement in insulin signalling

The potential involvement of a pertussis toxin (PTX) sensitive G protein as a mediator of insulin-dependent response was first reported by Heyworth et al. (1986). The ability of insulin to inhibit adenylate cyclase activity in hepatocytes was blocked by pretreatment of cultures with PTX. Later, studies by others showed that insulin-stimulated PI-glycan hydrolysis was inhibited by PTX (Luttrell et al. 1988) and that hydrolysis of PI-glycan by phospholipase C is coupled to the insulin receptor by a PTX-sensitive G\textsubscript{i} protein (Hoffman et al. 1991). In contrast to the above studies, Chuprun et al. (1997), showed pretreatment with PTX had no effect on insulin induction of c-fos. Similarly, Linder et al. (1994), showed that the closely related IGF-I receptor does not use pertussis toxin-sensitive G proteins in stimulating DNA synthesis in MG-63 cells. The use of pertussis toxin has thus given conflicting results in the literature and has lead to uncertainty about the role of G\textsubscript{i} in receptor tyrosine kinase signalling. Therefore alternate approaches should be utilized.

Using a biochemical approach, two groups have suggested an association between G protein binding and activation with the insulin receptor (Okamoto et al. 1993, Jo et al. 1993). Jo et al. (1992) identified two G-proteins of 67 and 41 kDa from wheat germ-purified insulin receptor preparations obtained from human placenta. Receptors were first purified by insulin-sepharose chromatography with wheat germ-binding proteins. The G protein depleted receptors were then prepared by modifying the insulin chromatography using an extensive washing protocol. From the column eluate the insulin receptor-bound proteins were separated by SDS-PAGE and blotted with a common G\alpha antibody, which
produced bands at approximately 41 and 67 kDa. The extracts were ADP-ribosylated with or without activated pertussis toxin, and the 41 kDa G protein was specifically modified in the presence of PTX. Furthermore, GTPγS binding activity was measured comparing the normal insulin receptor with the G protein depleted insulin receptors. In this experiment, GTPγS binding was significantly decreased in the G protein depleted insulin receptors and there was a loss of ability of the insulin receptor to stimulate kinase activity. Jo et al. (1992) further found that the PTX catalyzed ADP-ribosylation of the 41 kDa G protein was enhanced by the addition of purified insulin receptors, indicating that an interaction between the insulin receptor and the Gi protein may have functional consequences. Whether this interaction takes place in intact cells was not clear from the study. In other studies, co-immunoprecipitation of the insulin receptor with anti-Gia antibody has been detected (Record et al. 1993, Sanchez-Margalet et al. 1999) suggesting a direct interaction but again there were no experiments to support a functional interaction between these proteins. Therefore, the significance of interactions between Gi proteins and insulin receptor remains unclear.

Genetic approaches to understanding the role of specific G proteins in insulin signalling, can be complicated by their wide distribution and function, often at early developmental stages. This can produce severe phenotypes in some cases. For example, creation of knock-out mice for Gs proved lethal in homozygous embryos, with a complex pattern of tissue-specific imprinting observed in heterozygotes with the null allele (Yu et al. 1998). Targeted deletion of the Giα2 gene in mice caused a runted phenotype and lead to ulcerative colitis and adenocarcinoma of the colon (Rudolph et al. 1995A, Rudolph et al. 1995B). To avoid developmental effects of complete Giα2
deficiency, Moxham and Malbon (1993A) introduced a $G_{i\alpha 2}$ antisense sequence inserted into a phosphoenolpyruvate carboxykinase (PEPCK) transgene into mice. This construct was expressed selectively in key insulin target tissues including fat, liver and skeletal muscle. Moreover, and consistent with the endogenous PEPCK gene, the antisense transgene was not expressed embryonically, but rather in the early neonatal period (Moxham et al. 1993B). This late expression prevented potentially deleterious effects from antisense-mediated suppression of fetal $G_{i\alpha 2}$ levels in utero. The transgenic mice generated showed a reduction in growth, significantly smaller skeletal frame and a runted phenotype compared to control littermates, but still developed in normal proportions (Moxham and Malbon 1993). Measurement of $G_{i\alpha 2}$ protein levels showed effective suppression in insulin target tissues (fat, skeletal muscle and liver) but normal levels in other tissues such as brain. Intriguingly, analysis of the conditional $G_{i\alpha 2}$ antisense mice in four founder lines revealed insulin resistance similar in many ways to patients with NIDDM (Moxham and Malbon 1996). Glucose tolerance and other insulin-dependent responses such as GLUT4 transporter recruitment, glycogen synthase activation and counter-regulation of $\beta$-adrenergic responses were all impaired. There was also an observed increase in protein-tyrosine phosphatase (PTP) activity in the liver, muscle and adipose tissues and attenuation of the insulin-stimulated tyrosine phosphorylation of IRS-1 in adipose tissue. Hence, the loss of $G_{i\alpha 2}$ proteins clearly had a profound effect on the insulin responsiveness in vivo.

The PEPCK- $G_{i\alpha 2}$AS mouse provides a genetic link between the G-protein $G_{i\alpha 2}$ and insulin receptor signalling cascades but certain potential caveats remain. First, expression of the PEPCK-$G_{i\alpha 2}$ antisense sequence may have effects on cellular function
unrelated to the suppression of $G_{\text{ia2}}$. Moxham and Malbon (1993) described transcript levels of PEPCK-$G_{\text{ia2}}$ antisense were extremely low and endogenous expression of PEPCK was not altered. But even with low transcript levels, they showed significant depletion of $G_{\text{ia2}}$ protein. This leads to questions about how they obtained such significant levels of depletion of the $G_{\text{ia2}}$ proteins in their system. In general, a high ratio of antisense to sense RNA is needed to deplete endogenous levels of a targeted protein. Their system described the levels to be about one-tenth the antisense expression compared to endogenous mRNA expression. This would be a very potent antisense mechanism to target and deplete $G_{\text{ia2}}$ proteins and with such a potent antisense, other non-specific proteins would be targeted and inhibited even without a perfect match. Even though use of the PEPCK promoter avoids developmental problems related to prenatal $G_{\text{ia2}}$ depletion, low promoter activity might still occur in utero. Early low expression of the $G_{\text{ia2}}$ antisense, with such a potent inhibitor action, could lead to non-specific alterations in cell signalling, which may indirectly affect insulin action.

A second concern about this in vivo model is the ability of $G_{\text{ia2}}$ replacement to "rescue" insulin resistance that was not demonstrated. The model therefore would support a role for $G_{\text{ia2}}$ for normal function and responsiveness of insulin in target tissues, but it does not. This would be significant, as restoration of the insulin response with wild-type $G_{\text{ia2}}$ proteins would better solidify the conclusion that $G_{\text{ia2}}$ proteins have a prominent role in insulin signalling. If the insulin response was not increased with the addition of wild-type $G_{\text{ia2}}$ proteins, then the insulin resistance may have resulted not
from depletion of $G_{i2}$ protein, but rather from a secondary effect related to antisense expression or the chromosomal integration site of the transgene.
A. 9. Strategies and Rationale

Pertussis toxin and "G protein antisense" studies have indicated a functional role of Gia2 proteins in the insulin-signalling pathway. However, due to the variable effects reported for PTX and the complexity of in vivo models, we used a molecular approach to explore the role of Gia2 in insulin signalling with a cell culture model. In this system we could manipulate and study the function of Gia2 on insulin signalling in a more quantitative manner and over a shorter time frame. L6 skeletal muscle cells were chosen for several reasons. First, L6 cells are a well characterized insulin responsive line in which both mitogenic and metabolic pathways can be regulated. Second, L6 cells are responsive to a number of hormones and growth factors, which allows assessment of the specificity of changes in insulin responses when Gi levels are manipulated. Thirdly, this system permits the analysis of "rescue" by replacement of wild-type endogenous Gi in Gi depleted cultures, a caveat not readily addressed in the antisense mouse thereby offering an additional advantage over the more complex in vivo model. Lastly, insulin responses are mediated by the endogenous insulin receptor, rather than by overexpressed recombinant receptors - frequently the case in studies using CHO or Cos cells, recombinant insulin receptors may lead to non-specific insulin responses.

One method for examining Gi effects is the use of several Gia2 mutants (Gia2 dominant negative or activated mutant) in co-transfection experiments with insulin responsive promoter constructs to determine if we can manipulate insulin responsiveness in our L6 wild-type cells. We will examine the effects of these mutants on other receptor tyrosine kinase responses (e.g. PDGF). We will also create an in vitro cell model with L6 skeletal muscle cells expressing a full-length Gia2 antisense RNA and compare the
hormone response to parental lines. A tetracycline-responsive promoter will control expression of the $G_i$ antisense RNA. Based on the transgenic studies of $G_{i\alpha 2}$ function, we hypothesize that in L6 myocytes, $G_{i\alpha 2}$ can regulate insulin responsiveness and that impaired $G_{i\alpha 2}$ function will contribute to an increase in insulin resistance. By looking at the effects of insulin on specific transcription, we hope to further the understanding of how $G_{i\alpha 2}$ proteins may contribute to the insulin response at either the receptor level or downstream in the signalling pathway.
B. Materials and Methods
B. 1. Materials

Cell culture reagents were purchased from GIBCO/BRL (Life technologies, Burlington, Ont. Can), included are Ham’s F-10 Nutrient Mixture, fetal bovine serum (FBS), penicillin G/streptomycin sulphate solution (5000 units/mL: 5000 mg/mL), trypsin (10X), Geneticin, trypan blue and Benchmark prestained protein markers. Alpha Modified Eagle Medium (αMEM) was purchased from Cellgro (Va, Ak. USA). Insulin was received from Lilly Canada. Restriction endonucleases and ligase were purchased from MBI Fermentas (Flamborough, Ont. Can), New England Biolabs (NEB) (Mississauga, Ont. Can) and Boehringer Mannheim (Laval, Que. Can). Luciferin and hygromycin B were purchased from ICN (Costa Mesa, Ca. Can). Wortmannin, rapamycin, poly-L-ornithine, lysophosphatidic Acid (LPA), 12-O-tetradecanoylphorbol-13-acetate (TPA), Platelet Derived Growth Factor-BB (PDGF-BB) were all purchased from Sigma-Aldrich Canada (Oakville, Ont. Can). PD98059 and SB 203580 was purchased from Calbiochem (San Diego, Ca. USA). Glycerol and DMSO were purchased from Caledon Laboratories (Georgetown, Ont. Can). Agarose and acrylamide was purchased from Bioshop Canada. All primary and secondary antibodies were purchased from Santa Cruz (Santa Cruz, Ca. Can) as well as the western blot Luminol (ECL) Reagent. The nitrocellulose membrane Hybond-C was purchased from Amersham Life Science (Oakville, Ont. Can). Our SRE/TK/Luc and TK/Luc promoters were from J. Pessin (University of Iowa). The “tet-operator” constructs were obtained from M. Gossen and H. Bujard (University of Heidelberg). Paul Albert (University of Ottawa) provided the Gio2wt and the Gio2*(Q205L) activated mutant was from H. Bourne (University of San Francisco). The Gio2(S48C) dominant negative was from M. Simon (California Institute of Technology).
B. 2. Bacterial Culture and Recombinant Techniques

B. 2.1. Enzymatic Manipulation of Plasmid DNA

The restriction enzyme digests were performed under standard conditions in a 37°C water bath for one hour. The reaction mixture contained 2 ul of plasmid DNA, 1 ul of restriction enzyme, 1 ul of 10x reaction buffer and 6 ul of distilled autoclaved water. For partial digests, the amount of time for the reaction to take place and enzyme concentration were varied until the conditions were optimal.

