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Signal Transduction in Adipocytes:

Regulation by insulin versus other growth factors

by Yuan-Ping Han

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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ABSTRACT**Signal Transduction in Adipocytes:****Regulation by insulin versus other growth factors****by****Yuan-Ping Han****Advisor: Professor Ronald A. Kohanski**

Protein tyrosine phosphatases (PTP) can play either negative or positive roles in signal transduction initiated by receptor tyrosine kinases. In adipocytes, stimulation of glucose uptake is the most prominent physiologically important signaling pathway under the acute regulation of insulin and its receptor. This pathway is inhibited by phenylarsine oxide (PAO), which is known to block PTP action. We have found that a 67kDa phosphotyrosyl-protein, pp67, present in the cytosol of 3T3-L1 adipocytes, became dephosphorylated in response to insulin, insulin-like growth factor-1, and epidermal growth factor. The kinetics of insulin-dependent pp67 dephosphorylation were similar to those of insulin-stimulated glucose transport in these cells. Dephosphorylation of pp67 was inhibited by phenylarsine oxide (PAO), but not by wortmannin, an inhibitor of phosphatidylinositol 3'-kinase and glucose transport activation. These findings are consistent with activation of a PTP by insulin as a potential mediator of glucose transport. In addition to showing that stimulation of PI3'-kinase was limiting for growth factor activation of glucose transport, we identified PI3'-

kinase as a target for inhibition by PAO. Therefore, the PI3'-kinase dependent pathway is adequate to explain the known effectors and inhibitors of glucose transport. While these data do not exclude the possibility, it is not necessary to invoke a PI3'-kinase independent, PTP-directed pathway, such as the one reported here by pp67 dephosphorylation.

In contrast to the persistent activation of a PTP for pp67, the insulin-stimulated MAP kinase pathway was transiently activated in 3T3-L1 adipocytes. We observed the transient formation of a membrane-associated complex including phosphotyrosyl-insulin receptor substrate-1 (IRS-1), SOS1, and possibly Grb2. We also found that this binary - and possibly ternary - complex was present at higher levels and for longer times in the cytosolic compartment. Based upon the known requirement for SOS1 activation in the membrane compartment, we conclude that the transient formation of the SOS1-IRS-1 complex in the membrane is the basis for the transient activation of MAP kinase tyrosine phosphorylation. Electrophoretic changes in SOS1, which occur following growth factor stimulation, were demonstrated to take place after MAP kinase activation had passed its peak, and therefore could not be the cause for the transient. Furthermore, we found a novel electrophoretic form of SOS1 which was induced by insulin and to a lesser extent by other growth factors. Unlike the known growth-factor induced shifts in electrophoretic mobilities of several signaling molecules, this novel SOS1 form was insensitive to phosphatase treatment. However, the underlying modification of SOS1 was not identified. .

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This thesis work could not be achieved without the help and guidance of my advisor, Dr. Ronald A. Kohanski. His inspiration, the highest scientific judgement and exceptional enthusiasm to penetrate the deepest inside of problems provided an atmosphere for my training. Although there are many ways to approach a goal, here I have been taught to use the strictest standard in judging the value of alternatives. To pursue unknown in science is just like gold rush, rewards are rare and not guaranteed. Temptation to find the novel intermediates for insulin signal transduction drove me to explore what I reported in this thesis. After all, I panned out a few grains of gold from much sand during five years.

I also appreciated other teachers, including Dr. Heng-Chun Li who was the member of my advisory committee and gave me valuable advice, Drs. Jie Chen for cell culturing and E. Wolf for technique help. I also thank Drs. Lu-Hai Wang, Irwin Gelman who both were examiners for my second examination. I greatly appreciated the time and attention given to the examination of this work by my thesis committee: Drs. Wang, Kohanski, Gelman, Sadowski, and Flores-Riveros. I also thank the Dean, Dr. Terry Krulwich.

I thank my father, Shilon Han who gives me philosophic guidance and enormous encouragement. I dedicate my finding to my past mother. I am greatly indebted to my wife Li-xiu Wang who gives me a warm family and love, and my son T.J. who always expects a bed-time story.

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CHAPTER ONE

pp67 DEPHOSPHORYLATION AND SIGNALING IN GLUCOSE TRANSPORT IN ADIPOCYTES

Abstract

Insulin and other growth factors initiate cascades of tyrosine, serine, and threonine phosphorylation. These are characteristic of the essential roles of protein kinases in the activation of signal transduction pathways. Protein tyrosine phosphatase would therefore be expected to function primarily in the deactivation phases of signal transduction. However, phosphatase can also be activators of signal transduction, and can serve this function downstream of tyrosine kinases. Where phosphatase activate signaling, it is possible that their substrates would be tyrosine-phosphorylated prior to engagement of growth factor receptor-tyrosine kinases. Therefore we have examined basal-state tyrosine-phosphorylated proteins in 3T3-L1 adipocytes for dephosphorylation in response to challenge by growth factors.

A 67kDa cytosolic phosphotyrosyl-protein, pp67, was found to be dephosphorylated following exposure of adipocytes to insulin. We observed less dephosphorylation in adipocytes challenged with insulin-like growth factor-1 (IGF-1), and epidermal growth factor (EGF). Because insulin is the primary regulator of adipocyte metabolism, its effects on pp67 dephosphorylation were more fully characterized: The insulin dose-dependence showed an

$EC_{50} \approx 2$ nM, and a short lag-period was observed before dephosphorylation of pp67 was detected. A steady-state was reached by about 6 min for pp67 dephosphorylation. The dephosphorylation of pp67 was inhibited by phenylarsine oxide (PAO) with an $IC_{50} \approx 5$ μ M. These four properties constitute correlations between pp67 dephosphorylation with the insulin-stimulated glucose transport in the adipocytes. Dephosphorylation of pp67 was, however, not blocked by wortmannin, and is therefore not downstream of phosphatidylinositol 3'-kinase (PI3'-kinase).

We have also shown that stimulation of glucose transport by each growth factor is proportional to the activation of PI3'-kinase. In contrast to pp67 dephosphorylation, the kinetics of PI3'-kinase activation did not display any lag-phase after insulin stimulation. By assaying PI3'-kinase activity associated with insulin receptor substrate-1 (IRS-1), we found that PAO inhibited activation of PI3'-kinase by insulin in intact cells, without inhibiting the co-immunoprecipitation of IRS-1 and the regulatory subunit of PI3'-kinase. Furthermore, PAO did not significantly inhibit PI3'-kinase when added *in vitro*, suggesting that PAO does not affect the catalytic subunit. Together, these findings indicate that PI3'-kinase activity is sufficient to account for the spectrum of growth factor and inhibitor effects on glucose transport in 3T3-L1 adipocytes.

Introduction

Insulin

The experiments of Von Mering and Minkowski at the close of the 19th century clearly demonstrated that the pancreas plays an important role in the prevention of diabetes (Degroot, 1989). The name "insulin" was based on the belief that the hormone was derived from the islets of Langerhans, as actually suggested as early as 1909 by deMayer. In the early 1920's Banting, Best, and Collip isolated the hormone for treatment of diabetes (Banting *et al.*, 1923). The determination of the primary structure of bovine insulin by Sanger and his associates provided the first complete structure of a protein. Insulin consists of an A and a B chain of 21 and 30 amino acid residues, respectively (Sanger, 1959). The two chains are linked through a pair of disulfide bonds; an intrachain disulfide bond connects the sixth and eleventh amino acids within the A chain. Despite the awarding of three Nobel Prizes - to F.G. Banting (1934) for the discovery of insulin, to Fred Sanger (1958) for determination of the primary structure of insulin, and to Dorothy Hodgkin (1964) for the tertiary structure of the hormone - the mechanisms by which this hormone regulates cellular processes are still not fully delineated. The strong driving force to study insulin action has always been an attempt to explain the underlying mechanism of diabetes mellitus.

Diabetes Mellitus

Diabetes mellitus was first described about 1500 B.C. in Egypt. *Mellitus*, the Latin word for honey, characterized the sweet taste of the urine of the patient. The word diabetes was derived from the Greek, referring to excretion of body water. Diabetes mellitus is considered to be a heterogenous group of diseases. However the hallmark of the disease is

that the patients have unusual high level of glucose in blood. The juvenile-onset form of the disease generally results from a deficiency of insulin production; therefore it is referred to as insulin dependent diabetes mellitus (IDDM). The possible etiology of this group includes virally induced β -cell destruction or production of cytotoxic auto-antibodies to β -cells. In contrast, the majority of diabetics who suffer from maturity-onset diabetes generally have above-normal levels of insulin but their tissues do not properly respond to the hormone; namely peripheral resistance to insulin. Therefore this group is referred to as non-insulin-dependent diabetes mellitus (NIDDM) which is often associated with obesity, hypertension, polycystic ovary syndrome, and atherosclerosis. The mechanisms underlying NIDDM are not clear and the major genes associated with NIDDM have not been identified. However, the phenomenon of insulin resistance in NIDDM strongly indicates that the defects are associated with the insulin-targeted cells, where insulin initiates actions through its transmembrane receptor.

Insulin Receptor

In mammals, insulin receptors are expressed at varied levels in almost all types of cells, ranging from hundreds of copies per cell to more than one hundred thousand per cell, for example in adipocytes. The insulin receptor is a 450,000 Da transmembrane glycoprotein composed of two α (135 kDa on SDS-PAGE) and two β (95 kDa) subunits, linked through disulfide bonds (reviewed by Zick, 1989). The cDNA of the receptor encodes a single protein of 1355 amino acid residues (Ebina *et al.*, 1985), although a 12 residue-shorter variant of the insulin receptor has been reported (Ullrich *et al.*, 1985). The extracellular portion of the receptor consists of the entire α subunit and about one third of the β subunit. This

extracellular portion of the receptor forms the insulin binding domain.

A single transmembrane domain is located in each of the two β subunits. This domain contains 23 hydrophobic amino acids, followed by a short stretch of basic residues (Arg-Lys-Arg), a feature of the primary sequence of the cytoplasmic boundary region in many membrane proteins. The cytoplasmic domain of the insulin receptor consists of the carboxyl-terminal 403 amino acids of the β subunit. A prominent feature of the intracellular domain is its protein tyrosine kinase activity, which was found before the receptor gene was cloned (Kasuga *et al.*, 1982). This kinase domain shows a high degree of amino acid sequence similarity to other growth factor receptors: 84% with IGF-1 receptor and 40% with EGF receptor (Ebina *et al.*, 1985; Ullrich *et al.*, 1985). The structural similarity among the receptor tyrosine kinase family additionally suggested that common mechanisms of signal transduction are likely for these receptor tyrosine kinases.

A gene product homologous to the mammalian insulin receptor was identified in *Drosophila*, called DIR (Ruan, 1995). The deduced amino acid sequence from DIR shows about 30% identity to the human insulin receptor. In the β -subunit there is about 50% identity between the fly and human genes. However a quite different feature is that *dir* encodes a novel, 400-amino acid carboxyl-terminal extension which contains four YXXM or YXXL motifs, which are the potential phosphorylation sites for recruitment of SH2 domain containing proteins. These tyrosine motifs are found in different substrates for mammalian insulin receptor (discussed below) instead of being fused directly to the receptor. What role the putative DIR has for the fly is still not clear.

The insulin receptor substrates

Since a protein kinase is the major feature of the cytoplasmic region of the insulin receptor, an obvious question was to identify its substrates. In fact the insulin receptor *per se* is one substrate for the receptor tyrosine kinase. The first detectable event after insulin binding is autophosphorylation of the receptor (Kohanski *et al.* 1986). Autophosphorylation of the insulin receptor probably achieves two purposes, creation of binding sites to recruit signaling molecules (discussed below), and, activation of the receptor kinase toward the downstream substrates. Seven tyrosine residues have been identified as *bona fide* autophosphorylation sites in the insulin receptor: two in the juxtamembrane region, three in the tyrosine kinase domain, and two in the C-terminal tail (Tornqvist and Avruch, 1988; Tavaré *et al.*, 1988; Kohanski, 1993, White *et al.* 1988). Despite the potential tyrosine phosphorylation sites for recruitment of SH2 containing proteins have been attempted to be mapped on the insulin receptor (Staubs *et al.*, 1994), and occasionally reports the association of SH2 domain containing proteins from cell (Kharitonov *et al.* 1995 and Van Hom *et al.*, 1994), it is still remain elusive for the significance of such interaction. However, unlike other growth factor receptors which directly recruit SH2 containing proteins, insulin receptor utilizes an alternative mechanism to transmit the signal.

A cytosolic protein with apparent molecular weight of about 185 kDa was shown to become tyrosine phosphorylated in response to insulin (White *et al.*, 1985). This tyrosine phosphoprotein was isolated from insulin-stimulated liver tissue by antiphosphotyrosine affinity chromatography. A cDNA encoding this protein was cloned and the inferred protein sequence designated IRS-1 for insulin receptor substrate-1 (Sun *et al.*, 1991). Multiple

potential phosphotyrosyl residues have been identified in IRS-1, among them nine with a common motif of YXXM. The tyrosine phosphorylation in such short motifs creates binding sites for certain SH2 domain containing proteins. The proteins include enzymes, such as PI-3' kinase (Yonezawa *et al.*, 1992; Myers *et al.*, 1992), a protein tyrosine phosphatase, Syp (also called SHPTP-2; Xiao *et al.*, 1994; Shen *et al.*, 1991) or adaptor proteins such as GRB-2 (Skolnik *et al.*, 1993).