For ligation reactions, the end of the vector DNA was dephosphorylated with calf intestinal alkaline phosphatase (CIAP). The CIAP was added for 5 minutes after the restriction digest reaction and the dephosphorylated DNA was immediately loaded into a 0.8% low melt agarose gel (BioRad). The fragment and vector were isolated from the low melt agarose gel under low-wave UV light and placed in a 65°C water bath for 10 minutes. The vector was diluted 20 fold with distilled water and the ligation reaction was setup with 1 ul of T4 ligase, 1 ul of ligase buffer, 1 ul of vector, 1 ul of fragment and 6 ul of distilled water. All ligation mixtures were incubated overnight at 14°C. The next day, the ligated construct was transformed into competent E. coli cells (DH5α strain).

B. 2.2. Transformation of DNA into Competent Cells

The plasmid DNA was mixed with 50 ul of bacterial DH5-α competent cells and allowed to sit on ice for 30 to 45 min. The mixture was heat shocked for 2 minutes in a 42°C water bath. After, the cells were placed back on ice and 450 ul of 1x LB with 20 mM glucose was added. The cells were incubated at 37°C for 1 hour with mild shaking and 200 ul were spread onto a LB agar plate containing 100 ug/ml ampicillin and
incubated overnight at 37°C. The next day, single colonies were selected and the plasmid DNA was isolated and purified.

B. 2.3.  PCR Sequencing

For sequencing reactions, the DNA sequence of interest was cloned into a vector containing a Universal Primer sequence. The manual sequencing was performed using the standard T7 Sequencing kit from Pharmacia Biotechnology. The T7 Sequencing Kit provided all of the solutions and reagents for dideoxy sequencing reactions with T7 DNA polymerase. Labeling of the sequence fragments was performed using 35-S labeled nucleotides.

B. 2.4.  Plasmid DNA Isolation:

a) Miniprep Isolation of DNA (boiling method)

Screening DNA clones for ligations, restriction analysis, and new plasmid clones were first isolated through miniprep DNA. The bacterial cultures were grown overnight in 3 ml of LB broth (10 g/L bacto-tryptone, 6 g/L bacto-yeast, 10 g/L NaCl) with 100 µg/ml ampicillin in a 37°C shaker. The bacterial cultures were then centrifuged at 12,000 rpm for 20 to 25 seconds at room temperature. The pellets were resuspended with 0.35 ml of ice cold STET buffer (6% sucrose, 0.5% Triton X-100, 50 nM EDTA pH 8.0 and 10 nM Tris-HCl pH 8.0). Then 25 ul of freshly prepared lysozyme solution (10 mg/ml) was added to the samples, which were then incubated for 5 minutes at room temperature. The suspensions were boiled for 40 seconds followed by immediate centrifugation at 12,000 rpm for 30 minutes at room temperature. A small white pellet was removed from the supernatant and an equal volume of isopropanol was added and the mixture was quickly
vortexed. The samples were then precipitated for 5 minutes at -20°C, after which they were centrifuged at 12 000 rpm for 15 minutes at 4°C. The supernatant was removed and 40 ul of water was added to dissolve the nucleic acid pellet. The DNA was re-precipitated with 2 ul of 10 M LiCl and 1 ml of ethanol at -20°C for 1 hour. The precipitates were then pelleted at 12 000 rpm at 4°C for 10 minutes. The supernatant was removed and the pellet allowed to air dry before it was redissolved in the appropriate amount of water i.e. 10-20 ul. The DNA was then run on an agarose gel to check the integrity.

b) **Cesium Chloride Plasmid Purification Method**

For larger amounts of DNA used in transfection experiments and for storage, a large-scale cesium chloride (CsCl) plasmid purification procedure was utilized. The method gives a large amount of pure and clean DNA to use in cell culture transfection experiments. An overnight bacterial culture was prepared in 3 ml of LB broth with 100 ug/ml ampicillin. The culture was added to 500 ml of LB with 100 ug/ml ampicillin (amp) and incubated until the culture was grown to an O.D. 600 ~ 0.6 which took approximately 6 hours. At this time 100 mg of spectinomycin was added to inhibit bacterial division but allow amplification of the plasmid copy number and the culture was incubated overnight. The next day, the culture was cooled on ice for 10 min and centrifuged twice in a 250 ml culture tube at 5000 rpm for 5 min at 4°C, discarding the supernatant after each spin. The pellet was resuspended in 5 ml of ice cold lysozyme solution (50 nM glucose, 25 mM Tris-HCl pH 8, 10 mM EDTA, 100 mg lysozyme) and transferred to a 40 ml oakridge tube and incubated for 5 min at room temperature. Then 10 ml of NaOH/SDS solution (0.2 N NaOH, 1% SDS) was added for the denaturation
process. The tube was mixed gently and allowed to sit on ice for 10 min. After 10 min, 7.5 ml of ice cold 3M/5M potassium acetate was added and mixed immediately by sharply inverting several times and placed back on ice for another 10 min. The tubes were then centrifuged at 12,000 rpm for 30 min at 4°C and the supernatant was transferred to a fresh 40 ml oakridge tube. Then 12.5 ml of isopropanol was added and incubated for 15 min at room temperature. The tubes were re-centrifuged at 12,000 rpm for another 30 min and the following supernatant discarded and the pellet air-dried for 5 min. The pellet was then dissolved in 8 ml of water for 30 min at room temperature and transferred to a 15 ml conical tube containing 8.0 g of cesium chloride powder. The solution was mixed until the cesium chloride was completely dissolved and then 0.75 ml of 10 mg/ml ethidium bromide was added. The solution was transferred to a 12.5 ml Quick seal ultracentrifuge tube (Beckman, Mississauga, Ont.), and topped off with paraffin oil before heat sealing. The tubes were centrifuged at 60,000 rpm in a Ti 70.1 rotor for 16 to 24 hours at 16°C. The resulting cesium chloride gradient separated the linear chromosomal (lower band) from the nicked plasmid DNA (upper band). The lower band was extracted with a #21 gauge needle and placed in a 15 ml conical tube. The ethidium bromide was removed with several extractions with water-saturated butanol until the liquid became colourless. The aqueous phase was transferred to a 40 ml oakridge tube and the volume was doubled with water and 2 volumes of 100% ethanol was added to precipitate the DNA at -20°C for one to two hours. The DNA was pelleted at 12,000 rpm for 30 min at 4°C and the supernatant was discarded and the pellet allowed to air-dry for 5 minutes. The DNA was re-dissolved with 400 ul of water and transferred to a 1.5 ml eppendorf tube. A final precipitation was done with the addition of 20 ul of LiCl and 1 ml of 100% ethanol. The
eppendorf tube was kept at -20°C for 2 hours and then centrifuged at 12 000 rpm for 10 min. The supernatant was discarded and the pellet was reconstituted in the appropriate amount of water (i.e. 200-500 ul) and the O.D. 260 was measured for quantification.

**B. 3. Cell Culture**

The L6 myoblast cells were maintained in αMEM with 10% FBS. For differentiation experiments, the L6 cells were grown until 95% confluent and then the medium was substituted with αMEM containing 2% FBS. Cell fusion took about 3 to 5 days until greater than 95% of the cells were fused into elongated myotubes. All media were supplemented with 1% penicillin/streptomycin. The muscle cells were grown in monolayers at 37°C in a 5% CO2 humidified atmosphere. The medium was changed every 3 days or 12-24 hours prior to experimentation. The cells were harvested from tissue culture flasks by incubating them in 1x trypsin for 5 min at 37°C. They were then transferred into a sterile conical tube and pelleted by centrifugation at 1000 rpm for 5 min. The harvested cells were then immediately plated for transfection experiments or frozen down for future use in liquid nitrogen. The antisense cell lines were first reselected with αMEM with 10% FBS and Geneticin (G418, 1.2 mg/ml) and then maintained in αMEM containing 10% FBS and hygromycin B (150 μg/ml).

**B. 3.1. Cell Storage**

Cells to be stored were harvested with 1x trypsin and pelleted by centrifugation at 1000 rpm for 5 minutes. They were resuspended with freezing solution (0.5 ml FBS, 10% DMSO) and aliquoted into Nunc CryoTube Vials. The vials were frozen by placing
them at 4°C for 10 min, then –20°C for 1 hour and finally –70°C for overnight to several months. For long term storage, the vials were placed in a liquid nitrogen tank.

B. 3.2. Transient Transfection by Calcium Phosphate

After purification by cesium chloride, the plasmid DNA was transfected into our L6 skeletal muscle cell lines by the calcium phosphate transfection method described by Sambrook et al. (1989). The DNA (luciferase reporter constructs +/- expression vectors) was aliquoted into luminometer tubes and 100 μl/plate of 2 M calcium chloride was added. The tubes were quickly vortexed to ensure complete mixing. Then the tubes were slowly vortexed while equal volumes of 100 μl/plate of 2x HEBs (16.4 g NaCl, 11.9 g HEPES, 0.21 g Na2HPO4, 800 ml H2O, pH 7.05) was added dropwise into the solution. The precipitation reaction was allowed to run for 20 min until the solution turned slightly cloudy. Meanwhile, during the precipitation reaction, the medium was aspirated from the plated cells and replaced with 1.8 ml of Ham’s F-10 with 8% FBS. Following the precipitation reaction, 200 μl of the DNA precipitate was added dropwise to the plates and mixed to ensure uniform distribution. The plates were then incubated for 4 hours at 37°C. Following the incubation, the plates were glycerol-shocked for 3 min.

The glycerol shock consisted of aspirating the media and replacing it with 1 ml of Ham’s F-10 with 15% glycerol for 3 min. After 3 min, the cells were washed once with Ham’s F-10 with 0% FCS and incubated overnight with αMEM, 0% FBS at 37°C. The cells were then harvested and luciferase activity measured as described in section (Treatments and Harvesting) below.
B. 3. 3. Poly-L-Ornithine (PLO) Transfection

Transfection of L6 myotubes was performed using the protocol set out by Robey et al. (1996). The cells were mixed with 2 ml of αMEM with 2% FCS, 10-15 ug/plate total plasmid DNA (luciferase reporter constructs +/- expression vectors), 30 ug PLO (10 ug/ml) and incubated at 37°C for 5 to 6 hours. After the incubation, the cell medium was aspirated and replaced with 1 ml of αMEM with 2% FBS and 25% DMSO for 5 min. After the transfection, the DMSO was removed and the cells were washed twice with PBS and incubated in serum-free αMEM overnight at 37°C. The cells were then treated, harvested and luciferase activity measured as described below.

B. 3. 4. Treatments and Harvesting

After transfection, the L6 cells were allowed to recover overnight. Cells were then equilibrated with inhibitor agents for 1 hour and hormone-treated for 3 to 5 hours. After the treatments, the medium was aspirated and the cells were harvested in 0.25 ml of ice-cold Harvest Buffer (50 mM Tris/MES (ICN) pH 7.8, 1 mM DTT, 0.1% Triton X-100). The cells were scraped off the plates with a rubber policeman and transferred to a 1.5 ml eppendorf tube. The cells were then lysed by vortexing for 10 seconds and centrifuged for 10 min at 12 000 rpm at 4°C. The supernatant was mixed with 15 ul of luciferase cocktail (0.75 M Tris/MES pH 7.8, 10 mM Mg Acetate, 6 mM ATP) and assayed using the Berthold Luminometer (Lumat LB 9501; Fisher) for luciferase activity.

B. 3. 5. β-Galactosidase Assay

For use as a transfection control, a β-galactosidase reporter gene under the control of the rous sarcoma virus-long terminal repeat construct (RSV-LTR) was co-transfected
into the L6 wild-type cells. After harvesting, the cell lysate was used to measure the β-gal levels. 90 ul of cell lysate was mixed with 3 ul 100x Mg buffer (100 ul 0.01 mM MgSO₄, 225 ul dH₂O, 175 ul β-mercaptoethanol), 201 ul 0.1 M NaHPO₄ buffer (4.416 g NaH₂PO₄, 23.86 g Na₂HPO₄, 250 ml dH₂O), and 66 ul o-Nitrophenyl-B-D-Galactopyranoside (ONPG) and vortexed. The tubes were incubated at 37°C for 30 min. to overnight until a colour change was detectable. Each sample was then read at 405 nm visible light on a plate reader.

**B. 3. 6. G₁ Antisense cell lines**

To create stable L6 lines, in which G₁ antisense RNA could be regulated under the control of tetracycline, parental cells were grown in tissue culture plates (150 mm) until approximately 70-80% confluent. They were transfected using the calcium phosphate technique (see above) with our tTA-VP16 fusion protein expression vector, which also contained a G418 resistance gene. The cells were allowed to recover for two days after transfection before the addition of Geneticin (1.2 mg/ml) to the medium. Then the cells were grown for 2 weeks until 100% of the L6 cells on the control plate cells had died and single foci of cells could be seen under a light microscope. Groups of up to 100 cells of the L6 stable line clones were isolated and removed using cloning rings. The edge of the ring was covered with vaseline and then placed over a colony of lightly trypsinized cells and the cells were removed with a glass pipette and placed in a 35 mm culture dish. The cells were then grown in αMEM with Geneticin, harvested and prepared for storage in liquid nitrogen. The cells were tested with a tet-operator luciferase construct to show which line had the greatest activity of tTA-VP16 fusion proteins. Once the cell line was selected, the stable line selection procedure was repeated using a second construct, which
contained our antisense sequence and a hygromycin resistant gene. The cells were selected using hygromycin [150 ug/ml] and individual resistant clones were expanded and tested for G1 protein expression using Western blot techniques.

**B. 3.7. Quantifying cell growth rate**

To measure the growth rates of the antisense L6 cells in comparison with parental clones, cells were visually counted with a hemacytometer. Cells were harvested from tissue culture flasks by incubating them in 1x trypsin for 5 min at 37°C. They were then transferred into a sterile conical tube and pelleted by centrifugation at 1000 rpm for 5 min. The pellets were then diluted in 1 ml of αMEM media at a ratio of 1:1 or 1:10 with trypan blue depending on the size of the pellet and vortexed for even mixing. Different dilution factors were used depending on the density of the cell population and the dilution factors were taken into account in the calculations. Then 10 ul of the mixture was loaded into each of the two compartments on the hemacytometer. Under a light microscope, the cells were counted in 8 different quadrants and averaged and the total number of cells was calculated. The cells were diluted and plated in a 60 mm culture dish at a density of 20 000 cells per plate in αMEM media with 10% FBS. The cells were then allowed to grow, harvested and counted at the pre-determined time point using the methods described.

**B. 3.8. Quantifying Protein in Cell Extracts**

The L6 cells were grown in a 10 cm culture dish until approximately 95% confluent and the protein extracts were prepared by the following procedure. The cells were washed three times with ice cold PBS and incubated on ice for 10 min with 1 ml of
RIPA buffer solution (150 mM NaCl, 50 mM Tris-HCl, 50 mM Na₂PO₄•7H₂O, 0.25% sodium deoxycholate, 0.1% Nonidet P-40, containing 1 mM Na₃VO₄, 1 mM NaF, pH 7.4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 mM EDTA, and 5 mM EGTA). Then the cells were harvested using a disposable cell scraper and transferred to a 1.5 ml eppendorf tube. Cellular debris was pelleted by centrifugation at 12000 rpm for 15 min at 4°C and the supernatant was transferred to a fresh 1.5 ml eppendorf tube. Protein concentrations were determined using Pierce BCA Protein Assay Reagent A and the samples were stored at -20°C.

B. 3.9. Western Blot Analysis

The Western blot experiments were performed using the Santa Cruz ECL Western blotting system described in the Santa Cruz Biotechnology protocols. The cell lysates were resolved on either 12.5% or 15% SDS-PAGE mini-gels (BIORAD) along with Gibco BRL pre-stained protein markers. The proteins were transferred from the polyacrylamide gel onto nitrocellulose membranes using a BIORAD transfer apparatus. The chamber was filled with 1 L of ice cold transfer buffer (15.1 g Tris base, 72 g glycine, 20% methanol) and the transfer reaction was carried out for 1-2 hours at 100 mA on ice. The nitrocellulose membrane was rinsed with water and stained with ponceau red (Sigma) for 10 min to check for even loading, even transfer and numbering of lanes. The nitrocellulose membranes were incubated with blocking buffer, 5% milk powder in TBST (20 mM Tris base, 137 mM NaCl, 1 M acetic acid, 0.1% Tween-20, pH 7.5) overnight on a shaker at 4°C. The next day, the membranes were rinsed and re-incubated with blocking buffer containing primary antibody (1:2000 dilution) overnight on a shaker at 4°C. The following day the membranes were washed 3 times with TBST for 15 min at
room temperature with constant agitation. Then the membranes were incubated with secondary antibody (e.g. 1:2000 dilution of anti-rabbit immunoglobulin-G horse radish peroxidase, Santa Cruz) at room temperature for 1 hours and then washed another 3 times with TBST for 15 min at room temperature. After a final rinse with PBS at room temperature for 10 min, the membranes were incubated with a mixture of equal volume of detection reagents for 1 min, wrapped in Saran Wrap and exposed to X-ray film for 1 second to 20 minutes.

B. 3. 10. Immunoprecipitation

The L6 cells were grown until 95% confluent and washed three times with ice cold PBS. They were then lysed and collected in 1 ml of cold RIPA buffer (see above) and transferred to a 1.5 ml eppendorf tube. The debris was removed by centrifugation at 12 000 rpm at 4°C for 10 min. The soluble cell lysates were then “pre-cleaned” with protein A-agarose (Santa Cruz Biotechnology) for 1 hour at 4°C. The beads were then centrifuged and discarded and the supernatant was incubated overnight with specific antibodies and protein A-agarose at 4°C. The next day, samples were centrifuged at 12 000 rpm for 10 min and the pellets were washed with 500 ul of ice-cold RIPA buffer. The washing was repeated 4 times with 10 minutes of washing and 10 minutes of centrifugation each time at 4°C. Finally, the precipitated samples were suspended in 20 ul of loading buffer (NEB) and boiled for 5 min. The proteins were resolved in a SDS-PAGE (12.5 or 15%) gel and analyzed through the standard ECL blotting system (see Western blot analysis).
B. 3. 11. Statistical analysis

All values are expressed as mean ± standard error. Data were subjected to analysis of variance (ANOVA) and probabilities of $p < 0.05$ were considered statistically significant.
C. Results
C. 1. Regulation of the promoter luciferase reporter constructs by insulin

To test the insulin response in a transcriptional readout and examine the effect of G_{ia} proteins on the insulin-signalling pathway, we used insulin responsive promoters fused to the firefly luciferase gene. One such promoter is derived from the c-fos gene. The c-fos promoter is highly sensitive to transcriptional activation by insulin in Chinese Hamster Ovary fibroblasts (Stumpo and Blackshear 1988) and regulation can be mapped to the serum response element or SRE. We therefore determined whether this well-defined transcriptional target could serve as a sensitive end-point for measuring insulin and growth factor activation in L6 cells. To obtain an adequate baseline of promoter activity, these studies were performed in the context of the Herpes thymidine kinase gene promoter (TK). The conditions were optimized using low serum conditions before the addition of hormone treatment. Transient transfections of the SRE reporter construct into L6 myoblasts demonstrated an approximate 5-fold insulin induction of luciferase activity compared to untreated controls (Fig 5A). In contrast, a TK/Luc reporter construct showed no response to insulin stimulation in the L6 cell line. The insulin activation maximally occurred after approximately 3 hours and luciferase activity remained elevated for about 24 hours (data not shown). The insulin response is also dose dependent with maximal induction about 100 nM (data not shown; see also Fig 18A). The SRE response to insulin in differentiated L6 myotubes showed the same insulin response of approximately 5-fold at 100 nM of insulin (Fig 5B). This demonstrates that insulin is able to activate the c-fos SRE in both proliferating and differentiated myocyte cultures.
Figure 5: Insulin regulation of the SRE/TK and TK promoter in both L6 myoblasts and L6 myotubes
L6 myoblasts (Fig 5A) and L6 myotubes (Fig 5B) were transfected by CaCl₂, using 1 ug/plate of SRE/TK/Luc and TK/Luc reporter constructs. After overnight serum starvation in αMEM (0% FCS) the cells were treated for 3 to 5 hours with 10 or 100 nM insulin and harvested for measurement of luciferase activity. The values represent the mean +/- S.E.M. of a minimum of 4 separate experiments. Basal expression was typically in the range of 10000 RLU and above for both promoters.
Hexokinases, which catalyze the phosphorylation of glucose to glucose 6-phosphate were originally categorized by Katzen et al. (1965). Insulin has been shown to regulate transcription of the hexokinase II (HEXII) gene in L6 cells (Printz et al. 1993). We used the PCR to amplify a 600 bp fragment of the rat HEXII gene containing the promoter region and ligated the promoter fragment to a luciferase reporter gene sequence. Transient transfections of the HEXII promoter into L6 cells showed approximately 4 to 6 fold stimulation in differentiated L6 myotubes. In contrast there was no significant induction of the HEXII promoter in L6 myoblasts by insulin under the same treatment conditions (Fig 6). The HEXII promoter showed equally strong basal activity in both L6 myoblasts and myotubes with relative light units above 5000 when read in the Bertold Luminometer.

C. 2. Alternate signalling pathways in insulin-regulated transcription

Signalling pathways in insulin regulation of the SRE/TK and HEXII promoters were defined using inhibitors to block specific steps in the insulin response. For a review of each inhibitor see sections A. 5. 2., A. 5. 3. and A. 5. 4.

These specific inhibitors block distinct insulin regulated events and are used to define the pathways that mediate insulin regulated transcription. Dose curves with each inhibitor determine the optimized amounts of inhibitors to be used. After the L6 myoblasts or myotube cultures were transfected with reporter constructs, they were allowed to recover overnight and each inhibitor was equilibrated for a minimum of 30 minutes to 1 hour before insulin treatment. As shown in Fig 7, with the SRE/TK
Luciferase Activity (fold stimulation)

Figure 6A: Luciferase Activity (fold stimulation)

Figure 6B: Insulin Regulation of the HEXII and TK promoter in L6 myoblasts and L6 myotubes

Figure 6: Insulin regulation of the HEXII and TK promoter in L6 myoblasts and L6 myotubes.