IRS-1 is not exclusively phosphorylated by insulin receptor or by the structurally related insulin-like growth factor-1 (IGF-1) receptor tyrosine kinase (Meyers *et al.*, 1993). Several, but not all, Interleukins such as IL-4, IL-9 and IL-13 can stimulate IRS-1 tyrosine phosphorylation in hematopoietic cells (Yi *et al.* 1995; Keegan *et al.*, 1994). However, in contrast to insulin and IGF-1 stimulation, IRS-1 is phosphorylated through a non-receptor tyrosine kinase, called JAK. In hematopoietic stem cells, interferon- α can stimulate IRS-1 tyrosine phosphorylation, in addition to activating the STAT signaling system (Uddin *et al.*, 1995). In 3T3-F442A fibroblasts, growth hormone (GH) as well as interferon- γ and leukemia inhibitory factor (LIF) were found to stimulate IRS-1 tyrosine phosphorylation in a transient manner, apparently through JAK2 (Argetsinger *et al.*, 1995). Growth hormone can stimulate IRS-1 tyrosine phosphorylation following by recruitment of PI-3' kinase in rat primary adipocytes (Ridderstrale, *et al.*, 1995). In parallel with IRS-1 tyrosine phosphorylation and PI-3' kinase activation, growth hormone also promoted insulin-like functions such as lipogenesis and anti-lipolytic action (Ridderstrale and Tornqvist, 1994). Further more, the GH-stimulated insulin-like actions were inhibited by wortmannin, which suggested shared the insulin stimulated PI-3' kinase pathway.

Phosphorylation of IRS-1 by insulin receptor *in vivo* is not as simple as a kinase recognizing the consensus motif of a substrate. The amino terminal region of IRS-1 has a pleckstrin homology (PH) domain. Expression of the PH domain-deleted mutant of IRS-1, together with insulin receptor in COS cells, showed that this mutated IRS-1 underwent reduction of insulin-dependent tyrosine phosphorylation compared with wild type IRS-1 (Voliovitch *et al.*, 1995). In contrast, both the PH-domain deletion mutant and wild type IRS-1 were readily phosphorylated by insulin receptor *in vitro*. This experiment indicated that unclarified component(s) or micro-environments in cells may be involved in the recognition between insulin receptor and IRS-1. This phenomenon may also explain the reason that substrates phosphorylated *in vitro* are often not detected *in vivo*.

In hematopoietic cell lines, a protein with a molecular weight close to that of IRS-1 was shown to become tyrosine phosphorylated in response to interleukin-4 and originally called 4PS (Wang *et al.*, 1993). An antibody against IRS-1 failed to recognize this protein, which indicated that 4PS was at least somewhat different from IRS-1. Homozygous deletion of the IRS-1 gene created "knockout mice" (Araki *et al.*, 1994). Surprisingly, the mice retained substantial responsiveness to insulin and largely maintained glucose homeostasis in this absence of IRS-1. In the IRS-1 knockout mice, another protein with a molecular weight slightly larger than IRS-1 was shown to become tyrosine phosphorylated in response to insulin and was designated as IRS-2. Like IRS-1, tyrosine phosphorylated IRS-2 was found to associate with PI-3 kinase and GRB2 (Tobe *et al.*, 1995). Subsequent studies showed that 4PS and IRS-2 are apparently the same protein (White, 1994). Recently, cloning of the IRS-2 cDNA was reported (Sun *et al.*, 1995). Highly similar to IRS-1, IRS-2 has the

consensus potential tyrosine phosphorylation sites essential for recruitment of PI-3' kinase, GRB-2, and SH-PTP2. The possible roles of these SH2 domain containing proteins will be reviewed in a later section.

Like other growth factors, insulin stimulates tyrosine phosphorylation of SHC, the cytosolic protein containing one SH2 domains and a collagen-like domains (Pelicci *et al.*, 1992). Growth factor stimulated tyrosine phosphorylation of SHC may be cell type dependent. In PC-12 pheochromocytoma cells both nerve growth factor (NGF) and epidermal growth factor (EGF), but not insulin, stimulated the tyrosine phosphorylation of SHC proteins followed by association with their receptors (Ohmichi *et al.*, 1994). Although insulin receptor was found expressed at a similar level as NFG and EGF receptors, insulin failed to stimulate SHC phosphorylation. In contrast, IRS-1 was found to be tyrosine phosphorylated. In another report, expression in CHO cell with a mutant insulin receptor, of which tyrosine sites Y¹¹⁶² and Y¹¹⁶³ were changed to alanines, leads to marked reduction of IRS-1 tyrosine phosphorylation (Ouwens *et al.*, 1994). However, SHC tyrosine phosphorylation was apparently unchanged. Thus it was suggested that the insulin receptor may have different specificity for diverse substrates. SHC may play an important role in insulin stimulated mitogenesis since the majority of GRB-2 and mSos proteins were found associated with SHC instead of IRS-1 in cells over-expressing insulin receptor (Sasaoka *et al.*, 1994).

Other potential substrates for the insulin receptor are reported in specific cell types. The vav proto-oncogene product (p95vav) containing a guanine nucleotide exchange factor domain as well as an SH2 and an SH3 domains is expressed in hematopoietic cell lines.

Insulin was found to stimulate the p95vav protein tyrosine phosphorylation (Uddin, 1995). Furthermore, the SH2 domain of p95vav can bind directly to the activated β -subunit of the insulin receptor *in vitro*. Thus it was suggested that p95vav may be a substrate for the insulin receptor tyrosine kinase. Many questions have remained to be answered: 1. Whether p95vav is expressed and phosphorylated by insulin stimulation in the major insulin responsive tissues like fat and muscle? 2. Whether the guanine nucleotide exchange factor functions like mSos to activate p21Ras, and, 3. What is role of the tyrosine phosphorylation?

A 60 kDa protein, pp60, which is tyrosine phosphorylated in response to insulin stimulation was identified in freshly isolated rat adipocytes but not in cultured mouse 3T3-L1 adipocytes (Lavan and Lienhard, 1993). The pp60 was found to be associated with PI-3 kinase. Interestingly, another protein with the molecular weight of 62 kDa, called p62, is tyrosine-phosphorylated in response to insulin in CHO cells over-expressing insulin receptor (Hosomi *et al.*, 1994). In HTC hepatoma cells over-expressing the insulin receptor, p62 was identified as p21Ras-GAP associated protein. Furthermore, p62 was found to be associated with the p85 subunit of PI-3 kinase. Surprisingly, however, the p110 catalytic subunit of PI-3 kinase was not detected in this complex (Sung *et al.*, 1994). Using the yeast two hybrid interaction-screening system, p62 was shown to associate with the SH3 domain of *c-src* kinase through the multiple proline-rich motifs of p62 (Richard *et al.*, 1995). Following tyrosine phosphorylation by the *src* kinase, pp62 recruits SH2 domain containing protein such as GRB-2 and PLC- γ in Hela cells (Richard *et al.*, 1995). There are no further report whether p62 is directly tyrosine phosphorylated by the insulin receptor or what function it play in normal insulin sensitive tissues.

In summary, insulin stimulates tyrosine phosphorylation of several proteins including the β -subunit of the receptor, IRS-1 and -2, SHC and other proteins. Tyrosine phosphorylation of these substrates by the insulin receptor in turn recruits different SH2 domain-containing proteins (Sun *et al.*, 1993). Thus, information from the excited receptor is transmitted to the SH2 domain-containing proteins through tyrosine phosphoproteins adaptor.

Activation of PI3'-kinase

Phosphatidylinositol-3 kinase (PI3'-kinase), which consists of an 85 kDa regulatory subunit containing two SH2 domains and a 110 kDa catalytic subunit. It is activated by many growth factors including insulin, through association with phosphotyrosine proteins, such as autophosphorylated growth factor receptors or docking proteins like IRS-1, IRS-2 and other proteins (Kahn and Folli, 1993). The phosphotyrosine motifs consisting of YXXM or YMXM were identified as p85 subunit recruitment sites in IRS-1 and IRS-2 (Sun *et al.*, 1991 and Sun *et al.*, 1995). A small portion of PI-3 kinase was reported in association with insulin receptor at its C-terminal region which contains a tyrosine phosphorylation sequence of Y¹³³⁴THM (Van Horn *et al.*, 1994). In addition to an SH2 domain, PI3'-kinase can also associate with *src* family kinase such as p56 lyn through the proline rich domain of the 85 kDa subunit and the SH3 domain in the kinase (Pleiman *et al.*, 1994).

The PI3'- kinase-initiated signaling pathway is not as well defined as the Ras/MAP kinase pathway. PI3' kinase catalyzes phosphorylation of phosphatidylinositol at the D-3 position in the inositol ring of phosphatidylinositol. Binding of PI3'-kinase to tyrosine

phosphorylated proteins like growth factor receptors or docking proteins like IRS-1 significantly increases the intrinsic lipid kinase activity. The function of the D-3 phospholipid product is still not defined. However, a growth factor regulated serine protein kinase, p70^{S6K} was shown to be functionally downstream of PI3'-kinase. Two lines of experiments supported such a notion: First, changing the tyrosine sites for recruitment of PI3'-kinase on the PDGF receptor concomitantly abolished PDGF stimulation of the recruitment of PI3'-kinase and p70^{S6K} activation; Secondly, wortmannin, an inhibitor of PI3'-kinase, also attenuated activation of p70^{S6K} by growth factors (Chung *et al.*, 1994). But how p70^{S6K} is regulated by PI3'-kinase is not clear. However recently it was reported that a serine/threonine kinase may mediate PI3'-kinase to p70^{S6K}. Protein kinase B (PKB) was previously isolated and shown similarity both protein kinase A (PKA) and protein kinase C (PKC). Both PKA and PKC are regulated by their respective second messengers cAMP and DAG. So the obvious question would be the identity of putative second messenger for PKB. A viral oncogene, v-akt, and its proto-oncogene c-akt were found to be homologous to PKB. It was shown that PKB is activated by growth factors like insulin, PDGF, EGF, and basic fibroblast growth factor (bFGF) (Burgering 1995). Several experiments suggested a possible role of PKB or the *c-akt* gene product in the PI3'-kinase signal pathway. Activation of PKB was inhibited by the PI3'-kinase inhibitor wortmannin and by co-expression of a dominant-negative mutant of PI3'-kinase. Mutation of tyrosine sites on the PDGF receptor that failed to recruit PI3'-kinase also abolished the growth factor-induced activation of PKB. The structurally related proto-oncogene product, c-akt is regulated in a similar way to PKB (Franke, T.F., 1995). Further more, the protein kinase activity of c-Akt is stimulated by

addition of phosphatidylinositol-3-phosphate, the product of PI3'-kinase, to Akt immunoprecipitates from serum-starved cells. These results suggested that the phosphatidylinositol product of the PI3'-kinase reaction may regulated like a classical second messenger, and PKB/Akt may be the target. The possible role of PI3'-kinase in insulin-stimulated glucose transport is reviewed in the late section.

Roles of Protein Tyrosine Phosphatase Activity in Insulin Signaling

The signaling information carried by phosphoproteins can be generated or removed as rapidly as a few seconds. This acute information transfer are acutely controlled by the activities of protein kinases and phosphatase. Because this thesis is focused on *phosphotyrosine* phosphoproteins, insulin regulated serine/threonine phosphatase are not reviewed here. Tyrosine phosphorylation states are controlled by the relative activities of PTKs and PTPs. Binding of growth factors and cytokines to their receptors usually activate either their intrinsic or associated PTKs. If tyrosine phosphorylation either at the growth factor receptor or the substrates initiates intracellular signaling, then removal of phosphate groups from phosphotyrosine sites by PTPs would functionally desensitize such signaling. It appears that the specificity of signaling is largely determined by the ligand binding to the respective extracellular portion of receptors. Therefore, dephosphorylation of the autophosphorylated receptor or the docking substrate seems not to be so highly restricted to a specific PTP. This also indicates that some PTPs are constitutively active. In the quiescent (such as serum-starved for cultured cells) state, the total PTP activities generally override PTK activities. This notion comes from the widely observed phenomenon that treatment of

quiescent cells with PTP inhibitors like vanadate, pervanadate or hydrogen peroxide, would globally increase tyrosine phosphorylation. These "housekeeping" or constitutively active PTPs maintain cells in a quiescent state and ready to accept extracellular stimuli, or facilitate cells to return to their basal states after the removal of the stimulant.

However, not all PTPs play negative roles in signal transduction. Instead, a few PTPs have shown positive or dominant roles in cell signaling. One example comes from a SH2 domain containing PTP designated variously as SH-PTP2, Syp, PTP1D and SHP1 (reviewed in chapter two). Another example of positively functioning PTP come from one possible pathway for activation in the proto-oncogene Src family PTKs. c-Src kinases are activated by dephosphorylation of a specific phosphotyrosine site at the carboxyl terminus. This dephosphorylation may lead to release of the internal binding of the SH2 domain to the phosphotyrosine site and provide more access for substrate binding to the active site (Courtneidge, 1994). Also, one transmembrane PTP, CDC45, may directly activate p56^{lck} and p59^{fyn} in lymphocytes (Trowbridge *et al.* 1994).

PTP1B, a non-receptor PTP, is ubiquitously expressed in many tissues. Osmotic loading of antibody for PTP1B in rat KRC-7 hepatoma cells was found to increase insulin signaling (Ahmad *et al.* 1995). Tyrosine phosphorylation of insulin receptor and IRS-1, PI-3' kinase activity, as well as DNA synthesis were raised by the antibody which can block PTP1B enzymatic activity but not by control antibody not directed against this PTP. These results showed that PTP1B has a negative role in regulation of insulin signaling and the target of its action is at least partly on the insulin receptor. The effects of insulin and insulin-like growth factor 1 (IGF-1) on PTP activity in rat L6 skeletal muscle cells was explored

(Kenner *et al.* 1993). In the basal state, about 85% of total cellular PTPase activity was associated with the particulate (Triton X-100-soluble) fraction. Incubation of the cells with insulin or IGF-I significantly increased particulate PTPase activity without altering activity in the supernatant or Triton X-100-insoluble fractions. PTPase activity was regulated by both acute and chronic insulin or IGF-I treatment. It was found that the chronic stimulation of PTPase activity was accompanied by enhanced expression of PTP1B mRNA and protein. Thus it shows that even the PTPs which apparently play negative roles are regulated.