Luciferase Activity (fold stimulation)

Range of 5000 RLU and above for both promoters. Basal expression in relative light units was very typical in these experiments. Basal expression in relative light units was very typical in these experiments. The values represent the mean ± SEM of a minimum of 4 separate experiments. The values represent the mean ± SEM of a minimum of 4 separate experiments. 10 or 100 nM insulin and harvested for measurement of luciferase activity. Overnight serum starvation in DMEM, cells were treated for 3 to 5 hours with 5 μg/plate of HEXII/luc and TK/luc reporter constructs. After overnight serum starvation in DMEM, cells were treated for 3 to 5 hours with 5 μg/plate of HEXII/luc and TK/luc reporter constructs. After overnigh overnight serum starvation in DMEM, cells were treated for 3 to 5 hours with 5 μg/plate of HEXII/luc and TK/luc reporter constructs.
Fig 7: Wortmannin and PD 98059 inhibit insulin stimulation of the SRE/TK/Luc promoter

L6 myoblasts were transfected with the SRE/TK promoter construct and serum starved overnight. The next day, they were incubated for 30 min with vehicle control, 100 nM wortmannin, 10 ug/ml PD 98059 or 100 nM rapamycin and then stimulated with 100 nM insulin for 3 hours. The cells were harvested and assayed for luciferase activity. The results are given as means +/- S.E.M. of 4 experiments performed in duplicate. P values were done by ANOVA: single factor.
promoter construct, stimulation by 100 nM insulin is inhibited by the addition of 100 nM wortmannin or 10 uM PD98059, while 100 nM rapamycin has no effect. These results contrasted sharply with those using the HEXII promoter construct (Fig 8). Insulin stimulation of the HEXII promoter is inhibited by 100 nM of wortmannin and 100 nM of rapamycin, whereas 10 uM of PD98059 has no effect. This shows that the SRE and HEXII promoter are both stimulated by insulin, but by different signalling pathways. This is most evident with the rapamycin inhibitor. The rapamycin inhibitor completely blocks insulin stimulation of the HEXII promoter while there is no affect on the SRE. This divergence in transcriptional regulatory pathways allowed us to address whether manipulation of Gi function would preferentially affect insulin regulation of MAPK or S6 kinase dependent genes.
Fig 8: Wortmannin inhibits and rapamycin blocks insulin stimulation of the HEKII/Luc promoter
L6 myotubes were transfected with the HEKII promoter and serum starved overnight. The next day, they were incubated for 30 min with DMSO vehicle control, 100 nM wortmannin, 10 μg/ml PD98059 or 100 nM rapamycin and then stimulated with 100 nM insulin for 3 hours. The cells were then harvested and assayed for luciferase activity. The results are given as means +/- S.E.M of 3 experiments done in duplicate.
C. 3. Regulation of SRE/TK and HEXII gene promoters by PDGF

Platelet-derived-growth-factor (PDGF) has been shown to be a stimulator of the p42/p44 MAP kinase pathway (Conway et al. 1999, Duckworth and Cantley 1997). Treatment of L6J1 myoblasts with PDGF-BB increased the rate of DNA synthesis and stimulated cell proliferation (Jin et al. 1991). In Fig 9, 100 ng/ml of PDGF stimulated the SRE construct approximately 6 to 8-fold after 3 hours. The 100 ug/ml PDGF is inhibited by both 10 uM PD98059 and 100 nM wortmannin and 100 nM of rapamycin has no effect on PDGF stimulation as expected (Fig 9). PDGF also stimulates the HEXII promoter in the same manner as insulin in L6 myotubes and is blocked by the same inhibitors (wortmannin and rapamycin) under the same conditions as previously described (Fig 10).
Wortmannin and PD 98059 block PDGF stimulation of the SRE/TK promoter
L6 myoblasts were transfected with the SRE/TK promoter and serum starved overnight. Cells were then incubated for 30 min with vehicle control, 100 nM wortmannin, 10 ug/ml PD98059, 100 nM rapamycin or 100 nM SB 203580 and then stimulated with 100 ug/ml PDGF for 3 hours. The cells were then harvested and assayed for luciferase activity. The results are given as means +/- S.E.M of 3 experiments performed in duplicate. ( p < 0.05, ANOVA)
Fig 10: Wortmannin and rapamycin block PDGF stimulation of the HEXII/Luc promoter

L6 myotubes were transfected with the HEXII promoter construct and serum starved overnight. Cells were then incubated for 30 min with vehicle control, 100 nM wortmannin, 10 ug/ml PD98059, or 100 nM rapamycin and then stimulated with 100 ug/ml PDGF for 3 hours. The cells were then harvested and assayed for luciferase activity. The results are given as means +/- S.E.M of 2 experiments performed in duplicate.
C. 4. Molecular manipulation of Gᵢ function in L6 cells

C. 4.1. Dominant Gᵢα2 mutants

To examine the role of Gᵢ proteins in insulin signalling, Gᵢ mutant expression vectors were co-transfected into L6 cells together with our reporter constructs and the effects on insulin signalling monitored.

A Gᵢα2(S48C) (or Gᵢα2DN) is a dominant negative mutant that interacts with Gᵦᵢ subunits and blocks GDP dissociation from Gᵢα2 subunit (Slepak et al. 1995). The Gᵢα2DN was transiently co-transfected into our L6 cells with the SRE reporter plasmid and luciferase activity was examined. The empty parental vector pCIS was used as a control, and also to balance the total amount of DNA in Gᵢα2DN transfected plates. The cells were allowed to recover after transfection and serum starved overnight followed by a 3 hour insulin (100 nM) treatment. As Fig 11A indicates, higher levels of the dominant negative (greater than 8 ug DNA per plate) caused up to a 70% decrease in insulin stimulation. The experiment was repeated a minimum of 4 times in duplicate for statistical analysis. The insulin response was not affected by the total amount of transfected DNA as shown with the control vector. Moreover the β-gal assay showed that transfection efficiency's were essentially equal under then conditions used (Fig 11B).

The Gᵢα2 mutation glutamine 205 to leucine, inhibited the activity of the Gᵢα2 GTPase resulting in a gain-of-function GTP-bound α subunit, Gᵢα2(Q205L). We co-transfected the Gᵢα2(Q205L) into L6 myoblasts in the same DNA amounts as before, together with our promoter construct and the luciferase activity was examined. Unlike the empty vector control and in sharp contrast to the dominant negative Gᵢα2 mutant,
increasing amounts of $G_{i22}(Q205L)$, caused an elevation in SRE transactivation that was comparable to the insulin response, Fig 11A. In the absence of the SRE, the TK promoter was unresponsive to either $G_{i22}(Q205L)$ or insulin stimulation, indicating that regulation of luciferase activity was conferred by a common promoter element.

To examine the effect of $G_{i22}$ dominant negative in differentiated muscle cells, L6 myotubes were co-transfected with either the SRE/TK or HEXII reporter gene together with a low (1 ug/plate) or a high dose (10 ug/plate) of our $G_{i22}$DN DNA construct. The results in Fig 12 are consistent with those in myoblasts and show that at 10 ug of $G_{i22}$DN per plate, the insulin stimulation is significantly diminished. The results indicate that blocking the activation of $G_{i22}$ with a dominant negative inhibits the insulin stimulation of the SRE/TK and HEXII promoters, whereas activating the same $G_i$ protein caused an elevation in SRE transactivation.
Fig 11A

Control vector  G\(\alpha_2\) (S48C)  G\(\alpha_2\) * (Q205L)
Dominant Negative  Activated Mutant
Fig 11B

- basal
- insulin

β-Gal units (ratio)

<table>
<thead>
<tr>
<th></th>
<th>Vector Control (10 ug/plate)</th>
<th>Gicα2DN (1 ug/plate)</th>
<th>Gicα2DN (10 ug/plate)</th>
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<tr>
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<tr>
<td>Insulin</td>
<td>0.8</td>
<td>0.7</td>
<td>1.4</td>
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**Fig 11A: Manipulation of the insulin response in L6 cells by Gia2 dominant negative or Gia2 gain of function mutants**

Myoblasts were transiently co-transfected with the SRE/TK or TK reporter constructs (1ug) and expression vectors (Gia2DN or Gia2*) from a range of 1 ug to 16 ug per plate as indicated by the dose triangles. An empty vector pCIS or pcDNA 3.1 were used for controls and to balance the amount of total DNA transfected. The cells were serum starved overnight before treatment with 100 nM insulin for 3 to 5 hours. They were then harvested and assayed for luciferase activity. The data are presented as mean values +/- S.E.M of a minimum of 3 separate experiments.

**Fig 11B β-gal assay as a control for co-transfection**

1 ug per plate of the beta-gal construct was co-transfected into the L6 cells along with the reporter gene and the indicated vector. The cells were serum starved overnight before treatment with insulin, then harvested and assayed for β-gal activity. The data presented are a mean +/- S.E.M. of a minimum of 3 separate experiments.
Fig 12 A

Fig 12 B

The Gα2DN inhibits insulin induction of the SRE/TK and HEXII promoter luciferase constructs in L6 myotubes

Cells co-transfected with 1 ug of the SRE promoter construct (Fig 12A) or 5 ug of the HEXII promoter construct (Fig 12B) together with 1 ug or 10 ug of the Gα2DN. The cells were serum starved overnight followed by insulin treatment for 3 hours before harvesting and measurement of luciferase activity. The results are presented as mean values +/- S.E.M. of at least three separate experiments.
C. 4. 2.  Loss-of function of $G_{ia2}$ - Characterization of L6 cell lines expressing $G_{ia2}$ antisense RNA

A full-length $G_{ia2}$ antisense cDNA construct was used to specifically deplete cells of $G_{ia2}$, a strategy used successfully in previous studies with GH4 pituitary tumor cells (Liu et al. 1999). To permit regulation of $G_{ia2}$ antisense expression we used a tetracycline-sensitive system, which allowed us to induce or repress the expression of the antisense RNA in L6 cell lines in the absence or presence of tetracycline respectively. Tetracycline binds to and inhibits the DNA (tet-operator) interaction of tTA-VP16. In the absence of tetracycline, this fusion protein, which contains the DNA binding domain of the E.coli tet repressor fused to the transactivation domain of Herpes VP16 protein, activates $G_{ia2}$ antisense mRNA synthesis via tet operator elements in the antisense expression vector. The tTA-expressing L6 cell line was established previously in our laboratory, by creating G418-resistant L6 cells expressing high levels of the tTA fusion proteins. Those cells were used as parental cells in a secondary round of selection. A tTA-expressing cell clone was transfected with our antisense construct using standard Calcium Phosphate methods (see Methods) and then challenged with hygromycin (100 ug/ml). Antibiotic resistant foci were cloned, expanded and tested with a $G_{ia2}$ antibody to look for suppression of $G_{ia2}$ proteins. In all, 13 lines were tested; the results showed that 8 of 13 lines had some degree of $G_i$ protein suppression (Fig 13A). The range of suppression was from 0% to almost 70% of the level in L6 parental cells (Fig 13C). The western blots were also tested with an ERK 1/2 antibody and a $G_o$ antibody (Fig 13A & 13B). No significant decrease in the levels of either of these proteins was observed. Our data therefore demonstrate that antisense RNA targeting in L6 clones was specific to $G_i$ proteins only. As $G_o$ is in the $G_i$ sub-family of $\alpha$ subunits (~88% identical) these results indicate that full-length antisense targeting is an effective means to suppress expression of individual members of the $G_\alpha$ family in these insulin target cells.
<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
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<tbody>
<tr>
<td>IB:</td>
<td>L6 tTA-4</td>
<td>1 2 3 4 5 7</td>
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<tr>
<td>Gi(\alpha)</td>
<td></td>
<td></td>
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<tr>
<td>ERK</td>
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<th>Control</th>
<th>G(\alpha)2 antisense clones</th>
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<tr>
<td>IB:</td>
<td>10-3 8</td>
<td>9 10 11 12 13 14</td>
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<tr>
<td>Gi(\alpha)</td>
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<tr>
<td>ERK</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig 13B

Gio2 antisense clones

IB:

Gioα

Go

Fig 13C

Densitometry units (fold ratio)

vs. tTA-4 parental L6 line

10-3 Hyg  Ga-AS2  Ga-AS3  Ga-AS4  Ga-AS7  Ga-AS10  Ga-AS13  Ga-AS14  tTA-4
Fig 13A: The expression of Gia2 antisense mRNA inhibited the expression of Gia proteins in the L6 tTA-expressing muscle cell line

Thirteen hygromycin resistant clones were isolated and tested for Gi depletion. 10-3 represents a clone carrying hygromycin resistant vector minus Gia2 sequences construct used as a negative control. Additional controls used were the tTA-expressing parental cell line and the L6 wild-type cell line. The cellular lysates were denatured in SDS containing buffer, subjected to electrophoresis on a 12% SDS polyacrylamide gel and proteins were transferred onto a nitrocellulose membrane (BioRAD). Membranes were probed with Gia or ERK antibody according to manufacturers specifications (Santa Cruz Biotech).