To examine whether a transmembrane PTPase, LAR, can modulate insulin receptor signaling *in vivo*, antisense RNA expression was used to suppress specifically LAR protein levels in the rat hepatoma cell line, McA-RH7777 (Kulas *et al.*, 1995). Results showed that insulin signaling from the tyrosine phosphorylation of insulin receptor and IRS-1 to PI-3' kinase activation were all decreased. Thus, it was suggested that LAR may be involved in the modulation of insulin receptor signaling in intact cells. However whether LAR is expressed to sufficient levels in insulin responsive tissues is not known.

Focal adhesion kinase (pp125FAK) was observed to undergo decreased tyrosine phosphorylation upon insulin stimulation in CHO cells over-expressing insulin receptor (Knight *et al.*, 1995). The PTP responsible for dephosphorylation of pp125FAK has not been identified. Dephosphorylation of pp125FAK occurred in an insulin concentration-dependent manner, with significant effects at approximately 0.3 nM and a maximal effect at 3 nM. Maximal dephosphorylation of pp125FAK was measured following 15 min of insulin stimulation. Dephosphorylation of pp125FAK requires the presence of the insulin receptor C-terminal tyrosine 1328 and 1334 (potential SH2 binding sites), which suggested a possible

SH2 domain-dependent interaction (Pillay, 1995). In contrast to insulin, PDGF was found to increase pp125FAK tyrosine phosphorylation (Knight *et al.*, 1995). Combining these results it was speculated that the PTP for pp125FAK dephosphorylation may not be Syp since both insulin and PDGF stimulate recruitment of Syp to either IRS-1 or PDGF receptor, respectively, while only insulin stimulates FAK dephosphorylation.

Insulin Signal Transduction in Acute Responses

Glucose Homeostasis

Like other growth factors, insulin stimulates cell proliferation through the mitogenic signaling pathways. However for the fully differentiated cells like muscle and adipocytes, which have lost the potential to enter cell cycle, insulin plays distinct roles in the regulation of metabolism. Distinguished from mitogenesis, regulation of metabolism is more often acute and reversible, despite the overall importance of gene expression to supply the enzymes for the metabolic processes. This difference implies that signal transduction in metabolic regulation may depend largely on reversible molecular modification. The plasma concentration of glucose in the human body, the most abundant circulating fuel, is maintained within a narrow range from 80 to 120 mg/dl throughout the day. One important feature of glucose homeostasis is that the brain cells rely on glucose as its almost sole energy supply in normal physiological situations. Even a brief under-supply of circulating glucose would be deadly. In contrast, a chronic over-supply of glucose in peripherals could cause Diabetes Mellitus. Thus the glucose must be maintain at a precise level.

Glucose homeostasis is largely achieved by the mutually balanced actions of insulin

and glucagon, which are both secreted from the pancreas, but from different cells. Although several hormones contribute to increase peripheral blood glucose levels, insulin is the only hormone that significantly decreases the blood glucose levels. The major target tissues of insulin for glucose homeostasis are muscle and adipose where insulin stimulates the uptake of glucose into the cells and its conversion into glycogen and fat for energy storage.

Glycogen Synthesis

Despite the fact that major mass of glycogen is stored in muscle, the reversible synthesis and breakdown of glycogen in liver provides the reservoir for maintaining glucose homeostasis. Muscle glycogen does not contribute to glucose homeostasis; glucose can not be released from muscle since phosphoglucose phosphatase is absent from muscle. Glycogen synthesis and breakdown are determined by the relative activities of glycogen synthase and phosphorylase. Activation of these two enzymes are reciprocally regulated by phosphorylation and dephosphorylation at specific sites on the enzymes. Glycogen mobilizing hormones such as epinephrine and glucagon trigger the intracellular cAMP-dependent protein kinase cascade through their respective transmembrane receptor and G proteins. Phosphorylation of glycogen phosphorylase and synthase at specific sites activates the former and inactivates the latter (reviewed by Cohen, 1993). In contrast, the mechanism of insulin stimulation of glycogen synthesis is mainly through activation of a serine protein phosphatase, PP1G, which has a catalytic subunit and a glycogen associated subunit. Dephosphorylation of glycogen synthase and phosphorylase activates the former and inactivates the latter, thus leading to a net synthesis of glycogen. PP1G is activated by

phosphorylation at a specific site through an insulin sensitive protein kinase, ISPK. On the other hand, ISPK was found to be inactivated by incubation with a protein serine phosphatase, PP2A, which suggested that phosphorylation was required for activation of the kinase. *In vitro* tests showed that MAP kinase was the only activator from muscle extracts. Thus it was suggested (but not demonstrated) that ISPK was activated directly by MAP kinase through phosphorylation. Thus the linkage between MAP kinase and insulin receptor was borrowed directly from the mitogenic signaling pathway; that is the MAPKK-MAPKKK and Ras activation system as reviewed in the previous part.

However, recent evidence has emerged to argue against the notion that the Ras-MAP kinase pathway plays an essential role in glycogen synthesis. First, in 3T3-L1 adipocytes, Ras and MAP kinase were activated to similar extent by insulin, EGF, and PDGF, while only insulin substantially increased glycogen synthesis, suggesting that activation of MAP kinase is not sufficient to activate glycogen synthesis (Robinson *et al.*, 1993; Wiese *et al.*, 1995). Second, co-expression of mutant mSos1 in CHO cells over-expressing the insulin receptor inhibited insulin activation of Ras and MAP kinase; however insulin-induced stimulation of glycogen synthase was not affected (Sakaue *et al.*, 1995). Therefore it is still an open question as to whether or not insulin shares some mitogenic signaling molecules to regulate the non-mitogenic metabolic actions. If Ras and MAP kinase are not sufficient to stimulate the glycogen signaling pathway, then what is the determining factor to initiate insulin specific metabolic actions?

Whether PI-3 kinase plays a role in the glycogen signaling pathway is inconclusive at the present time. The PI-3 kinase inhibitor, wortmannin, abolished both insulin activation

of glycogen synthesis as well as glucose transport. However, over-expression of a dominant negative mutant of the 85-kDa subunit of PI-3 kinase that lacks the binding site for the catalytic 110-kDa subunit in CHO cells over-expressing insulin receptor showed no effect on insulin stimulated glycogen synthesis, whereas the insulin regulated glucose transport and PI-3 kinase activity were attenuated (Sakaue *et al.*, 1995). These results suggested the existence of a Ras-independent and wortmannin-sensitive pathway for activation of glycogen synthase and this wortmannin-sensitive component may not be PI-3 kinase. Also, the results suggested that insulin stimulated glycogen synthesis and glucose uptake may be regulated by divergent pathways. A few evidences support that p70^{S6K}, one of the downstream effectors of PI-3' kinase is involved in the insulin regulation of glycogen synthesis. It was reported that rapamycin can block both the insulin activation of p70^{S6K} and glycogen synthesis in adipocytes (Shepherd et al, 1995). Consistently, the originally proposed regulatory kinase, ISPTK for PP1G activation, is structurally homologous (if not identical) to p70^{S6K}. One mechanism for insulin activation of glycogen synthase is through inactivation of glycogen synthase-3 (GSK3). Based on the concomitantly inhibition of wortmannin on PI3'-kinase, protein kinase B and insulin-induced inhibition of GSK3, it was suggested that such inhibition of GSK3 was mediated through protein kinase B (Cross et al., 1995)

As reviewed in the section on mitogenic pathways, the SH2 domain-containing protein tyrosine phosphatase, SH-PTP2, is required for activation of the Ras/MAP kinase pathway (Sakaue *et al.*, 1995). However, even after co-expressing the catalytically inactive mutant SH-PTP2 in a CHO-IR cell line, insulin still stimulated glycogen synthase to the similar extent as in the parental CHO-IR cell line. This suggested that SH-PTP2 and its

dependent Ras/MAP kinase pathway may not be necessary for insulin to regulate glycogen metabolism.

Facilitative Glucose Uptake.

Glucose is transported into cells by two distinct mechanisms: 1. The energy-dependent mechanism, such as the intestinal Na⁺/glucose transporter, and 2. The energy-independent pathway, namely the facilitative glucose transporter (Glut; reviewed by Bell *et al.*, 1993). The first facilitative glucose transporter was isolated from erythrocytes and its gene was cloned and designated as GLUT-1 (Mueckler, 1985). Since GLUT-1 was found at low level expression in liver and muscle, this suggested that glucose uptake by liver and muscle cells was carried out by other facilitative glucose transporters. Based on the belief that the facilitative glucose transporters might have conserved transmembrane regions, screening cDNA from different tissues with the GLUT-1 probe led identification of other facilitate glucose transporter-like genes, designated GLUT-2 through GLUT-5 (Burant, 1991). A model was proposed for the orientation of GLUT-1 in the plasma membrane which consists of 12 membrane spanning α -helical segments with intracellular carboxyl- and amino-termini (Mueckler, 1985). This model has been extended to all members of the GLUT family. The most conserved amino acid residues are located within the putative transmembrane domains and the *intracellular* loops between the transmembrane domains. Thus the facilitative glucose transporter is basically a glucose specific channel.

Of the five types of glucose transporter (GLUTs), only GLUT-4 is regulated by insulin and is expressed in skeletal muscle, heart and adipose tissues. Attempts to map the

insulin-response domains in GLUT-4 showed that the 30 amino acid residues at the carboxyl terminus are important for responsiveness in insulin stimulated translocation to the plasma membrane, while the amino-terminus of GLUT-4 functions as an internalization motif (Piper et al, 1993 and Garippa et al, 1994). It was originally proposed that insulin stimulated glucose transport by promoting translocation of a glucose transporter from cytoplasmic pools to the plasma membrane (Suzuki and Kono, 1980; Cushman and Wardzala, 1980). This model was well demonstrated by many experiments, including measuring distribution of an epitope-tagged GLUT-4 after insulin stimulation (Kanai et al, 1993). In adipocytes, both GLUT-4 and the brain type (GLUT-1) transporters are expressed, at different levels depending on the species organ of the adipocytes. In the mouse 3T3-L1 adipocytes, there are about 950,000 and 280,000 copies of GLUT-1 and GLUT-4, respectively (Calderhead *et al.*, 1990). This was determined by quantitative immunoblot analysis with specific antibodies for GLUT-1 and GLUT-4. Insulin was found to increase translocation of GLUT-1 and GLUT-4 about 6.5- and 17-fold, respectively. For rat adipocytes, most of the GLUT isotype expressed is GLUT-4 (90%) with a small amount of GLUT-1 (Bell et al., 1993). The signal transduction pathway of insulin regulated glucose transport has yet to be elucidated. However a few phenomenological facts have been well documented.

Insulin stimulated glucose transport is an acute and reversible response process. So the active components involved in the recruitment of GLUT-4 must fit into a very restricted time-frame between activation of the insulin receptor and the steady state of GLUT-4 translocation to plasma membrane (the detail kinetics will be discussed in section). Some of the possible components involved in this signal transduction leading to glucose transport are

reviewed here.

The initial component to be examined obviously would be the insulin receptor itself, and more specifically, whether the RTK activity of the insulin receptor is required for GLUT-4 translocation. Kinetic evidence showed that activation of the insulin receptor measured by autophosphorylation occurred prior to elevation of the insulin stimulated glucose transport rate (Kohanski *et al.*, 1986). Over-expression of tyrosine kinase-deficient mutant insulin receptor (Met-->Ile) failed to increase GLUT-4 translocation in rat adipocytes, confirming the essential role of the RTK in this signal transduction pathway (Quon *et al.*, 1994). The known immediate event after insulin receptor would be IRS-1. A few evidences have suggested IRS-1 has essential role in insulin stimulation of glucose transport (discussed late), however whether IRS-1 is indispensable in this action has been examined.

The targeted deletion of IRS-1 in mice showed intrauterine retardation of growth and reduction of insulin stimulated glucose transport both in liver and muscle cells (Araki *et al.*, 1994; Tamemoto *et al.*, 1994). The surprisingly large residual response to insulin was suggested to be due to another substrate, IRS-2, which may compensate for the loss of IRS-1. Indeed, IRS-2 is structurally functionally like IRS-1, and also serves to recruit PI-3 kinase and GRB2 (Araki *et al.*, 1994; Tobe *et al.*, 1995). From different angle to view these facts indicated that IRS-1 might be so important that retarded genes have been evaluated for preparing the possible lose of IRS-1. The major fact link IRS-1 to glucose transport is the recruitment of PI3'-kinase.

PI3'-kinase associates with tyrosine-phosphorylated IRS-1, IRS-2 and other tyrosine-phosphorylated proteins after insulin stimulation (Giorgino *et al.*, 1993; Myers *et al.*, 1992).

The intrinsic lipid kinase activity is dramatically increased after binding to tyrosine phosphorylated IRS-1. Several lines of evidence indicate that PI-3 kinase participates in signal transduction from insulin to GLUT-4 translocation. Wortmannin inhibits both insulin stimulated PI-3 kinase activation and GLUT-4 translocation with the same IC_{50} (Okada *et al.*, 1994; Kotani *et al.*, 1995). Micro-injection of the SH2 domain-deleted p85 subunit of PI-3 kinase blocked insulin stimulated glucose transport in 3T3-L1 adipocytes (Kotani *et al.*, 1995). Thus PI-3 kinase is presumably an intermediate in the signaling pathway of insulin-regulated glucose transport. However, can this conclusion be extended to say that activation of PI-3 kinase is sufficient for translocation of GLUT-4? Two reports came out with quite different conclusions. In CHO cells stably over-expressing the PDGF receptors, and in 3T3-L1 cells expressing epitope-tagged GLUT-4, PDGF was found to stimulate GLUT-4 translocation (Kamohara *et al.*, 1995). Using mutant PDGF receptors that altered the binding sites for either GAP, PLC- γ , or PI-3 kinase, respectively, only the PI-3 kinase binding-site mutant failed to activate GLUT-4 translocation in CHO cells. In this report, however, the activity of PI-3 kinase was not mentioned; moreover, insulin showed a much greater potential than PDGF to stimulate GLUT-4 translocation. Another report showed activation of PI-3 kinase in 3T3-L1 adipocytes and found that insulin, but not PDGF, markedly increased glucose uptake, whereas both hormones stimulated total tyrosine phosphoprotein-associated PI-3' kinase activities to similar extent (Wiese *et al.*, 1995). Thus it was suggested that activation of PI-3 kinase is not sufficient for the full stimulation of glucose transport in adipocytes, indicating that another factor is required to define insulin stimulated-glucose transport in adipocytes. However, their data is inconsistent with the conclusion. In the time

course of PI-3 kinase activation, only at 5-min point, PDGF and insulin stimulated PI-3 kinase to a similar extent. Insulin stimulated PI-3 kinase kept in its steady-state for up to 30 min (Wiese *et al.*), which is consistent with the kinetic pattern of insulin stimulated-glucose transport (Kohanski *et al.*, 1989). However, PDGF stimulated PI-3 kinase activity declined after 5 min and yielded levels of activation significantly lower than insulin stimulated PI-3 kinase activity. Because of this discrepancy, the issue of whether or not PI-3 kinase activation is sufficient to stimulate glucose transport in adipocytes remains unresolved. However, it has been shown that PI-3 kinase activation is required.