Fig 13B The Goc-specific antibody was used to show that antisense RNA expression had no effect on a closely related member of the Goc family.

Fig 13C Standard densitometry analysis of the Gia signal was performed on several of the antisense cell lines. The data was shown as one representative of several western experiments
C. 5. Measuring growth rates in G\textsubscript{ia2} antisense cell lines

To determine whether G\textsubscript{ia2} antisense RNA expression has an affect on cell proliferation, we determined the relative growth rates of the stable L6 clones. We compared growth rates of two highly depleted cell lines, Ga-AS3 and Ga-AS14, to L6 wild-type and tTA parental myoblasts. The cells were initially plated at an even density of 20,000 per plate and their growth rates were measured over a period of several days. In Fig 14, we show on a logarithmic scale, that there were no significant differences between the control and antisense expressing cells. This would argue that G\textsubscript{i} depletion does not affect normal mitogenesis in L6 myocytes in the presence of serum. It further demonstrates that prolonged selection of clones in the presence of hygromycin and chromosomal integration of recombinant vector sequences has not affected normal growth of these cells.

C. 6. Testing basal promoter function in G\textsubscript{ia2} antisense cell lines

To examine whether our antisense cells exhibited any changes in general transcription function, they were transfected with the SRE, TK and HEXII luciferase promoter constructs. The activity of each promoter was recorded for the various cell lines. In Table 2, we show that basal luciferase light units were not significantly different when comparing the antisense cell lines to the control cell lines. The levels for a given promoter can vary considerably when comparing different clones, but that this variability does not correlate with the level of G\textsubscript{i} (see Fig 13). Moreover, inter-experimental fluctuations in basal promoter activity were anticipated due to transfection efficiency and a change in the basal readings by 30 to 40% was observed. Such differences were seen in
both our antisense cell lines and L6 control cells, and lie well within the normal parameters of our luciferase assay. This allows us to conclude that general transcription mechanisms are not altered when G\textsubscript{i} protein levels are suppressed by G\textsubscript{i} antisense RNA expression.
Fig 14  Comparison of growth rates between control and Ga-AS antisense cells
The control and antisense cells were plated onto a 60 mm cell culture dish at an initial density of 20 000 cells per plate. The cells were allowed to grow in αMEM (10% FBS) until the appropriate time point within a six day period. The cells were then harvested and counted using a hemacytometer to determine the total number of cells per plate.
Table 2

<table>
<thead>
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<th>L6 Myoblasts</th>
<th>L6 Myotubes</th>
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<tbody>
<tr>
<td></td>
<td>SRE/TK/Luc</td>
<td>HEXII/Luc</td>
</tr>
<tr>
<td>tTA-4</td>
<td>74108</td>
<td>12127</td>
</tr>
<tr>
<td>L6</td>
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<td>19102</td>
</tr>
<tr>
<td>10-3</td>
<td>143502</td>
<td>n/a</td>
</tr>
<tr>
<td>Ga-AS2</td>
<td>99749</td>
<td>n/a</td>
</tr>
<tr>
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<td>41075</td>
</tr>
<tr>
<td>Ga-AS4</td>
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</tr>
<tr>
<td>Ga-AS14</td>
<td>140645</td>
<td>7139</td>
</tr>
</tbody>
</table>

Table 2  Relative light units (RLU) of basal expression of SRE/TK and HEXII promoters in L6 myoblasts and L6 myotubes
Antisense cell lines were transfected with 1 ug/plate of each luciferase promoter reporter construct. The numerical values are an average of the basal promoter activity of a minimum of 3 different experiments done in triplicate. Promoter activity varies by up to 40% in transfection experiments performed on different days. This fluctuation range is commonly observed and can be corrected by use of an internal standard (RSV-β-gal).
C. 7. Insulin responsiveness in G_{i2} antisense cell lines

The SRE luciferase construct was transfected into our antisense expressing L6 clones to test for hormone responsiveness in a G_{i} protein-suppressed background. The cells were hormone treated with 10 μg/ml LPA (Lysophosphatidic Acid), 100 nM insulin or 10 ng/ml PDGF for 3 hours. Lysophosphatidic acid stimulates the SRE promoter through a G_{i} protein-coupled receptor expressed endogenously in many cell types (Perkins et al. 1994). Each line was tested a minimum of three times and all luciferase activity determinations were done in triplicate. As shown in Fig 15A, the control lines showed approximately 5-fold increase in promoter activity in the presence of insulin or PDGF and about a 2-fold increase with LPA. Interestingly, in the antisense cell lines, the degree of inhibition appeared to correspond to the relative amount of G_{i} proteins detected by western analysis and this was verified by correlative analysis. The correlation plots Fig 15B, 15C, 15D show a good correlation (R approximately 0.9) between the amount of G_{ia} protein and SRE/TK promoter activation with both G_{i} protein coupled receptors and receptor tyrosine kinases. In lines with the greatest suppression of G_{i} proteins (approx. 70%), we saw about a 60 to 70% decrease in insulin and PDGF stimulation (e.g. Ga-AS3, Ga-AS14). The stimulation effect of LPA was almost abolished. Table 3 is our statistical analysis with each reading compared to tTA control. Values of p < 0.05 were deemed statistically significant and several of our depleted cell lines were highly significant in terms of increased resistance, especially to LPA and insulin.

For analysis of the HEXII promoter construct, two cell lines with the lowest level of G_{i}, Ga-AS3 and Ga-AS14 were chosen. Under differentiation conditions (low serum) there were no noticeable differences in myotube formation in antisense lines compared to
wild-type controls (data not shown) showing that Gi depletion did not generally interfere with the differentiation response of L6 myocytes. Control cells transfected with HEXII/Luc promoter showed a 3 to 4 fold stimulation with insulin and PDGF and about a 2-fold response to LPA. The two antisense cell lines showed significant inhibition when compared to the control line. There was about a 50 to 60% decrease in insulin and PDGF stimulation. The LPA stimulation showed only a slight decrease after a 3-hour treatment in the Ga-AS3 line (Fig 16). Whether this indicates that LPA signalling can proceed via a "non-Gi" protein in L6 myocytes as has been suggested in NIH3T3 cells (see Mao et a. 1998A) is not yet clear.

C. 8. 12-O-tetradecanoylphorbol 13-acetate (TPA) responsiveness in Giα2 antisense cell lines

TPA has been shown to be an activator of c-fos through a protein kinase C – mediated mechanism (Heinrich and Kraiem 1997). Soh et al. (1999) showed that TPA can activate PKCα or PKCe, and that SRE activation occurs through either a MEKK1-SEK1-JNK-TCF or Rho-SRF pathway. In Fig 17, we show that control cells transfected with the SRE/TK promoter, is stimulated approximately 2-fold by 100 nM TPA. There was no significant inhibition in our antisense cell lines compared to the significant inhibition of the insulin stimulation. PDGF at 100 ng/ml was used as a positive control (data not shown). The inhibition by insulin stimulation and not TPA stimulation shows us that depletion of Gi proteins in our cell line is specific to the insulin signalling pathway and other pathways, such as PKC, remains unaffected.
Figure 15A: Regulation of the SRE/TK promoter is inhibited in Gi-deficient L6 antisense cell lines

Antisense cell lines were transfected with 1 ug/plate of SRE/TK/Luc reporter construct. After overnight serum starvation in αMEM (0.5% FCS), the cells were treated for 3 to 5 hours with 100 nM insulin, 2 ug/ml LPA, or 10 ng/mL PDGF. They were then harvested for measurement of luciferase activity. The values represent the mean +/- of S.E.M. of a minimum of 4 separate experiments each done in triplicate.
Table 3

<table>
<thead>
<tr>
<th>P-Values</th>
<th>LPA</th>
<th>Insulin</th>
<th>PDGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>tTA</td>
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<td>1.0000</td>
<td>1.0000</td>
</tr>
<tr>
<td>L6</td>
<td>0.9647</td>
<td>0.9845</td>
<td>0.9686</td>
</tr>
<tr>
<td>10-3</td>
<td>0.4458</td>
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</tr>
<tr>
<td>Ga-AS2</td>
<td>0.1410</td>
<td>0.1082</td>
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</tr>
<tr>
<td>Ga-AS3</td>
<td>* 0.0084</td>
<td>* 0.0010</td>
<td>* 0.0269</td>
</tr>
<tr>
<td>Ga-AS4</td>
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</tr>
<tr>
<td>Ga-AS7</td>
<td>* 0.0145</td>
<td>0.2236</td>
<td>0.3898</td>
</tr>
<tr>
<td>Ga-AS10</td>
<td>0.0620</td>
<td>* 0.0468</td>
<td>0.6615</td>
</tr>
<tr>
<td>Ga-AS13</td>
<td>0.1608</td>
<td>* 0.0322</td>
<td>0.4432</td>
</tr>
<tr>
<td>Ga-AS14</td>
<td>* 0.0219</td>
<td>* 0.0013</td>
<td>* 0.0433</td>
</tr>
</tbody>
</table>

Table 3 Calculated p values for fold change. The fold increase in each growth factor-stimulated antisense line was compared to the tTA control cells. The values were calculated using Anova: Single Factor and p* values < 0.05 were considered statistically significant.
Fig 15B

LPA (2 ug/ml)

R = 0.9502

Luciferase Activity (fold stimulation)

Ratio of G proteins (Ga-AS /control)
Fig 15C

Insulin (100 nM)

Luciferase Activity (fold activation)

Ratio of G proteins (Ga-AS/Control)

R = 0.9246
PDGF (10 ng/mL)

Luciferase Activity (fold stimulation) vs. Ratio of G proteins (Ga-AS/control)

R = 0.9558
Fig 15B-D Correlative graphs illustrating responses of an SRE directed TK promoter to LPA (B), insulin (C), and PDGF (D) as a function of Gα protein level
The amount of Gα protein is in a ratio = 1 compared to the parental L6 line. Luciferase activity is measured 3 hours after hormone treatment. Each Gα-AS antisense expressing clone is indicated by its designated number (see Fig 13). C1 is the tTA parental control, and C2 is a hygromycin resistant empty vector control.
Figure 16: Regulation of the HEXII promoter is inhibited in Gi-deficient L6 antisense cell lines

Antisense cell lines were transfected with 5 ug/plate of HEXII reporter construct. After overnight serum starvation in αMEM with 0.5% FCS the cells were treated for 3 to 5 hours with 100 nM insulin, 2 ug/ml LPA, and 10 ng/ml PDGF. They were then harvested for measurement of luciferase activity. The values represent the mean +/- S.E.M. of a minimum of 3 separate experiments each done in triplicate. P values were using ANOVA: single factor.
Fig 17 Activation of SRE/TK by TPA is unaffected by depletion of Gi proteins. The antisense cells were transfected with 1 ug of SRE reporter and stimulated with increasing doses of both insulin and TPA for 3 hours. The cells were then harvested and luciferase activity was measured. The results are the mean values +/- S.E.M. of three separated experiments done in triplicate. The statistical significance was done using ANOVA: single factor.
C. 9.  Conditional inhibition of PDGF signalling in G\(_i\) antisense cells