Another question would be whether the known components in mitogenic pathways are also used in the insulin stimulated glucose transport. Individual components of the Ras/MAP kinase pathway have been examined in order to evaluate their possible roles in the insulin stimulated glucose transport. As mentioned in last section, Ras is the activator for Raf. Introducing an oncogenically activated Raf-1 mutant into 3T3-L1 fibroblasts by retrovirus, leads to the expression of activated Raf-1 in differentiated 3T3-L1 adipocytes (Fingar *et al.*, 1994). Hexose uptake was found to be markedly increased in comparison to control adipocytes, or to those infected with the retroviral vector without the cDNA. It was found that total cellular GLUT-1 protein was increased about 5-fold, but in contrast, activated Raf-1 did not effect either expression of GLUT-4 or its cellular distribution. Thus it was shown that activation of Raf is not involved the insulin stimulated GLUT-4 translocation.

In the protein kinase cascade from Raf to MEK to MAPK, the dual specificity kinase MEK (that phosphorylates MAPK on threonine and tyrosine), shows unusually high

specificity because MAP kinase is the only substrate found so far. A specific inhibitor for MEK, PD98059, completely abolished insulin activation of MAPK and the downstream event of transcriptional activation of c-fos, measured in both 3T3-L1 adipocytes and L6 myotubes. (Lazar *et al.*, 1995). However phosphatidylinositol 3'-kinase as well as insulin-stimulated glucose uptake, lipogenesis, and glycogen synthesis were not affected. This shows that MEK is not involved in the glucose transport signaling pathway.

In adipocytes, EGF and PDGF were found to stimulate deoxyglucose transport by about 2-fold with an EC₅₀ of 0.1 and 0.4 nM, respectively. By contrast, insulin stimulated deoxyglucose transport about 16-fold. Both EGF and PDGF induce a 2-fold increase in GLUT-1 at the cell surface, but both these growth factors were no effect on GLUT-4 levels at the cell surface. In contrast, insulin induced a 13-fold increase in cell surface GLUT-4. However, insulin, EGF and PDGF all activate MAP kinase to similar extent. Thus it was suggested that activation of MAP kinase is not required for this process (Robinson *et al.*, 1993; Wiese *et al.*, 1995). P70^{S6K} was activated through PI3'-kinase pathway by insulin and many growth factors. However, p70^{S6K} does not appear to participate in the insulin stimulated glucose transport pathway, since rapamycin did not inhibit insulin activated glucose transport, despite the fact that the hormonal activation of p70^{S6K} was completely blocked by the drug (Fingar *et al.*, 1993).

An SH2 domain containing PTP named as Syp, SH-PTP2, PTP1D, was ubiquitously expressed including in adipocytes (extensively reviewed in chapter 2). The possible role of Syp in insulin stimulated glucose transport has been examined. Micro-injection of either the SH2 domain of Syp or anti-Syp antibodies into 3T3-L1 adipocytes inhibited the

insulin-stimulated expression of GLUT-1 (Hausdorff *et al.*, 1995). This indicated that the role of Syp is in the insulin stimulated mitogenic pathway, which regulates gene expression levels. Also, it suggested that insulin stimulates glucose transport by two distinguishable pathways: One is the Syp-Ras-MAP kinase dependent pathway, which functions by increasing glucose transporter synthesis. In contrast, the second pathway is the acute pathway, functioning through stimulated translocation of GLUT-4 to the cell surface, and may be independent of Syp. Furthermore, several growth factors, including EGF and PDGF, stimulated SH-PTP2 association with tyrosine phosphorylated proteins, while only insulin can stimulates GLUT-4 translocation. Thus SH-PTP2 may not be essential for activation of glucose transport.

Diverse inhibitors have been applied to probe the possible components in the signal transduction pathway of insulin-stimulated glucose transport. Okadaic acid, the inhibitor for protein phosphatase was a potent stimulator of glucose transport in 3T3-L1 adipocytes, however it prevented insulin stimulation of glucose transport (Jullien *et al.*, 1993). Surprisingly, in the presence of this phosphatase inhibitor, tyrosine phosphorylation of IRS-1 and association to PI-3 kinase were reported not to be observed. These results suggest that okadaic acid interferes with insulin's stimulation of glucose transport by inhibiting IRS-1 phosphorylation and its association with PI3-kinase and/or other signaling molecules. The rational behind this was not clear, because the tyrosine phosphorylation of IRS-1 is the key element in its role as a signal transducing molecule. Perhaps, okadaic may act through the serine phosphorylation of IRS-1. ML-9, an inhibitor of myosin light chain kinase, was reported to inhibit insulin stimulation of 2-deoxyglucose transport activity by 80% at 100

μM ($\text{IC}_{50} = 27 \mu\text{M}$) without affecting 2-deoxyglucose transport activity in the basal state (Inoue et al., 1993). ML-9 inhibited insulin-induced translocation of both GLUT-4 and GLUT-1 in a dose-dependent manner, but was without effect on the insulin receptor kinase activity. The process of insulin stimulated translocation of glucose transporters is similar to that of secretion which involves movement, docking and fusion of vesicles to the plasma membrane. This compound, ML-9, has been reported to block diverse vesicle movements, including platelet degranulation and catecholamine secretion (Saitoh *et al.*, 1986; Nagatsu *et al.*, 1987). Although the real target of the inhibitor to block glucose transporter is not identified, ML-9 was shown to inhibit p70^{S6K} which was shown to be dissociated from the insulin regulated glucose transporter, as discussed above.

In addition to insulin, the structurally similar insulin-like growth factor I (IGF-I) also stimulates glucose transport in adipocytes. The receptors for insulin and insulin-like growth factor I (IGF-I) have in common a high sequence similarity. Binding of either insulin or IGF-1 to their own receptors is highly specific as compared with binding to the respective other receptor (insulin receptor: $K_d = 3.6 \text{ nM}$ insulin *versus* a $K_i > 500 \text{ nM}$ IGF-I. IGF-I receptor, $K_d = 1.1 \text{ nM}$ IGF-1 *versus* a $K_i = 190 \text{ nM}$ insulin; Weiland et al, 1991). Although insulin is the only hormone has been found to stimulate glucose uptake in physiological condition (*in vivo*). For the cultured cell lines, both hormones have equal potentials to stimulate metabolic pathways such as glucose transport as well as stimulate a mitogenic response.

How the hypothesized insulin regulated GLUT-4 containing vesicles translocate between the plasma membrane (PM) and cytosolic light density microsome (LDM) is totally

unclear at the present time. Recently, a protein named celubrevin was shown to co-localize with GLUT-4 (Volchuk *et al.*, 1995). Cellubrevin and GLUT-4 both increase their expression in 3T3-L1 adipocytes after differentiation. Both cellubrevin and GLUT-4 were found to reside in the LDM in the basal state. Insulin stimulation decreased the levels of these two proteins in the LDM, and both proteins were found at increased levels in the PM. Immunopurified GLUT-4-containing vesicles were found to contain cellubrevin, and immunopurified cellubrevin-containing vesicles contained GLUT-4 protein. Another protein termed GTBP70 from rat adipocytes was found to bind to the GST fusion protein with GLUT-1, GLUT-2, and GLUT-4 but not to irrelevant fusion proteins (Liu *et al.*, 1995). GTBP70 was found to bind to the C-terminal domain and the central loop, but not to the N-terminal domain of GLUT-4. However cellular extracts from rat hepatocyte did not contain such a glucose transporter binding protein, which suggested its tissue specificity is correlated to insulin action in adipocytes. As mentioned in previous, the carboxyl terminal of GLUT4 was responsible for translocation of the transporter (Piper *et al.*, 1993).

In summary, the signal transduction pathway for the insulin specific acute actions such as glucose transport and early steps of glycogen synthesis, are largely undefined. It is known that the protein tyrosine kinase of the insulin receptor is required, and that the terminal step is GLUT-4 translocation to the plasma membrane. In this process, IRS-1 and/or IRS-2 are needed to recruit and activate PI-3 kinase, but they may have other functions. It is also not certain that activation of PI3'-kinase is sufficient, and that a parallel and necessary pathway remains to be discovered. We have attempted to shed light on possible answers to these questions.

MATERIALS AND METHODS

Culture and Differentiation of 3T3-L1 Adipocytes:

The following materials were purchased from GIBCO BRL: Dulbecco's Modified Eagle's Medium (DMEM, #12100-061), fetal bovine serum (FBS, #26140), and calf serum (CS, #). The following were purchased from Sigma: Streptomycin sulfate (.#S9137), dexamethasone (#D4902), methyl isobutylxanthine (#I5879) and penicillin G (#PEN-NA). The bovine insulin (Zn salt) at 28 U/mg was from E.Lily Co (#170BA5Q). Cell culture dishes were from Falcon (15-cm, #3005; 10-cm, #3003; 3.5-cm, #3001)..

The base medium (DMEM) was prepared from powder for 20 liters: after the powder dissolved, 74g sodium bicarbonate, 2g streptomycin sulfate, and 1.05g of penicillin G were added. The pH was adjusted to 7.3- 7.5 with HCl, and then sterile-filtered (0.22 μ bell filters) under positive pressure from a 10% CO₂/air gas tank; 0.9 or 1.8 liters were collected in autoclaved bottles. The medium was stored at 4 °C.

Additional supplements to DMEM required for differentiation were prepared as follows (per 0.9 liter of DMEM): 100 ml FBS, 1.0 ml of insulin at 1mg/ml (dissolved in 0.01 N HCL, sterile-filtered and stored at 4 °C), 1.0 ml 0.25 mM dexamethasone (dissolved in 95 % ethanol), and 10 ml of methylisobutylxanthine dissolved at 11.5mg/ml in water by titration of the initial suspension with small aliquots of 1N KOH, and then sterile-filtered. The methyl isobutylxanthine solution is not stored but is used immediately.

Growth factors:

Bovine serum albumin (Sigma, RIA-grade, #A-7888) was used as carrier for dilutions

of growth factors, at a concentration of 1mg/ml in Kreb's Ringer's Phosphate Buffer (KRP, described under the assay for glucose transport).

Insulin(Zn salt, 28 U/mg), from E.Lily Co.(#170BA5Q), was prepared at 1 mg/ml in 0.01 NHCl, giving a final stock concentration of 183 μ M, and was stored at 4°C. To make a final concentration at 100 nM for stimulation: the 183 μ M stock was diluted into 1/10, and 17 μ l of the diluted solution was added per 3 ml KRP/BSA.

Epidermal growth factor (EGF) was from Upstate Biotechnology Inc., and was dissolved to give a stock concentration of 100 ng/ μ l (about 1.67 μ M) in 10% acetic acid. This stock solution was stored frozen in aliquotes at -80°C; each aliquot was thawed only once. To prepare a 200 nM solution for stimulating cells, 36 μ l of stock was added to 3 ml KRP/BSA.

Insulin-like Growth Factor-1 (IGF-1) was purchased from Upstate Biotechnology Inc. (human recombinant, # 01-141). The lyophilized powder was supplied in 25 μ g aliquots and was dissolved in 250 μ l 0.2 M acetic acid, which gave a stock solution at 100 ng/ μ l (14.3 μ M). To make a solution at 100 nM for stimulation of cells, 21 μ l of the stock was added to 3 ml KRP/BSA.

Platelet Derived Growth Factor (PDGF, the bb isoform) was from Upstate Biotechnology Inc.(human recombinant, # 01-305). The 10 μ g lyophilized powder in each vial was dissolved in 1000 μ l 4 mM HCl with 1 mg/ml BSA (RIA-grade), which gave a stock concentration at 10 ng/ μ l (0.33 μ M). To make a final solution at 3 nM for stimulation of cells, 30 μ l stock was added to 3 ml KRP/BSA.

Inhibitors:

Wortmannin (CalBiochem, Cat.#681675), phenylarsine oxide (Aldrich, Cat.#40,249-4), and rapamycin (CalBiochem, Cat.#553210) were dissolved in DMSO (Fisher, Certified-grade, Cat.#D128-500) solution. Wortmannin was prepared as a 1 mM stock and stored in -20°C. PAO was prepared fresh before each use at a stock concentration of 24 mM. Before these drugs were applied to 3T3-L1 monolayers, the stock solutions were diluted into KRP containing BSA at 1mg/ml, to yield 80-fold concentrated solutions of each drug; typically 50 µl were used per 4ml on a 10-cm monolayer.

Cell Culture

The cell culture conditions and differentiation protocol of Frost and Lane (1985) was used, but cells were treated with the differentiation “cocktail” for longer than two days. 3T3-L1 fibroblasts were grown in monolayer cultures at 37 °C in 10% CO₂ in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% calf serum. Two days after confluence (day 0), monolayers were treated with a cocktail containing 1µg/ml insulin, 0.25µM dexamethasone, 1 mM methyl isobutylxanthine, in DMEM supplemented with 10% fetal bovine serum (FBS) for 2-3 days, followed by refeeding for with the same mixture for 2 days (day 5). Then the medium was replaced with DMEM supplemented with 10% FBS and containing insulin at 1µg/ml for 2 days (day 7), followed by another 2-day feeding with DMEM supplemented only with 10% FBS (day 9). Adipocyte morphology was well established by day 6. Cells were typically used on day 9 from start of differentiation; this protocol can produce more than 95% conversion of fibroblasts into adipocytes. The prolonged induction was used because it could “force” differentiation in monolayers where

the degree of confluence had been misjudged; *i.e.*, for those times when cell growth had not been fully contact-inhibited. This modified protocol was determined by trial-and-error after testing the solutions of insulin, dexamethasone, methyl isobutylxanthine, or resupplementing DMEM with fresh glutamine, and trying FBS from different suppliers. Different solutions of each component with different lots of each reagent did not promote uniform differentiation of 3T3-L1 monolayers, and therefore changing the length of treatment with the differentiation cocktail was tried and found to produce adipocytes in which insulin stimulated the uptake of glucose and where insulin receptor autophosphorylation and insulin receptor substrate -1 tyrosine phosphorylation were easily detected, as described below.

Treatment of 3T3-L1 Adipocytes with Growth Factors and Drugs:

3T3-L1 adipocyte monolayers in 10-cm dishes were washed three times with 8 ml PBS, each time, and then incubated in 4.0 ml serum-free DMEM for 2-3 hours at 37°C in an atmosphere of 10% CO₂. Monolayers were then transferred to a 37°C warm-room, and the DMEM was replaced by washing monolayers three times with 8ml pre-warmed KRP, each time. One of two protocols was followed to stimulate the adipocytes: Growth factor at the desired final concentration was present in the KRP/BSA applied to each monolayer after the washing, or growth factor was added to the KRP/BSA from a more-concentrated stock solution. Monolayers were incubated with growth factor for a selected time at 37°C. Monolayers for the basal-state condition were treated as above, but no growth factor was added.

Drugs were administered to the adipocytes by adding aliquots of concentrated stock solutions to give the desired final concentrations. Two control treatments of the monolayers

were done; *First*, neither carrier nor drug were used, and, *second*, a monolayer was treated with carrier (DMSO) in exactly the same dose and for the same time as in the test samples with growth factor present. For treatment of monolayers with or without drugs, controls were done that bracketed the time-frame of the experiment; *i.e.*, control monolayers were taken first and last over the period that an experiment was performed in the 37°C warm room

Preparation of Cytosolic and Membrane Fractions, and Whole Cell Lysates:

After treatment of adipocyte monolayers with growth factors and/or drugs at 37°C, all subsequent steps employed ice-cold buffers and/or were done on ice or in a 4°C cold-room. To harvest, monolayers were washed twice with ice-cold saline-tris-vanadate buffer (STV) containing 1.0 mM Na_3VO_4 , 50 mM NaCl and 50 mM Tris-base at pH 10.5 (STV/10.5) or with STV adjusted to pH 7.6 with HCl (STV/7.6) if co-immunoprecipitation experiments were to be performed after harvesting the cells; 5-ml washes were done for 10-cm dishes, and 10-ml washes were done for 15-cm dishes.

To prepare separate cytosolic and membrane fractions, cells were scraped from the dish in ice-cold STV (4ml per 10-cm dish, or 6 ml per 15-cm dish) and then homogenized with 15 strokes at high speed in a Potter/Elvehjem teflon-on-glass homogenizer. Cellular components were fractionated by centrifugation at 250,000xg (55.2Ti Beckman rotor in a Beckman L6 or L7 model centrifuge) for 1.5 hour at 4°C. The fat layer was removed by gentle aspiration. Supernatant were collected and immediately aliquotted for storage (-80°C), or immunoprecipitation, or mixed with sample preparation buffer for SDS-PAGE, as described below. These soluble fractions are referred to as cytosolic fractions.

The pellets in each centrifuge tube were extracted in 2 ml 1% Triton X-100 in STV

buffer by homogenization in a teflon-on-glass homogenizer, and the residual Triton-insoluble material removed by centrifugation at 250,000xg for 30 min. Additional fat solubilized by this procedure was aspirated and the Triton-solubilized material was aliquotted as described above. These Triton-soluble fraction are referred to as the membrane fractions.

To prepare “whole cell lysates” by detergent extraction, adipocyte monolayers that had been treated with or without growth factor for the desired time were washed twice with STV and then collected in STV with the following additions: 1% Triton X-100, 5 mM EDTA, 1 mM PMSF. Four ml of this lysis buffer was used for a 10-cm dish, and 6 ml was used for a 15-cm dish. Cells were gently homogenized by three hand-driven strokes of the pestle in a teflon-on-glass homogenizer, and then kept on ice for one hour. Lysates were then cleared by centrifugation at 8000 rpm in a Sorvall SS-34 rotor at 4°C. The solubilized fat layer was removed by aspiration. From each soluble infranatant (below the fat and above the insoluble pellet) 0.5-1.0 ml aliquots were taken for either immunoprecipitation or storage, and aliquots were also taken and mixed immediately with sample buffer for SDS-PAGE, as described below.

Immunoprecipitation and Co-immunoprecipitation Using Specific Antibodies:

Proteins were immunoprecipitated or co-immunoprecipitated using rabbit polyclonal antibodies or antisera raised against the entire protein or defined regions of each protein, as listed in Table 1. For samples prepared in STV/7.4, with or without non-ionic detergent, antibodies were added directly. For samples prepared in STV/10.5, the pH was lowered to 7-8 by the addition of 1M Tris HCl, and the pH was monitored by spotting small samples on pH test strips (Sigma); the exact pH of these samples was not determined. All steps were

performed at 4°C or on ice.

Immuno complex was formed by incubating samples taken from growth factor-treated or control cells (prepared as described above) with 2 µg of polyclonal antibody or 2 µl of antiserum. Incubations were done in 1.5 ml microcentrifuge tubes placed on a rocking platform at 4°C for 1 hour. Then 60 µl of a 50% suspension of Protein-A agarose, pre-washed and swollen in PBS containing 0.02% NaN₃, were added and rocked for an additional 1 hour. The Protein A-adsorbed immune complexes were washed 6 times with 1-ml each time of STV/7.4 containing 1% Triton X-100 and the residual solution from the last wash removed as thoroughly as possible by carefully aspiration through a gel-loading pipet tip. Bound proteins were eluted by heating in a boiling water bath for 3min in 80µl 1xLaemmli sample buffer containing 100 mM DTT. Proteins were resolved by SDS-PAGE and detected by Western blot with selected antibodies.

Detection of Specific Proteins by Western Blot:

Buffers for Western Blot: Tween-20 was from Sigma (#P7949) without any detectable differences in results. BSA for the blocking solutions was from Sigma (fraction V, #A6793) Methanol was HPLC-grade from Fisher (#UN1230). Other reagents have been listed in other sections.

Transfer buffer: The buffer composition is 25 mM Tris base, 192 mM glycine and 20 % methanol at a pH= 8.2-8.5. The buffer is stored at 4°C, and may be used for up to twelve blots. However, in the 4-liter Hoeffer electrotransfer tank, if the current is higher than 1.5 A at 100 volts then the buffer should not be used again.

TBST: The buffer composition is 50 mM NaCl, 20 mM Tris base at pH 7.4. Tween-20 was

present at 0.15 %. This buffer is prepared as a 10-fold concentrated stock solution and stored at -20°C.

TBS: The buffer composition is 50 mM NaCl, 20 mM Tris, pH 7.4.

Blocking Solutions: For antiphosphotyrosine Western blots using the monoclonal antibody "4G10" from Upstate Biotechnology Inc. (#321), the blocking solution was 10% BSA (Sigma, #A6793) in TBST. For a large nitrocellulose membrane (12x16 cm), 20 ml of blocking solution is required. For other monoclonal and/or polyclonal antibodies, the blocking solution was 10% (w/v) nonfat dry milk (Carnation brand; the powder stored at -20°C).

Enhanced Chemiluminescence: agents were from Kirkegaard & Perry Laboratory (Cat.#50-59-00). Five ml of each reagent were mixed and used per 12x16 cm membrane. Exposure times were varied empirically. All other steps were performed exactly according to the manufacturer's instructions.

The immunoprecipitation or co-immunoprecipitation of specific proteins were determined by Western blot with rabbit polyclonal or mouse monoclonal antibodies raised against selected proteins, as listed in Table 2. Details for detection of phosphotyrosyl proteins are given separately. All protocols are described for detection on nitrocellulose membranes (NCM) after electrotransfer from a standard 16x12cm slab SDS-PAGE. All incubation and rinsing steps were done at room temperature on a rocking platform with a fixed rocking speed (Techline, with a piece of flat cardboard taped to the rocker surface; about 40 "rocks" per minute) or an orbital shaker (New Brunswick Scientific) set at about 100 orbits per minute.

For detection with rabbit polyclonal antibodies, NCM were blocked with 25-50ml fresh 5% non-fat milk in TBST/0.1 for 1h at room temperature. The NCM were rinsed 3x20ml TBS, allowing 5 min for each rinse. Binding of primary antibodies - at the dilutions listed below - was done for 2h in 15 ml of 2.5% milk in TBST/0.1. The NCM were rinsed 3x20ml TBST/0.1, allowing 10 min for each rinse. Binding of 20µg of HRP-conjugated goat anti-rabbit secondary antibody was done in 15ml of 2.5% milk in TBST/0.1 for 1 hour. The NCM were rinsed 3x20ml TBST/0.1 and 2x20ml TBS, allowing 15 min for each rinse. Detection of bound antibody was done by enhanced chemiluminescence by mixing the two reagents in the kit in a final volume of 8ml; the NCM was treated with the ECL reagent in a 13x16.5cm flat container (Nalgene) and visualization was done on Kodak XS film for variable periods of time. The film was developed in a Kodak X-OMat 20 automated X-ray film processor. For detection with mouse monoclonal antibodies, the above protocol was followed, but the horseradish peroxidase conjugated second antibody was different.

The amounts of primary antibodies diluted in 15ml (see above) varied according to the individual antibodies used but were typically less than the manufacturer's suggestions when the antibody was from a commercial source; *i.e.*, the antibodies were more dilute than recommended and this generally gave a lower background with adequate detection. The amounts of each antibody or antiserum used are listed in Table 1.

Detection of Tyrosine Phosphorylation by Western Blot: Proteins from treated adipocyte monolayers containing SDS-PAGE sample buffer were resolved by SDS-PAGE (gel composition of 8%T and 3%C) at 170v for 4.5h or at 50v for 14h, at room temperature, followed by electrotransfer to NCM, as described below. All subsequent step are at room

temperature. The NCM was rinsed in water and blocked with 10 % BSA (Sigma Fraction V) in TBST/0.2 for one hour. Then the NCM was incubated with 0.2 μ g of anti-phosphotyrosine monoclonal antibody (Upstate Biologicals, Inc., clone 4G10) in 20 ml of 2.5 % BSA in TBST/0.2 for 2 hours. The NCM was washed with 2x20ml TBST/0.2, allowing 5 min for each wash. The rinsed NCM was incubated for 1.5h with horseradish-peroxidase conjugated goat anti-mouse IgG2b (Caltag, Catalog No. M32407) at 2 μ g in 20 ml of TBST/0.2 containing 2.5% BSA (w/v). The NCM was then washed 4x50ml TBST/0.2, once with water, and then once with TBS to reduce the amount of residual Tween-20 on the NCM. Enhanced chemiluminescence, as described above, was used to detect the bound α -PTyr. With this primary antibody, 6 ml each of reagents A and B from the kit were sufficient for two full-sized filters.

SDS-PAGE and Electrotransfer:

SDS-PAGE Stock Solutions: All chemicals are from Boehringer/Mannheim, except EDTA which was the tetrasodium salt from Sigma.

Acrylamide stock solutions: The standard stock solution was 50 % T (3 % C). Weigh 3 g bisacrylimide and 97 g acrylamide, were added to deionized water to 206.8 g, which gives a final volume of 200 ml. Variations in %C are made in a similar fashion.

pH 8.8 Buffer: 113.55 g of Tris base were dissolved in 450 ml water and 18ml of concentrated HCl. After cooling, adjust the pH to 8.7-8.9 with HCl and the volume to 500ml.

pH 6.8 Buffer: 37.85 g of Tris base were dissolved in 210 ml water and add 23 ml of concentrated HCl. After cooling, adjust the pH to 6.7-6.9 and the volume to 250 ml.

3X Sample Buffer: This three-fold concentrated buffer is mixed with aliquots of cell lysates

or extracts prior to SDS-PAGE. It is prepared from 0.27 g EDTA (6 mM) dissolved in 55ml water and 15ml of pH 6.8 buffer (above). 6g of SDS are dissolved, then 20ml of glycerol and 1ml of 1% bromphenol blue in water (w/v) are added. The volume is brought to 100ml, and the buffer stored at -20°C in aliquots. When present, DTT is added to a concentration of 100mM. One volume of buffer is mixed with two volumes of sample.

Running Buffer: The pH of this buffer is approximately 8.3; it is prepared without adjustment from the following reagents: Glycine, 259 g; Tris base, 54g; SDS, 18g; water to 18 liters.

Gel Compositions: The preparation of separating gels at the various densities used in this work and based on the system of Laemmli (1970), are composed from the above buffers and solutions as given in Table 3, below. After “casting” the separating gel, the solution is overlaid with water-saturated n-butanol and polymerization allowed to proceed for at least 30min. This layer and residual unpolymerized gel solution are removed before casting the stacking gel. To prepare the stacking gel for one of these separating gels, the following are mixed to give a volume of ~10 ml: 7.6 ml water, 0.8 ml 50%T, 1.0 ml Buffer pH 6.8, 0.1ml 10% SDS, 0.5ml 1% ammonium persulfate, and 0.02ml TEMED. The final acrylamide concentration is 4%T. These gels may be used 90 min after casting. Special conditions for gels are listed in the Figure legends; otherwise the standard separating gel was 8%T (2%C). Electrophoresis was typically 4-4.5h at 180-200v. Although the slab gels became warm during this procedure, the resolution of ERKs and most other proteins was typically improved, compared to overnight electrophoresis at low voltage.