Using a dose curve for both insulin and PDGF, we stimulated control L6 muscle cells and the Ga-AS14 antisense cells for three hours with increasing amounts of each hormone. In control cells, insulin stimulation of the SRE was maximum at 100 nM with no further increase even at ten fold higher hormone levels. In the G\(_i\) antisense cells, the inhibitory effect on insulin signalling was observed across the entire dose range (Fig 18A). In contrast, PDGF stimulation was significantly suppressed only at ligand concentrations at the low end of the dose curve (200 nM). At higher PDGF concentrations, the magnitude of the SRE response was comparable between control and G\(_i\) antisense cells (Fig 18B). This indicates a selective difference between the insulin and PDGF stimulated responses in G\(_i\) depleted L6 myocytes. Since both hormones activate MAPK and S6 kinase-dependent transcription, but through different RTKs, this result suggests that Gi dependence may involve early events associated with receptor activation.
Fig 18A

Luciferase Activity (fold stimulation)

basal  insulin 100nM  insulin 300nM  insulin 1uM

* p = 0.0006
** p = 0.0193
*** p = 0.0285

Control
Ga-AS14
Fig 18B

Luciferase Activity (fold stimulation)

* p = 0.0396

- Control
- Ga-AS14

basal, PDGF 10ng/ml (200nM), PDGF 100ng/ml (2uM), PDGF 300ng/ml (6 uM)
Fig 18 PDGF resistance but not insulin resistance in Ga-AS cells is ligand dose dependent

The antisense cells were transfected with 1 ug of SRE reporter construct and stimulated with increasing doses of either insulin or PDGF for 3 hours. The cells were then harvested and luciferase activity was measured. The results are the mean values +/- S.E.M. of four separated experiments done in triplicate. The statistical significance was done using ANOVA: single factor.
C. 10. Tetracycline-dependent rescue of the insulin response in Ga-AS cells

The addition of tetracycline (100 nM) to our antisense cell line inactivates the tTA-VP16 protein thereby lowering expression of Giα2 antisense RNA. This should allow the depleted Gi proteins to return to L6 wild-type levels and restore insulin responsiveness. To test the tet-responsiveness of the Ga-AS cells, we transfected a construct containing a tet-operator-containing promoter linked to a luciferase gene. The light units measured from our luciferase assay showed high levels of luciferase activity, approximately 200 000 light units in our L6 tTA expressing cells. As shown in Fig 19, after treatment with 100 nM of tetracycline, the luciferase activity was reduced by 60 to 80% in both control and antisense cells. These results demonstrate a regulatable system for controlling the promoter of the Gi-AS expression vector.

After L6 controls and Ga-AS cells were grown for 5 days in the presence of 100 nM tetracycline, the cells were harvested and a western blot with our Giα antibody was used to detect whether the Gi protein level had returned back to wild-type levels. Fig 20 shows that there was indeed an increase in the amount of Giα proteins in our antisense cell line in the presence of tetracycline, whereas the control cell lines that lacked a Gi expression cassette, showed no significant difference in Gi levels in the presence or absence of tetracycline.

Hormone responsiveness was measured in Ga-AS cells before and after tet treatment using the SRE/TK promoter construct. As shown in Fig 21, in the presence of tetracycline, the insulin and PDGF responses were restored to wild-type levels.
Restoration of the insulin response in our antisense lines show that G\textsubscript{i} proteins have a direct effect on insulin signalling. These experiments therefore identify a novel system in which the degree of insulin resistance can be regulated by altering the level of G\textsubscript{i}. 
Fig 19 Addition of tetracycline inhibits transcriptional activation by tTA proteins

The control and antisense cells were grown in the presence of 100 ng/ml of Tetracycline. They were transfected with a tet-operator/luciferase reporter vector that is activated by the tTA protein. Tetracycline inhibits tTA by preventing its binding to DNA transactivation of our tet promoter. The cells were then harvested and luciferase activity was measured. The results are the mean +/- S.E.M. of three separate experiments done in triplicate.
Fig 20A

**IB:**

<table>
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<tr>
<th>Protein</th>
<th>tTA-4</th>
<th>Ga-AS14</th>
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<tr>
<td>Actin</td>
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<td></td>
</tr>
<tr>
<td>Giα</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tet (100nM)</td>
<td>-</td>
<td>+</td>
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</table>

Fig 20B

**Densitometry**

**Ratio of Gi levels**

- no tet
- tet
- no tet
- tet

**Graph**

- Y-axis: Ratio of Gi levels
- X-axis: Densitometry
Fig 20  Tetracycline treatment restores Gi levels in antisense cell lines

The tTA parental and antisense cells were grown for up to 5 days with or without the presence of 100 ng/mL of tetracycline. They were then harvested in RIPA buffer and the cellular lysates were denatured in SDS, subjected to electrophoresis on a 12% SDS polyacrylamide gel, transferred and immunoblotted with Giα, ERK and actin antibodies according to manufacturer’s specifications (Santa Cruz Biotech) Fig 20A. Densitometry was done with the Giα western blot using standard densitometry techniques and comparing each sample to the tTA parental cell line (first lane) Fig 20B.
Fig 21

- basal
- LPA 2μg/ml
- Insulin 100nM
- PDGF 10ng/ml

* p = 0.05
** p = 0.04

Luciferase Activity (fold stimulation)

No tet | +tet (100nM) | No tet | +tet (100nM)

control | Ga-AS14
Fig 21  Addition of tetracycline restores hormonal stimulation of transcription in Ga-AS cell lines

The control and antisense cells were grown in the presence of 100 nM of tetracycline for up to 5 days before transfection. They were transfected with the SRE reporter vector and serum starved overnight. The cells were then harvested and luciferase activity was measured. The results are the mean +/- S.E.M. of three separate experiments done in triplicate. The statistics were done using Anova: single factor.
C. 11. Reduced hormone dependent phosphorylation of IRS-1 and MAP kinases in G\textsubscript{i}-AS cells

Upon stimulation of the insulin receptor tyrosine kinase, a multitude of phosphorylation events occurs along the insulin-signalling pathway. The insulin RTK phosphorylates proteins such as IRS-1 and 2, and in turn the IRS protein activates a cascade of signalling pathways, such as the MAP kinase pathway. The SRE/TK promoter is dependent on the phosphorylation of IRS-1 and ERK proteins as a result of insulin stimulation. To determine if the depletion of the G\textsubscript{i} proteins has a direct affect on the insulin phosphorylation of these proteins in L6 cells, we used specific primary antibodies to determine the activation state of each protein.

The pERK antibody recognizes the phosphorylated form of ERK1/2. The western blot (Fig 22A) showed similar basal levels of ERK phosphorylation in parental and Ga-AS L6 clones and a robust 10-20 fold increase in phosphorylated ERK minutes after insulin stimulation (Fig 22B). Densitometry data indicated a 50 to 60% lower level of phosphorylated ERKs in insulin-stimulated Ga-AS3 and Ga-AS14 cell lines when compared to L6 wild-type control. Shown in Fig 23, the antisense clone Ga-AS14 also exhibited impaired insulin-dependent phosphorylation of IRS-1, indicating that the signalling defect is proximal to the insulin receptor. Consistent with our transcriptional data, several proteins along the insulin signalling pathway show a remarkable decrease in activation after insulin stimulation in our G\textsubscript{i} depleted cells. This decrease is significant as it indicates the G\textsubscript{i} proteins must play an important role in the activation of insulin stimulation, and that the presence of G\textsubscript{i} proteins is needed for optimal stimulation.
**Fig 22A**

<table>
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<th>Ga-AS14</th>
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<td>+</td>
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<tr>
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<tr>
<td>pERK</td>
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**Fig 22B**

- L6
- Ga-AS3
- Ga-AS14

**Fig 22C**

- L6
- Ga-AS14
Fig 22A  Decreased phosphorylation of ERK proteins in Gi antisense cells
The L6 wildtype and antisense cells were serum starved overnight before a 15 min insulin treatment. They were then harvested in RIPA buffer and the cellular lysates were denatured in SDS, subjected to electrophoresis on a 12% SDS polyacrylamide gel, transferred to membranes and immunoblotted with pERK and ERK antibodies according to manufacturer's specifications (Santa Cruz Biotech).

Fig 22B  Quantification of phospho-ERK in insulin-stimulated Gi antisense cells.
Densitometry of the pERK level was measured using standard densitometry techniques as described in the methods, comparing each sample to the L6 wild-type unstimulated control. The results are the mean +/- S.E.M. of three separate experiments.

Fig 22C Quantification of the phospho-ERK over total ERK in insulin stimulated Gi antisense cells
The densitometry of pERK was corrected for total ERK and compared the L6 wild-type to insulin stimulation using the Ga-AS14 antisense cell line. The results are a mean +/- S.E.M. of two separate experiments.
Fig 23 Inhibition of IRS-1 protein phosphorylation in the Ga-AS14 antisense cells.

The L6 wildtype and antisense cells were serum starved overnight before insulin treatment. IRS proteins were immunoprecipitated with an IRS-1 specific antibody and immunoprecipitated proteins were denatured in SDS, subjected to electrophoresis on a 8% SDS polyacrylamide gel, transferred to membranes and immunoblotted with pTyr and IRS-1 antibodies according to manufacturer's specifications.
D. Discussion and Future Directions
D. 1. Discussion

Data from this study strongly supports the view that heterotrimeric Gi proteins can play an important role in insulin responsiveness in target cells. This model is supported by several studies from other labs (Moxham and Malbon 1996, Chen et al 1997). In this work, we showed that depression of Gi proteins increased insulin resistance in L6 Gi antisense cells. Similarly insulin resistance was observed in L6 cells transiently transfected with a Gi22 dominant negative mutant. We showed that insulin activation of both SRE/TK and HEXII promoters are affected by Gi depletion indicating that mitogenic and metabolic pathways may involve insulin receptor recruitment of heterotrimeric G proteins. Our model has provided a more quantitative approach to analyzing Gi function in insulin signalling in that a close correlation (R = 0.9) between Gi levels and insulin responsiveness was demonstrated. Lastly, our data show that Gi replacement in insulin resistant clones can restore a full hormonal response. The results lead to several conclusions and interpretations about the role of Gi proteins on receptor tyrosine kinase signalling.

D. 2. Evidence for G proteins in Insulin signalling

Heterotrimeric G proteins are key elements in the transmission of hormone and neurotransmitter signals in a wide variety of systems and are typically activated by GPCR-type receptors (review Neer 1995). Insulin signalling through receptor tyrosine kinases has not traditionally been associated with heterotrimeric G protein, but as was shown in the Gi22 depleted mouse, several insulin-induced pathways were inhibited
including GLUT4 translocation and glycogen synthase activation. While $G_{ia2}$ depletion lead to an impaired insulin response, the expression of a PEPCK-$G_{ia2}(Q205L)$ activated mutant mimicked insulin action through enhanced glucose tolerance \textit{in vivo} (Chen et al. 1997). These studies suggest a role for $G_{ia2}$ in the regulation of insulin signalling and glucose homeostasis. Interestingly, a third "PEPCK-directed" transgenic mouse expressing $G_q$ antisense RNA in insulin target tissues had increased body mass and hyperadiposity (Galvin-Parton et al. 1997). The absence of $G_q$ abolished the stimulatory control of lipolysis and caused the mice to accumulate fat. Given the very different phenotypes of PEPCK-$G_{iAS}$, PEPCK-$G_{i(Q205L)}$ and PEPCK-$G_{qAS}$ mice, these data provide further evidence for the specific effects of $G_i$ depletion on insulin signalling in an \textit{in vivo} system.