2-deoxy-[U-¹⁴C]glucose uptake:

The method was described by Frost and Lane (1987), but all manipulations were conducted in a 37 °C warm room. Adipocyte monolayers in 35 mm culture dishes were washed 3 times with 3 ml of Krebs-Ringer phosphate-buffered saline (KRP: 128 mM NaCl, 4.7 mM KCl, 1.25 mM Mg₂SO₄, 10 mM Na₃PO₄, adjusted to pH 7.4, and then CaCl₂ was added from a concentrated stock solution to give a final concentration of 1.25mM), and then ≤0.95ml of KRP containing RIA-grade BSA at 1mg/ml was used to cover each monolayer; the final transport assay was done in a volume of 1.0ml, and therefore the exact volume was determined by the total number of additions to be made before adding 50μl of the stock 2-deoxy-[U-¹⁴C]-glucose. For controls in experiments involving growth factors only, 0.95ml was used; for controls involving both drugs and growth factors, 0.90 ml was used which allows for 50μl of DMSO “carrier” to be added; for monolayers to which only growth factor would be added, 0.90ml was used; for monolayers that would require pre-incubation with drug *and* then treatment with growth factor, 0.85ml was used.

When present, as indicated in the Figure Legends, drugs were added to monolayers 5-10min before the addition of growth factor, or 15-20min before the addition of 2-deoxy-[U-¹⁴C]-glucose for monolayers without growth factors (controls including the time of exposure to the drug). Insulin or other growth factors were then added to give final concentrations indicated in the Figure Legends. After a 10-min pre-incubation with growth factor, steady-state transport rates were determined by adding 2-deoxy-[U-¹⁴C]-glucose (0.5m C/mmol) from a 20-fold concentrated stock solution to a final concentration of 0.2 mM, followed by incubation for additional 10 min. Cells were then washed rapidly with

3x1.5ml ice-cold PBS and then dissolved in 1 ml of 1% Triton X-100 in water, and uniform aliquots were transferred to clean scintillation vials. 10 ml Liquescent was added and 2-deoxy-[U- ^{14} C]-glucose uptake was determined by scintillation counting in a Beckman LS9000 liquid scintillation counter.

PI-3 kinase assay:

The method of Fukui *et al.* (1989) was used. Adipocyte monolayers were treated with growth factors and/or drugs exactly as described above and in specific detail in the Figure Legends. Whole cell lysates were prepared in the following ice-cold buffer: 150 mM NaCl, 20 mM Tris-HCl, 1mM PMSF, 1mM Na₃VO₄, 1% Nonidet P-40, and 5 mM EDTA, pH7.5. Cytosolic fractions were prepared in the above lysis buffer without Nonidet P-40; in this case, after the lysates were vortexed for 10 sec, followed by centrifugation at 12,000 rpm in an SS-34 rotor in a Sorvall RC-5B high speed centrifuge and the dissolved fat was removed by aspiration, a 200 μ l aliquot of supernatant was mixed with 200 μ l of the Nonidet P-40 lysis buffer before adding antibodies.

Different antibodies were used for immunoprecipitation, depending upon the stimulating growth factor and the association to be monitored. Antibodies, such as α -PTyr (the affinity-purified antibody or the agarose conjugate), α -IRS1, α -EGFR or α -PDGFR (A/B) (2 μ l serum for α -IRS1, 4 μ g for other antibodies) were added individually to lysates and incubated for 2 hours at 4°C. Except for the pre-coupled anti-PTyr immunoadsorbed proteins, the other immune complexes were adsorbed to protein A-agarose as described above for immunoprecipitations prepared for SDS-PAGE and Western blots.

To prepare samples for PI3-kinase assays, the immune complexes adsorbed to Protein

A-agarose were washed by the following procedure: 4 times with 1ml Nonidet P-40 buffer as described above; once with 1ml PBS; once with 1ml LT buffer (0.5M LiCl in 20 mM Tris-HCl pH7.5); once with 1ml water; once with 1ml TEN buffer (20mM Tris-HCl, 1mMEDTA, 100mM NaCl, pH7.5) and once with 1ml TGN buffer (20mM Tris-HCl, 0.5mM EGTA, 100mM NaCl, pH7.5). PI-3 kinase activities in these washed immunoprecipitates were measured as follows: The immunoprecipitates were suspended in 25µl of "substrate solution" containing 24.5 µl of TGN buffer and 0.5 µl phosphatidylinositol at 20 mg/ml in DMSO. Reactions were started by adding 10µl of [γ - ^{32} P]ATP/MgCl₂ solution prepared from 0.5µl of [γ - ^{32} P]-ATP (>3000 Ci/mmol) and 9.5µl 77mM MgCl₂. After 15 min the reaction was stopped by addition of 150 µl extraction solution (chloroform:methanol:37% HCl, 100:200:2) and vortexed, followed by addition of 100 µl chloroform. The lower organic layer was taken and washed with 150 µl methanol:1N HCl (1:1). The lower layer was taken and vacuum dried in a Savant Speed Vac. Samples were dissolved in 20 µl of chloroform and spotted onto silica gel plates (Watman). Chromatography was performed in chloroform:methanol:30% ammonium hydroxide:H₂O (43:38:5:7). ^{32} P-labeled phosphatidylinositol was quantified on the dried plates using a Phosphorimager.

***In vitro* phosphatase treatment (for chapter II).**

Cytosolic and membrane fractions prepared as described above in STV/pH10.5. For the heat inactivation of endogenous phosphatase, DTT was added to a final concentration of 1 mM and samples were then heated at 80°C for 2 min. The pH was then titrated to 7.5 by addition of small aliquots of 1 M Tris-HCl; the change was monitored by spotting aliquots on broad-range and then narrow-range pH paper. Dephosphorylation reactions were done in

phosphatase buffer (PP Buffer) containing 20 mM Tris at pH7.4 with 0.5 mM $\text{Mn}(\text{CH}_3\text{CO}_2\text{H})_2$, 1 mM DTT and 0.5 mg/ml BSA (RIA grade); some adjustments to this buffer were made for different phosphatase. 30 μl aliquots of heat-inactivated samples were mixed with 5 μl of the different stock solutions of phosphatase at the dilutions stated below, or with 5 μl of PP Buffer as a control, and these mixtures incubated at 30°C for 20-30 min. The dephosphorylation reactions were terminated by adding 15 μl of 3-fold concentrated SDS-PAGE sample buffer. The different electrophoretic mobility forms of Sos1 and of IRS-1 were resolved by the specialized low-density crosslinked SDS-PAGE described below (8%T, 0.3%C), and detected by the Western blot protocol given above. Additional details pertinent to each experiment are described in the Figure Legends. The stock protein-phosphatase PP1, PP2A, PP2B were kindly provided by Dr. H.-C. Li, and stored frozen in 40% glycerol.

Image Processing and Quantitation: Figures were prepared by scanning on an Agfa color scanner and aquired through Photoshop. Quantification was done using several different programs that gave integrated densities over an area selected by the user.

Table 1**Antibodies for Immunoprecipitations**

The antibodies used for immunoprecipitations are given according to the amounts used per 0.5 ml of whole lysate. These amounts were determined from systematic trials with each antibody, or using a standard 2 μ g per immunoprecipitation if the resulting Western blot produced a sufficiently strong signal.

Antibody	Quantity ^a	Species ^b	Source
anti- β (IR) ^c	2-5 μ l	r-pAs	This lab with Dr. L.-H. Wang
anti-IRS1	2 μ l	r-pAs	Dr. J.Pierce (SA-70914)
anti-PDGFR	2 μ l	r-pAs	Upstate Biotechnology (Cat#06-132)
anti-EGFR	2 μ g	s-pAb	Upstate Biotechnology (Cat#06-129)
anti-SHC	2 μ g	r-pAb	Transduction Labs (Cat#S14630)
anti-Grb2	2 μ g	r-pAb	Santa Cruz (Cat#SC-255)
anti-Sos1	2 μ g	r-pAb	Santa Cruz (Cat#SC-256)
anti-SHPTP2	1.5 μ g	r-pAb	Santa Cruz (Cat#SC-280)
anti-ERK1	2 μ g	r-pAb	Santa Cruz (Cat#SC-94)
Anti-p85(PI3'K)	1 μ g	r-pAb	Santa Cruz (Cat#SC-423)
anti-PTyr (4G10)	2 μ g	m-mAb	Upstate Biotechnology (Cat#05-321)

^a Antisera were aliquoted by volume; antibodies from Transduction Labs were supplied at 2.5 μ g/ μ l; antibodies from Santa Cruz and from UBI were supplied at 0.1 μ g/ μ l. All antibodies were stored either at 4°C or at -20°C, according to the supplier's instructions.

^b r-pAs, rabbit polyclonal antiserum; r-pAb, rabbit polyclonal antibody (purified IgG); m-mAb, mouse monoclonal antibody (purified IgG); s-pAb, sheep polyclonal antibody).

^c The antigen was the purified cytoplasmic kinase domain of the human insulin receptor.

Table 2

Preparation of antibodies for Western blots

The primary and horseradish peroxidase-conjugated (HRPO) secondary antibodies used in Western blots are given according to the amounts used per 20ml of solution. The anti-PTyr antibody 4G10 was diluted in 4% BSA in TBST, and all other primary antibodies were diluted in 4% nonfat dry milk in TBST. The HRPO secondary antibodies were diluted in 4% BSA in TBST for use with 4G10 anti-PTyr antibody, or in 4% nonfat dry milk in TBST for all others. These dilutions were determined from systematic trials with each antibody.

Antibody	Quantity ^a	Species ^b	Source
Primary			
anti- β (IR) ^c	2-5 μ l	r-pAs	This lab with Dr. L.-H. Wang
anti-IRS1	2 μ l	r-pAs	Dr. J. Pierce (SA-70914)
anti-p70 ^{S6k}	2 μ l	r-pAs	Dr. J. Blenis
anti-Grb2	1 μ g	m-mAb	Transduction Labs (Cat.#G16720)
anti-Grb2	3 μ g		Santa Cruz (Cat.# SC255)
anti-SHC	5 μ g	r-pAb	Transduction Labs (Cat.#S14630)
anti-SHC	1 μ g	m-mAb	Transduction Labs (Cat.#S14620)
anti-Sos1	1.5 μ g	r-pAb	Santa Cruz (Cat.#SC-256)
anti-SHPTP2	1.5 μ g	r-pAb	Santa Cruz (Cat.#SC-280)
anti-ERK1	0.5 μ g	r-pAb	Santa Cruz (Cat.#SC94-C)
anti-PTyr (4G10)	0.3 μ g	m-mAb	Upstate Biotechnology (Cat.#05321)
HRPO Secondary			
anti-rabbit IgG	15 μ g	goat	CALTAG (Cat.#L42007)
anti-mouse IgG	3 μ g	goat	CALTAG (Cat.#M32007)
anti-mouse IgG-2b	3 μ g	goat	CALTAG (Cat.#M32407)

^a Quantities for dilution into 15ml of TBST with either BSA or nonfat dry milk, as described in the text. Antisera were aliquoted by volume; antibodies from Transduction Labs were supplied at 2.5 μ g/ μ l; antibodies from Santa Cruz and from UBI were supplied at 0.1 μ g/ μ l; HRPO secondary antibodies from CALTAG were supplied at 1 μ g/ μ l. All antibodies were stored either at 4°C or at -20°C, according to the supplier's instructions.

^b r-pAs, rabbit polyclonal antiserum; r-pAb, rabbit polyclonal antibody (purified IgG); m-mAb, mouse monoclonal antibody (purified IgG).

^c The antigen was the purified cytoplasmic kinase domain of the human insulin receptor.

Table 3**Laemmli Slab Gels: Separating**

To achieve slab gels for SDS-PAGE of specific crosslinking densities, solutions were mixed according to the amounts listed here. Each solution gives a final volume of 40ml, which is sufficient to prepare one standard slab gel of dimensions 1.5mm x 12.5cm height x 16cm width (or a volume of ~33 ml). Compositions are given for a fixed %T, and the %C can be varied according to the needs of the experiment.

Solution	Final Acrylamide Concentration (%T)				
	6	8	9	10	15
	Volume (ml)				
H ₂ O	24.6	23.2	22.8	21.6	17.6
Buffer pH 8.8	8.0	8.0	8.0	8.0	8.0
Acrylamide (50%T)	4.8	6.4	7.2	8.0	12.0
10%SDS (w/v)	0.4	0.4	0.4	0.4	0.4
1% AmPer ^x (w/v)	2.0	2.0	2.0	2.0	2.0
TEMED	0.03	0.02	0.02	0.02	0.01

^x AmPer, ammonium persulfate.

HYPOTHESIS AND EXPERIMENTAL DESIGN

The initial goal of this study was to identify insulin-regulated tyrosine phosphoproteins in 3T3-L1 adipocytes. The expected outcome after insulin stimulation would have been increased tyrosine phosphorylation of substrates of the insulin receptor, in addition to the autophosphorylation of the receptor, itself. Indeed, a few substrates of the insulin receptor such as IRS-1 and the structurally related molecule IRS-2, as well as Shc proteins, have been established in this and other cell types. In contrast, we have attempted to identify the opposite event in 3T3-L1 adipocytes: The insulin stimulated dephosphorylation of phosphoproteins by PTP activation. Thus we looked for basally tyrosine-phosphorylated proteins that would undergo a decrease in their levels of tyrosine phosphorylation during insulin stimulation. Since insulin and growth factors share many but not all signal transduction pathways, it was thought that an insulin-specific dephosphorylation might be part of, or the distinguishing event in an insulin-specific pathway. Stimulation of glucose transport is a well-documented acute action of insulin in adipocytes. Therefore we have attempted to analyze whether any observed insulin-stimulated phosphotyrosine dephosphorylations could fit criteria that would permit it to be included in that signal transduction pathway.

To assay for phosphotyrosine dephosphorylations, we took advantage of a sensitive monoclonal anti-phosphotyrosine antibody to recognize phosphotyrosyl proteins, and thus to observe their dephosphorylation. We also examined the growth factor specificity

and the effects of various inhibitors on the observed dephosphorylation, as the best available criteria for assessing a potential role in the signal transduction pathway leading to accelerated glucose transport. Because some of our observations differed from those already reported in the literature, we re-examined inhibitor effects and growth factor specificity for stimulating glucose transport in 3T3-L1 adipocytes.

RESULTS

1. pp67 Dephosphorylation.

1.1. Tyrosine Phosphorylation in Adipocytes.

To monitor the changes in phospho(tyrosyl)proteins in adipocyte proteins after various treatments of the cells in monolayer culture, we used a monoclonal antiphosphotyrosine antibody for Western blots. In looking for insulin-stimulated changes that might be important for glucose transport activation, we challenged cells with insulin long enough for glucose transport to reach a steady-state, by which time any changes required for that activation process should have been complete. It was shown previously that this time would be 10-20 min for both the glucose transport rate (Frost et al., 1987; Frost and Lane, 1985) and the autophosphorylation of the insulin receptor (Kohanski et al., 1986). To maintain a consistent set of experimental conditions, we also stimulated adipocytes with other growth factors for the same time period.

Our initial attempt to detect phosphotyrosine dephosphorylation was from whole lysates made in the presence of detergents. The results, shown in the anti-PTyr Western

blot in Figure 1, revealed multiple tyrosine-phosphorylations when compared to the basal state. Some of these tyrosine phosphorylations have been reported in this and other cell systems. The evidence for the identifications listed here will be given later, but we know that the following phospho(tyrosyl)proteins co-migrate at the positions marked on the Figure: PDGF receptor at position a; EGF receptor at position b; IRS-1 at position c; β -subunit of the insulin receptor at position d. These are important because they demonstrate that the receptors for each hormone in these cells became tyrosine-phosphorylated (presumably autophosphorylated) when challenged with its respective ligand, and not with another hormone. The absence of IGF-1 receptor tyrosine phosphorylation, but the occurrence of IRS-1 tyrosine-phosphorylation after incubating with IGF-1, might suggest either that IGF-1 acts through the insulin receptor, or its own receptor is not present at a high level. This will be considered in more detail below. However, we did not find clear evidence for phosphotyrosine-*de*phosphorylation using these experimental conditions for lysing the cells.

The cell-lysis conditions were then altered in an attempt to reveal possible dephosphorylations. Two factors governed our decision of what changes to make because we anticipated a need to use two-dimensional iso-electric focusing/SDS-PAGE to resolve phosphotyrosyl proteins. *First*, sample-loading for the first dimension would be done at the "basic" end of the isoelectric focusing gel. Therefore the samples were prepared at elevated pH so that an adjustment would not be necessary later. Also, this should reduce changes, after lysis, in phosphorylation states generated in the cells since there are not many kinases or phosphatases that function efficiently at $\text{pH} \geq 10$. *Second*, we separated

the cytosolic and membrane fraction because membrane proteins are not likely to be well-focused since they are variably glycosylated. We also hoped that this would reduce the background immunoreactivity in a one-dimensional analysis. The results of these two changes are shown in Fig.2 for samples prepared from basal and insulin-stimulated cells. In general, the patterns of tyrosine-phosphorylation were more distinct in the cytosolic fractions. At pH 10.5, there appeared to be a greater abundance of phospho(tyrosyl)proteins; this may either be due to the assumption stated above about reduced phosphatase or/and protein kinase activities, or it might be due to the improved cell - and possibly organelle - lysis that could occur under strongly basic conditions. Most importantly we observed the apparent dephosphorylation of a 67kDa protein.



Figure 1. Growth Factor Stimulated Changes in Tyrosine Phosphorylation of 3T3-L1 Adipocyte Proteins Analyzed in Triton X-100 Lysates. 3T3-L1 adipocyte monolayers were serum-starved for 2.5h and then challenged with the indicated growth factors for 20 min. The monolayers were lysed in STV buffer containing 1.2% Triton X-100 (w/v), the insoluble material removed by centrifugation, and the solubilized proteins resolved by SDS-PAGE (8% T, 3% C). Phosphotyrosyl proteins were detected by Western blot with a monoclonal antibody against phosphotyrosine, as described in detail under Experimental Procedures. The relative molecular masses are given on the right (kDa) and the relative mobilities of selected prominent phosphotyrosyl proteins are indicated by lower case letters on the left, and are discussed in the text. The concentrations of growth factors used were: Basal, no growth factor added (i.e., control); insulin, 100 nM; EGF, epidermal growth factor at 200 nM; PDGF, platelet-derived growth factor at 3 nM; IGF-1, insulin-like growth factor-1 at 100 nM.

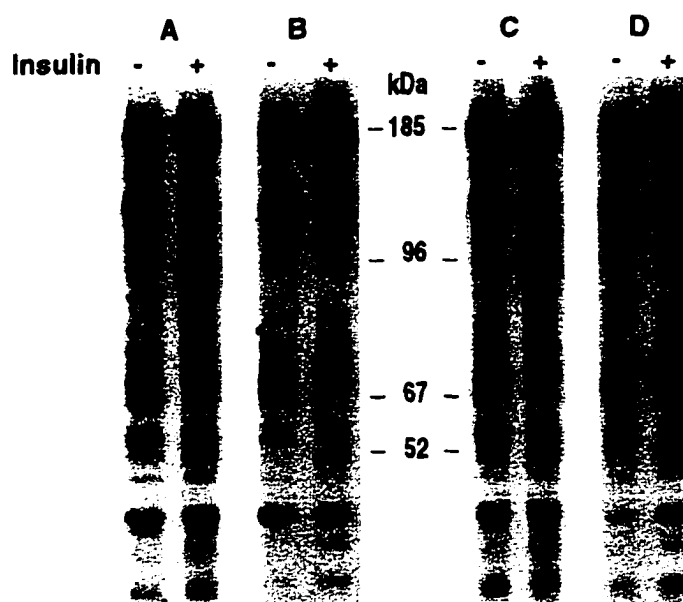


Figure 2. Comparison of Phosphotyrosyl Proteins in Triton X-100 Lysates versus Cytosolic Proteins, Prepared at pH 7.6 versus pH 10.5. 3T3-L1 adipocyte monolayers were serum-starved for 2.5h and then challenged - or not - with 100nM insulin for 20 min. Cells were lysed in Triton X-100 containing STV buffer (*panels A and C*) and the cleared infranants (below the layer of fat near the meniscus and above the pellet of insoluble material) were referred to as "lysates." Cytosolic fractions were prepared in STV buffer (*panels B and D*) as described in detail under Experimental Procedures. The pH of the STV buffer was either 7.6 (*panels A and B*) or 10.5 (*panels C and D*). Phosphotyrosyl proteins were detected by Western blot after separation of proteins by SDS-PAGE.

1.2. A Survey of Growth Factor Effects on the Dephosphorylation of pp67.

The detection of phospho(tyrosyl)proteins benefitted tremendously from the separation of cytosolic and particulate fractions, and we have obtained clear results showing several insulin-induced changes in tyrosine phosphorylation. Furthermore, we have observed an apparent dephosphorylation of a 67kDa cytosolic phospho(tyrosyl)protein, stimulated by insulin. We then tested for growth factor specificity in pp67 dephosphorylation (Fig. 3). The Western blot shows that in the basal state, pp67 was clearly tyrosine- phosphorylated. In response to insulin stimulation, the tyrosine phosphorylation state of pp67 decreased significantly, whereas EGF and IGF-1 had weaker effects. Because of the background tyrosine-phosphorylation observed in response to PDGF it is difficult to determine clearly whether or not PDGF promoted pp67 dephosphorylation. However, preliminary work on the purification of this protein indicates that PDGF also stimulates the dephosphorylation of pp67 about as well as does EGF (Kohanski and Dao, unpublished observations). The experiment shown in Fig. 3 has been repeated several times, and we have consistently obtained the same spectrum of pp67 dephosphorylation by this panel of growth factors. Furthermore, pp67 appears to be the only phospho(tyrosyl)protein observed, by this single-dimensional SDS-PAGE analysis, to undergo significant dephosphorylation among proteins recovered in the cytosolic fraction.

We then examined the membrane fractions from these stimulated cells to determine if pp67 was present in the membrane compartment either before or after stimulation, or even if we could detect pp67 dephosphorylation in this compartment

as well as in the cytosolic fractions. As shown in Fig. 4, we did not detect pp67 in either the basal-state membrane fraction, nor did it appear following stimulation with any of the four growth factors tested. Also, as indicated in the Figure, we did observe the tyrosine-phosphorylation of the insulin receptor's β -subunit in response to insulin, as well as proteins later identified as the EGF receptor and the PDGF receptor following stimulation with EGF or PDGF, respectively (see Chapter 2). In addition, it appears that some phosphotyrosyl-IRS-1 was also present in the membranes after insulin stimulation. The phospho(tyrosyl)protein migrating at about 96 kDa, following stimulation with IGF-1, is likely the phosphorylated IGF-1 receptor β -subunit.



Figure 3. Growth Factor Stimulated Changes in Tyrosine Phosphorylation of 3T3-L1 Adipocyte Cytosolic Proteins: Dephosphorylation of pp67. 3T3-L1 adipocyte monolayers were serum-starved for 2.5 h and then challenged with the indicated growth factors for 20 min. The monolayers were then harvested in STV at pH 10.5, the cytosolic fraction prepared, and phosphotyrosyl proteins detected by Western blot, as described in the Experimental Procedure. Relative molecular masses are indicated on the right (kDa), and one prominent basally tyrosine-phosphorylated protein of 67kDa is indicated by the arrow (pp67). The concentrations of growth factors used were: Basal, no growth factor added (i.e., control); insulin at 100 nM; EGF, epidermal growth factor at 200 nM; PDGF, platelet-derived growth factor at 3 nM; IGF-1, insulin-like growth factor-1 at 100 nM.

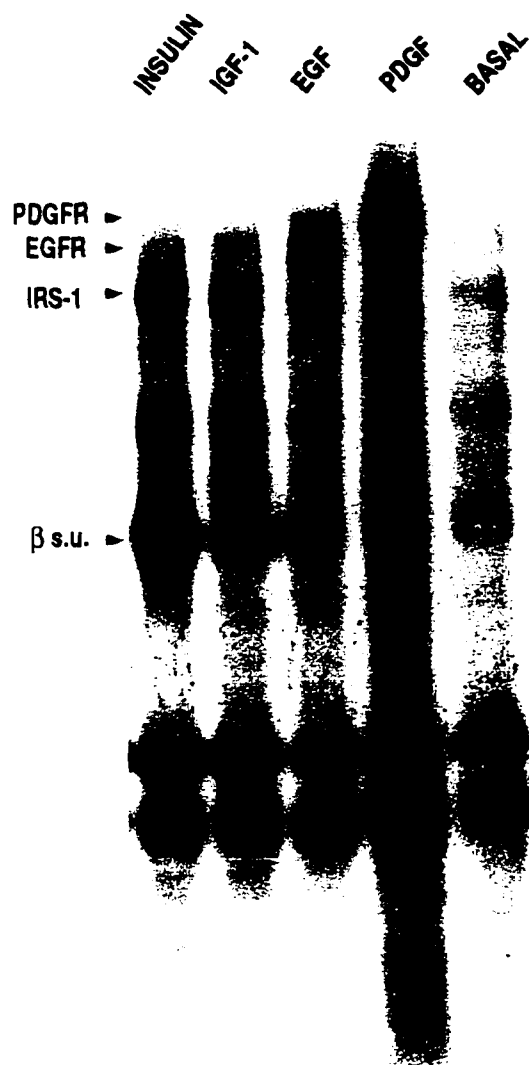


Figure 4. Growth Factor Receptor Autophosphorylation Analyzed in Membrane Fractions of 3T3-L1 Adipocytes. Membrane fractions were prepared from the growth factor-stimulated 3T3-L1 adipocyte monolayers described in Figure 3. These were solubilized in 1.2 % Triton X-100 (w/v) and the phosphotyrosyl proteins were detected by Western blot after SDS-PAGE. Mobilities of the respective phosphotyrosyl-receptors and IRS-1 are indicated.

1.3. Growth Factor Effects on IRS-1.

Since autophosphorylation of the IGF-1 receptor β -subunit was relatively weak, we re-examined effects downstream of IGF-1 receptor. Specifically, we measured the tyrosine-phosphorylation and the electrophoretic mobility-shift of IRS-1 after growth factor stimulation (Fig.5). Our findings demonstrated that IGF-1 was as potent as insulin in promoting both the tyrosine-phosphorylation and the change in electrophoretic mobility of the substrate IRS-1; the conversion of the "a" to "b" forms (indicated in Figure 5) was nearly complete in the presence of either insulin or IGF-1. These electrophoretic mobility differences have been reported by others to be due to Ser/Thr phosphorylation (Sun et al., 1992). The fact that receptor β -subunit tyrosine-phosphorylation was weak in response to IGF-1 and strong in response to insulin, but that each growth factor produced equally strong responses in IRS-1, clearly supports the conclusion that IGF-1 and insulin were acting through their respective receptors. In addition, the selective phosphorylation of the 52kDa isoform of Shc in response to insulin but not in response to IGF-1 (Fig. 3) further demonstrated that these two related hormones acted through their respective receptors. As expected, EGF and PDGF did not stimulate either the tyrosine phosphorylation or the electrophoretic mobility shift of IRS-1.