Recent data in cultured cells support a role for $G_q$ in GLUT4 translocation in 3T3-L1 adipocytes (Imamura et al. 1999). In this study insulin "utilized" $G_q$ to stimulate glucose transport and GLUT4 translocation. The data indicated that $G_q$ transmitted signals from the insulin receptor to the p110alpha subunit of PI3-kinase for GLUT4 translocation, hence $G_q$ action is upstream of and dependent on PI3-kinase activation. Based on these studies, $G_q$ has an important role in insulin stimulated mechanism involving glucose regulation. This supports the view that $G_q$ is a critical component of the insulin signalling pathway leading to GLUT4 translocation and establishes a role for a second G protein in the regulation of insulin signalling.

Pharmacological studies have previously suggested the involvement of $G_i$ proteins in insulin action, but such studies have at times been controversial. Butler et al. (1996) has demonstrated that a pertussis-toxin blocked insulin-stimulation of phospholipase C
and also dramatically inhibited the induction of c-fos mRNA. In contrast to these studies, Chuprun et al (1997), showed insulin-stimulated c-fos induction (using a fos-lacZ reporter gene) was completely unaffected by pertussis toxin. Therefore, even though sensitivity to pertussis toxin can be an indicator of Gi involvement in insulin actions, its successful use may depend on a variety of conditions (treatment times, dosage, cell types, etc). Our own experience with pertussis toxin in transfection experiments suggests its effects may not be restricted entirely to Gα-subunits. We have found that the basal promoter activity in luciferase assays can be non-specifically inhibited by prolonged treatment with pertussis toxin. Whether this involves effects on promoter function, luciferase RNA stability or activity of the reporter protein is not clear. Therefore, a molecular approach may provide a better understanding of the role of Gi proteins on insulin stimulation.

D. 3. Insulin responsive target gene promoters

To investigate the role of Gia2 on insulin signalling, we used transcriptional targets including the SRE element and the HEXII promoters in transient transfections. The significance of having two independently inducible promoters was to determine if a Gi protein has a narrow specific effect on perhaps one insulin signalling pathway, or whether it has a more general effect, possibly at the receptor level. Insulin activates the SRE promoter through the MAP kinase pathway, whereas the HEXII promoter is stimulated by the PI3K/p70S6K pathway. The SRE element is activated by a wide variety of extracellular signals such as different growth factors and phorbol esters. Our data shows that insulin, PDGF, LPA and TPA all induced the SRE in both L6 skeletal myoblasts and myotubes. The HEXII promoter was activated by insulin, LPA and PDGF but in
myotubes only. This suggests that the p70s6K dependent transcriptional pathway may become activated during myocyte differentiation.

The level of activation is important to establish and better understand the different effects observed in our Gi antisense lines. Whereas insulin and PDGF both stimulate our promoter elements through distinct receptor tyrosine kinases, TPA activates the SRE promoter most likely through direct activation of protein kinase C (Soh et al. 1999, Johansen and Prywes 1995, Treisman 1995), independent of receptor tyrosine kinase like the insulin receptor or PDGFR. TPA was therefore a good control to demonstrate that our promoters can respond normally in the antisense cells. LPA stimulation served as a positive control for the "Gi antisense effect" as it is known to activate a Gi-coupled receptor (Jalink et al. 1994, Moolenar et al. 1997). Accordingly in the absence of Gi proteins, LPA showed decreased induction of the SRE element. The LPA GPCR is a member of the recently identified Edg subfamily of receptors and several members have now been cloned. They show distinct properties in ligand specificity, Ca^{2+} response, modulation of adenylyl cyclase, and MAP kinase activation (Bandoh et al. 1999).

The SRE/TK and HEXII promoters were shown to be activated by distinct pathways in the presence of either insulin or PDGF. The inhibitor experiments showed that the SRE stimulation was specifically blocked with PD 98059 (MEK inhibitor) and the HEXII promoter was sensitive to rapamycin (p70s6k inhibitor). Wortmannin (PI-3K inhibitor) was able to inhibit the stimulation of both the SRE and HEXII promoters in our L6 cells indicating that PI3-kinase may be a common element needed for the activation of both promoters. Others have shown PI3-kinase to be upstream of both promoter regulatory pathways (SRE and HEXII) (Cross et al. 1994, Yamauchi et al. 1993, Osawa
et al. 1996). Although the role of PI3-kinase is not normally thought to have a role in MAP kinase dependent activation of SRE promoter by tyrosine kinases, wortmannin was able to block the activation in this study leading to a conclusion that PI3-kinase had a role in MAP kinase activation. However, Wennstrom and Downward (1999), recently showed that wortmannin has the ability to reduce the activity of PI3-kinase to below a threshold level of activity, and at that level, the activity of MAPK can be is inhibited indirectly. This evidence may suggest that PI3-kinase is not directly involved in the activation of the Ras/MAP kinase pathway for the stimulation of our SRE promoter, but its presence may be required.

D. 4. $G_{ia2}$ dominant negative inhibits insulin response

To address the role of $G_{ia2}$ in insulin RTK signalling, we used mutants capable of altering the normal functions of the $G_{ia2}$ protein in our L6 myocytes. The actions of altered $G_{ia2}$ on our transcriptional regulatory elements were examined on basal function and after insulin stimulation compared to wild-type cells. Our study was the first to show that expression of a $G_{ia2}$DN (dominant negative) can result in a greater than 50% decrease in insulin activation of two specific target genes (Fig 11A, 12). This indicates that the inhibition by the $G_{ia2}$DN acts on proteins that may be common to both pathways involved in insulin stimulation of our promoter elements. The mechanism of $G_{ia2}$DN-mediated inhibition of insulin signalling still needs to be elucidated. One possibility is that the $G_{py}$ subunits are needed for activation of signalling components such as Ras or PI3-kinase by insulin. The substitution for serine 47 to cysteine on the $G_{ia2}$ subunit prevents the release of $G_{py}$ (Slepak et al. 1994). The role of $G_{py}$ in MAPK activation by
GPCRs has been previously demonstrated in LPA stimulation of G\textsubscript{i}-coupled receptors leading to the activation of MAP kinase and appears to be dependent on the activation of p21ras (Ras) (Howe and Marshall 1993). Indeed, it was shown that G\textsubscript{\beta\gamma} is the primary mediator of Ras activation and subsequent MAP kinase activation in response to stimulation of G\textsubscript{i}-coupled receptors. (Koch et al. 1994). It has also been demonstrated that G\textsubscript{\beta\gamma} may be needed for the activation of PI3-kinase (Kurosu et al 1997). Sequestration of a pool of \beta\gamma by G\textsubscript{i}DN could therefore block activation of proteins such as Ras or PI3-kinase.

Whereas the G\textsubscript{i}DN dominant negative irreversibly binds to the \beta\gamma subunits, the activated G\textsubscript{i} mutant is constitutively bound to GTP and does not interact with \beta\gamma subunits. In our study, transient co-transfections with the G\textsubscript{i}DN (Q205L) activated mutant resulted in stimulation of the SRE element (Fig 11A), indicating that it has the ability to mimic insulin stimulation in our cells. This suggests that G\textsubscript{i}DN (Q205L) may activate various signalling molecules, such as Ras or PI3-kinase required for activation of the c-fos response element. This data complements work by Mao et. al. (1998A), showing that activated mutants of several other G\textsubscript{\alpha} subtypes (G\textsubscript{q}, G\textsubscript{11}, G\textsubscript{14}, G\textsubscript{16}, G\textsubscript{12} and G\textsubscript{13}) can activate the serum response factor (SRF) mediated gene transcription in NIH3T3 cells. Interestingly these investigators found no effect of G\textsubscript{i} or G\textsubscript{\alpha} activated mutants on the SRF component of the SRE element. This could be explained by the fact SRF mediated gene transcription can be independent of MAP kinase activation. The SRF is more commonly activated by a Rho-dependent pathway (Mao et al. 1998B). The members of the Rho family of small GTP-binding proteins including RhoA, Rac and Cdc 42 all have the ability to stimulate the SRF element, whereas MAP kinase activates the SRE element.
through phosphorylation of TCF-Elk (Hill and Treisman 1995). Experiments by Mao et al. (1998A) provided evidence of the involvement of different \( G_\alpha \) subunits to activate the SRE element through SRF proteins but they utilized the SRF/Luc construct, which does not contain the TCF binding site present in our promoter construct. This may explain the lack of stimulation when they used the \( G_i \) activated mutant. Our results directly complement the study by Chen et al. (1997) that conditional, tissue-specific expression of using \( G_{i2}(Q205L) \) \emph{in vivo} mimics insulin action. Their results show that an activated \( G_{i2} \) protein has the ability to activate hexose transporters, that the recruitment of GLUT4 was elevated in adipocytes, and that hepatic glycogen synthase was found to be activated in the absence of administered insulin. Their study concluded that early events in insulin stimulation were activated, while our study shows that late events (insulin stimulated transcription) were mimicked by the activated \( G_i \) mutant. Together the evidence suggests a permissive role of \( G_{i2} \) in the insulin receptor tyrosine kinase linked pathway and supports a model in which \( G_{i2} \) may be involved in both early and late effects of insulin signalling.

**D. 5. \( G_i \) depletion results in increased insulin resistance: comparison of \emph{in vivo} and cell culture models**

Although the PDPCK-\( G_{i2} \)AS mouse showed no obvious defects in longevity or fecundity (Moxham et al. 1993), it was extremely runted in addition to having pronounced insulin resistance (Moxham and Malbon 1996). This was observed in several founder animals, which should minimize the possibility that it was the result of disruptive chromosomal integration events by transgene sequences. However, because insulin resistance in these animals was observed in two tissues where expression of the
Gia antisense RNA was undetectable, even by PCR (Moxham and Malbon, 1996), this could suggest that mechanisms other than G\(_i\) depletion were involved in the development of the hormone-resistant phenotype. Expression of an activated G\(_{ia2}\) mutant in transgenic mice (Chen et al. 1997) provided further insight into the role of G\(_{ia2}\) in insulin responsiveness, but chronic overexpression of such mutants may affect signalling pathways that are not normally regulated by endogenous G\(_{ia2}\).

Due to the potentially complex effects associated with such an in vivo approach, we wanted to examine the role if G\(_i\) proteins in insulin signalling in a homogeneous cell population in which the effects of antisense-mediated G\(_i\) depletion could be tied more directly to changes in insulin responsiveness. We created an L6 cell line expressing a full-length G\(_{ia2}\) antisense RNA, a strategy distinct from that used in the PEPCK- G\(_{ia2}\)AS mouse. Suppression of the G\(_{ia2}\) by a full-length antisense RNA was selected because it has been used successfully previously in pituitary tumor cell lines (Liu et al., 1999). These investigators demonstrated specific reduction of G\(_{ia2}\) levels without loss of other G proteins such as G\(_{ia1}\), G\(_{ia3}\) and G\(_o\).

Our antisense system was designed to use a tetracycline-sensitive promoter to drive expression of antisense G\(_{ia2}\) RNA, which allowed us to reduce G\(_{ia2}\) levels and then restore them in the presence of 100 ng/ml tetracycline. The dose of tetracycline required to regulate tet operator elements is substantially lower than that normally used as a cell culture antibiotic. Therefore, non-specific effects of tetracycline on insulin signalling are likely minimal. Our data shows that depleted levels of G\(_i\) are associated with impaired insulin stimulation, but in the presence of tetracycline expression of G\(_i\) and hormone responsiveness are restored (Fig 21). This data agree well with our correlative analysis of
multiple Ga-AS clones, where a quantitative relationship was seen between the level of $G_i$ and the magnitude of the hormone response.

As a final comment on our cell culture system, it should be noted that all insulin effects were measured in the presence of endogenous receptors only. Other cell culture models for studying insulin signalling often resort to overexpression of recombinant insulin receptors to obtain a strong insulin response. Expression of high levels of insulin receptors may result in the recruitment of signalling pathways that would normally not mediate insulin actions. The L6 myocyte cultures may therefore offer an insulin-responsive system that more accurately reflects the insulin function in normal tissues.

D. 6. Insulin Receptor and PDGFR responses in $G_i$ antisense cells

Transcriptional activation by insulin, LPA, and by PDGF at lower doses, were all inhibited by the absence of $G_i$ proteins in our antisense cell lines (Fig 15). LPA is known to signal specifically through a GPCR (Jalink 1994, Moolenar 1997), and at low $G_i$ protein levels, an increase in resistance to LPA is expected and was observed. Interestingly however, PDGFR function can also be affected by $G_i$ depletion (Fig 18B). PDGF regulation was significantly inhibited at lower concentrations (10 ng/ml) and after increasing the amounts of the hormone, the inhibition was no longer seen. This conditional repression contrasts with that observed with the insulin receptor, in which case the level of inhibition was significant at all levels of the hormone dose curve. This leads us to suggest that $G_i$ proteins may have a more specific interaction with the insulin receptor or with associated signalling components than with PDGF receptor signalling complexes.
Although the insulin receptor and PDGFR require autophosphorylation for signalling, only the insulin receptor is capable of binding and phosphorylating tyrosyl residues of the IRS proteins (Staubs et al. 1998). The differences seen in the insulin and PDGF dose curves (Fig 18A, B) leads us to suggest that the activation by each receptor tyrosine kinase is differentially affected by Gi depletion. There are a number of functional differences between insulin receptor and PDGFRs that could explain, in part, different requirement for Gi proteins. Both receptors can activate similar downstream pathways such as the MAP kinase pathway (review Avruch 1998, Conway et al. 1999), but unlike the insulin receptor, PDGF does not require docking proteins such as IRS-1 for maximal activation. Although insulin and PDGF stimulate PI3-kinase activity, only insulin is capable of stimulating certain events like GLUT4 translocation and glucose transport (Staubs et al. 1998).

In our study, G\textsubscript{i22} deficiency resulted in marked insulin resistance whereas PDGF resistance was not as pronounced and was observed only at lower ligand concentrations. This might suggest a weaker association of Gi proteins with PDGFR signalling complexes than with the insulin receptor. However, Conway et al. (1999), has shown that PDGF activation of the MAP kinase involves PI3-kinase dependent activation of Ras and Raf kinase and that the PI3-kinase interaction with Ras is mediated by a Gi protein. One possible explanation of our data is that at low levels of PDGF, dimerization of receptors is less extensive and activation of PI3-kinase and the Ras-Raf-MAP kinase pathway is dependent on recruitment of Gi. However, at higher PDGF levels, increased oligomerization of PDGFRs facilitates new protein interaction that can activate PI3-kinase and MAP kinase pathway in a Gi-independent manner.
Because the insulin receptor, but not the PDGFR, phosphorylates IRS-1 leading to activation of both MAPK and p70S6K, we examined tyrosine phosphorylation of IRS-1 in our antisense lines. The western blot data indicated that tyrosine phosphorylation of IRS-1 was indeed impaired after insulin stimulation (Fig 23), consistent with the transgenic study of Moxham and Malbon (1996). Impairment of IRS-1 phosphorylation could block many downstream events in the insulin signalling pathway, and could account at least in part for the reduction we observed in transcriptional activation. In an alternate pathway to IRS-1, the adapter protein Shc which is expressed in L6 myoblasts (Klint et al. 1995) can independently recruit Grb2-SOS to the insulin receptor, thereby activating ras and the MAPK pathway (review White 1997). As insulin stimulation of MAPK phosphorylation was inhibited in our Gi depleted cells, we can presume that Shc binding and phosphorylation by the insulin receptor is equally inhibited to IRS-1. Another possibility though is that Shc is not efficiently recruited to the insulin receptor in our Gi-deficient L6 cells, but preferentially interacts with the activated PDGFR. It would be interesting to determine whether such a difference in receptor/adapter association contributes to the functional differences of insulin receptors and PDGFRs in the Ga-AS cells.

D. 7. Direct Interaction between the Insulin Receptor and G proteins

Inhibition of insulin stimulated IRS-1 phosphorylation following G1a2 depletion suggests the possibly close association of the Gi at the insulin receptor/IRS signalling complex. Interestingly a physical association of the insulin receptor with G1a1,2 has been demonstrated in co-purification studies (Sanchez-Margalet et al. 1999, Jo et al. 1993, Record et al. 1993). Jo et al. (1992) found an insulin receptor peptide contains two
putative G-protein-binding motifs GPBP2 (1135-1156) and GPBP1 (1319-1333) (see section A. 8.). Their studies provide evidence for a potential regulatory site for G-protein interaction with the insulin receptor in the tyrosine kinase domain. Unfortunately, no follow-up studies to this work have been published and the functional significance for binding of the 41 kDa and 67 kDa proteins to the insulin receptor remains unknown.

Recently, Hallak et al. (2000) has shown an association of the heterotrimeric Gi protein with the insulin-like growth factor-1 (IGF-1) receptor. The homologous IGF-IR is structurally very similar to the insulin receptor and could be co-immunoprecipitated with a Gi protein. Remarkably, IGF-1 was shown to trigger the release of Gβγ subunits and the IGF-1 mediated activation of MAPK was pertussis toxin sensitive. These data indicate a striking and direct association of Gi proteins with the IGF-IR and demonstrate how components of the G protein trimer may mediate specific responses to the IGF-1 stimulation. Moreover, the results of Hallak et al. (2000) complement work done by Sanchez et al. (1999), who also co-immunoprecipitated Gi proteins with the insulin receptor. Both research groups suggest a functional relationship between the Gi protein and receptor tyrosine kinases through a direct binding interaction, and propose that these receptor have the ability to activate heterotrimeric G proteins.

D. 8. Role of PTPases in insulin resistance

In the transgenic mouse study by Moxham and Malbon (1996), insulin resistant Giα2 deficient tissues showed an increase in protein tyrosine phosphatase (PTPase) activity. PTPases have been implicated in the physiological regulation of the insulin signalling pathway (review Goldstein et al 1998, Byon et al 1998). They generally act to
negatively regulate signals generated by RTKs by dephosphorylating the activated RTK and key RTK substrates. PTPases tend to be ubiquitously and abundantly expressed. Several PTPases have been cloned and examined, PTP-1b and PTP-LAR (leukocyte common antigen related molecule) have been shown to dephosphorylate the insulin receptor (Kenner et al 1996, Hashimoto et al. 1992). In the G_i depleted mouse, total cellular phosphotyrosine phosphatase activity and PTP-1b protein levels in adipose, liver and skeletal muscle cells was markedly increased compared to control littermates, while another PTPase, Syp, was not significantly altered. In addition to an increase in total PTPase activity, fractionation experiments showed that in G_{ia2} deficient cells, PTP-1b was released from its membrane-associated endoplasmic reticular location to the cytosol.

Moxham and Malbon (1996) described their G_i-depleted transgenic mice as having an overall increase in PTP-1b activity. They suggested that increased phosphatase activity may be the mechanism for increased insulin resistance. However, a recent study by Bleyle et al., (1999) suggested that expression levels of relevant PTPases may not be sufficient to control insulin receptor kinase activity, but rather restricted accessibility of the PTPase to the insulin receptor is more critical. There might be a more preferential dephosphorylation of the insulin receptor (rather than PDGFR) by the PTPase that would explain the greater insulin resistance. Since PTP-1b dephosphorylates both the insulin receptor and PDGFR, there may be involvement of accessory proteins, which would target the PTP to a specific receptor tyrosine kinase.

There may be in vivo evidence in favour of preferential interaction of PTP-1b with insulin signalling. A mouse model was recently created in which the PTP-1b gene was disrupted (Elchebly et al. 1999). The PTP-1b knockout mice had increased insulin
sensitivity and were resistant to high fat-induced obesity. PTP-1b has been implicated in the attenuation of the insulin signal (Cicirelli et al. 1990) and this phosphatase has been shown to interact directly with the activated insulin receptor (Seely et al. 1996). Its inhibitory effects on insulin action therefore are likely by dephosphorylation of both the insulin receptor and IRS-1. Elchebly et al. (1999), showed that in PTP-1b deficient mice, the insulin-stimulated tyrosine phosphorylation of IRS-1 in muscle was increased compared to control. This effect was judged to be specific as autophosphorylation of another receptor tyrosine kinase, the epidermal growth factor receptor (EGFR) was not affected in the PTP-1b deficient animals. These results appear consistent with those of the G_{i\alpha 2} deficient mouse model, where higher concentrations of PTP-1b and elevated phosphatase activity were detected.

Although insulin receptors appear able bind G_{i\alpha 1,2} and PTP-1b (Bandyopadhay et al. 1997) it is not known whether these two proteins can bind to the receptor at the same time or within the same domain. It was shown that PTP-1b could be displaced by other SH2 domain binding proteins, their activity could be regulated by such action (Milarski et al. 1993). If G_{i} and PTPases do interact with insulin receptor in a mutually exclusive manner, one possible mechanism to explain our data would be that in the absence of G_{i\alpha 2} proteins, PTP-1b could interact readily with the insulin RTK resulting in dephosphorylation and decreased insulin signalling. PTP-1b would bind and dephosphorylate the insulin receptor as well as any IRS-1 proteins that rapidly associated with the activated receptor, however, if in L6 cells G_{i} heterotrimers or activated G_{i\alpha} subunits can bind and occupy PTP-1b sites on the insulin receptor, receptor phosphorylation and signalling may be prolonged. Mapping of interaction domains of G_{i}...
proteins and the IGF-1 receptor (Hallak et al. 2000) may provide insight into how such an interference mechanism may function.

D. 9. Future Directions

More research is needed to understand the actions between Gi proteins, insulin receptor tyrosine kinase and protein tyrosine phosphatases. PTP-1b and other PTPases need to be measured in our antisense clones to determine how their levels are altered after Gi depletion. Co-immunoprecipitation experiments could be performed to determine whether insulin receptors, PTPases, and Gi proteins associate with each other in vitro, what the interactive domains are, and how such interactions are regulated. Lastly, given that PTPases target most RTK, including the PDGFR, our antisense cells should be used to determine why hormone resistance is most pronounced for insulin.

D. 10. Conclusion

We conclude that Gi proteins have a significant role in insulin signalling and activation of target genes in the L6 muscle cell line. We have shown that replacement of Gi rescues insulin signalling in Gi deficient antisense clones demonstrating that the insulin resistance is specifically due to antisense-directed depletion of Gi proteins. We also conclude that the hormone resistance is selective, as PDGF and TPA stimulation are less affected by Gi antisense RNA expression than is insulin stimulation. Our results showing the importance of Gi proteins in insulin target cells should stimulate thinking towards the development of additional therapeutic targets in the treatment of type 2 diabetes.
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