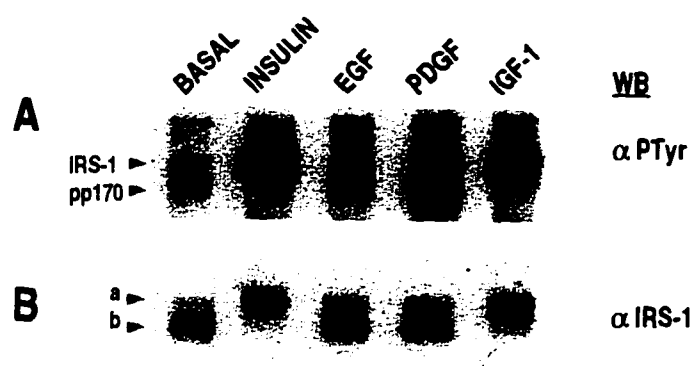


Figure 5. Growth Factor Effects on IRS-1 Tyrosine-Phosphorylation and Electrophoretic Mobility. Cytosolic fractions from growth factor-treated 3T3-L1 adipocytes were the same as those shown in Figure 3. *Panel A.* Tyrosine-phosphorylation of IRS-1 was examined by Western blot with antiphosphotyrosine monoclonal antibody. *Panel B.* The above blot was stripped and reprobed with a rabbit polyclonal antibody against IRS-1. The two different mobilities of IRS-1 under these electrophoretic conditions are indicated as "a" and "b" on the left. Further details are given in the text.

2. Characterization of Insulin-Stimulated pp67 Dephosphorylation.

2.1. The Insulin Concentration Dependence for pp67 Dephosphorylation.

A major criterion for any biochemical event to be downstream of receptor autophosphorylation and upstream of the physiological endpoint is the dose-response curve for hormonal stimulation. The dose-response for insulin-stimulated dephosphorylation of pp67 showed an $E_{50} \approx 1$ nM (Fig. 6). In the membrane fractions from these same samples, we measured an $EC_{50} \approx 5$ nM for insulin-stimulated autophosphorylation of its receptor's β -subunit, monitored with anti-PTyr from the immunoprecipitated β -subunit. These observations are in good agreement with the previously published data from 3T3-L1 adipocytes (Kohanski et al., 1986): an $E_{50} \approx 2$ nM for stimulation of the glucose transport rate, and an $E_{50} \approx 5$ nM for receptor autophosphorylation, which was measured by incorporation of $^{32}P_i$ in the receptor's β -subunit, whereas here we have used immunoreactivity with anti-PTyr antibodies. Therefore, pp67 dephosphorylation has a dose-response curve for insulin similar to that already published for insulin-stimulated glucose transport, which is a physiologically significant endpoint downstream of receptor autophosphorylation. This conclusion therefore meets an important criterion that is necessary but *not* sufficient to link pp67 with insulin-stimulated glucose transport.

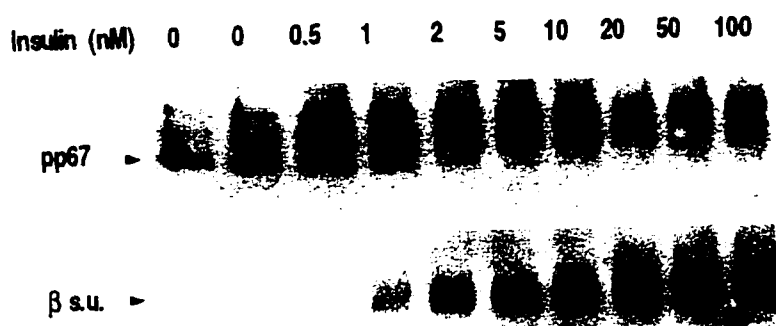


Figure 6. Titration of pp67 Dephosphorylation and Insulin Receptor Autophosphorylation. The insulin dose-dependence of pp67 dephosphorylation, and as a control the dose-dependence of insulin receptor β -subunit autophosphorylation, were assayed by anti-phosphotyrosine Western blot. 3T3-L1 adipocytes were serum-starved for 2-2.5 h and then stimulated with the indicated concentrations of insulin for 15 min. *Upper panel:* pp67 dephosphorylation was detected in cytosolic fractions by Western blot with anti-PTyr. The cytosolic fractions were prepared at pH 10.5, as described in the text. *Lower panel:* Autophosphorylation of the insulin receptor β -subunit detected after immunoprecipitation from whole lysates prepared in STV at pH 10.5 in 1.2% Triton X-100. The pH of the lysates was adjusted to 7.8 before immunoprecipitation of the insulin receptor with rabbit polyclonal anti-kinase domain antibodies. The immunoprecipitated proteins were resolved by SDS-PAGE, and autophosphorylation of the β -subunit was detected by Western blot with anti-PTyr.

2.2. The Time Course for Insulin-Stimulated pp67 Dephosphorylation.

Another major criterion for a functional relationship between biochemical events and physiological processes is the time-frame in which they occur. The time-window during which regulatory intermediates could change or accumulate is “open” between the time insulin is added to cells and the time when the steady-state for glucose transport rate is reached (Kohanski et al., 1986): Receptor autophosphorylation reaches its steady-state level within one minute after the addition of insulin, and the increase in glucose transport rate is not observable during the first 1-2 minutes after insulin is added. The steady-state rate of glucose transport is typically achieved between 5-10 min after stimulation with insulin. The dephosphorylation of pp67 apparently took place within this window (Fig. 7). In fact, there was a definite delay of 2 min before the dephosphorylation was observed in the anti-PTyr Western blot. The steady state of pp67 dephosphorylation was reached at 6 min after insulin was added and remained up to 20 min. In addition, we noted that other proteins became tyrosine-phosphorylated after some delay, as well (Shc and ERK tyrosine-phosphorylation are described in Chapter 2). However, the tyrosine-phosphorylation of IRS-1 appeared to commence without any lag period. To confirm that insulin stimulated these cells normally, we determined the course of insulin receptor β -subunit tyrosine-phosphorylation in the membrane fractions from these cells (Fig. 8). A 96 kDa protein, shown to be the β -subunit of the insulin receptor by immunoprecipitation with anti-kinase domain antibodies (see Chapter 2) was observed to become immediately tyrosine phosphorylated within 30 second after stimulation, which was the first time-point taken. Furthermore, the steady-state of β -subunit phosphorylation was maintained up to 20 min in the continued presence of insulin.

(The appearance of phosphotyrosyl-IRS-1 in the membrane fractions will be discussed in Chapter 2). The time-courses of insulin receptor autophosphorylation in the membrane compartment, and the progress of IRS-1 phosphorylation and pp67 dephosphorylation are summarized for comparison in Fig. 9. The relative amounts used for presentation of these results are used only for clarity in the Figure and do not reflect the different amounts of tyrosine-phosphorylation of each phosphoprotein. From this analysis, it is clear that insulin-stimulated dephosphorylation of pp67 meets a second criterion for the signaling pathway leading to activation of glucose transport: The time course of pp67 dephosphorylation occurs within the time-window between stimulation of insulin receptor autophosphorylation and the increase in the glucose transport rate.

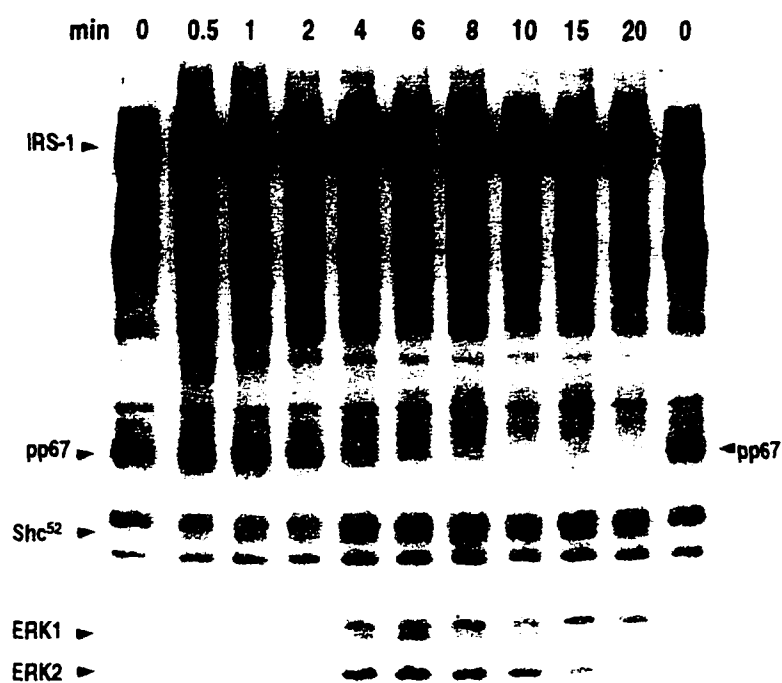


Figure 7. Time Course of Insulin-Stimulated pp67 Dephosphorylation. 3T3-L1 adipocytes were stimulated with insulin for times ranging from 0-20 min, and then cytosolic fractions were prepared in STV/10.5. The proteins were resolved by SDS-PAGE and the presence of phosphotyrosyl proteins was assessed by Western blot with antiphosphotyrosine antibodies. Relative molecular masses are indicated on the right (kDa) and the mobilities of pp67, IRS-1, and several other phosphotyrosyl proteins, established in the text, are indicated on the left. This Western blot is representative of two experiments.



Figure 8. Time Course of Insulin-Stimulated Insulin Receptor β -Subunit Autophosphorylation. 3T3-L1 adipocytes were stimulated with insulin for times ranging from 0-20 min, and then membrane fractions were prepared in STV/10.5. These were solubilized in 1.2 % Triton X-100 (w/v) in STV/10.5, the proteins were resolved by SDS-PAGE, and the presence of phosphotyrosyl proteins assessed by Western blot with antiphosphotyrosine antibodies. Relative molecular masses are indicated on the right (kDa) and the mobilities of the insulin receptor β -subunit, IRS-1, and one unidentified phosphotyrosyl protein are indicated on the left. This Western blot is representative of two experiments.

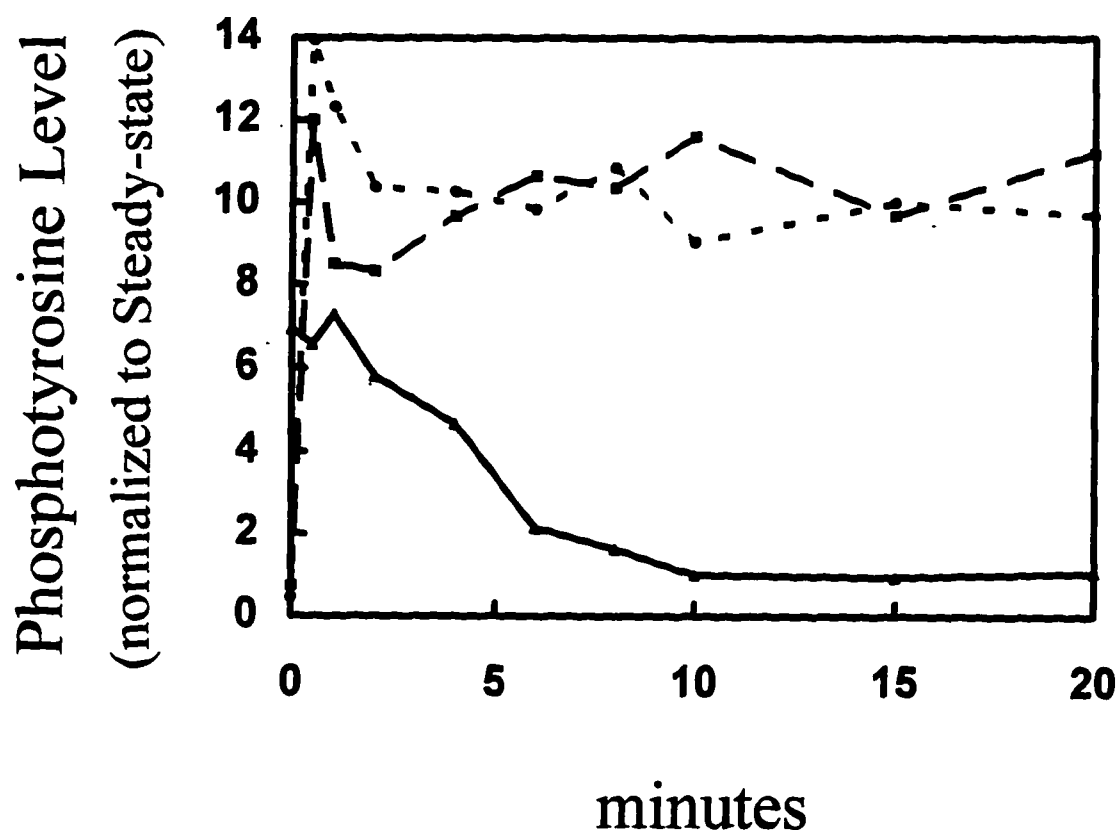


Figure 9. Summary of Time-dependent Insulin-Stimulated Changes in Tyrosine Phosphorylation. The relative levels of phosphotyrosine of the insulin receptor β -subunit (■), of IRS-1 (●), and of pp67 (▲) were determined by integrated densitometry of the Western blots (as shown in Figs. 7 and 8, but at different exposures to be within the estimated linear range of the X-ray film used in the ECL protocol). For each time point, the densities were normalized to a maximum value: zero-time for pp67; 0.5min for IRS-1 and insulin receptor.

2.3. The Inhibition of pp67 Dephosphorylation by Phenylarsine Oxide.

If the apparent decrease in tyrosine phosphorylation of pp67 is due to a PTP, it would be expected that a PTP inhibitor would block the insulin-stimulated dephosphorylation of pp67. We did test the effect of pervanadate, a powerful inhibitor for a wide spectrum of PTPs. We found that tyrosine phosphorylation was globally increased. However, since antibodies for pp67 are not available at the present time, we could not determine whether pp67 dephosphorylation was inhibited by pervanadate, due to this high background of tyrosine phosphorylation (results not shown).

We have also tested the effect of phenylarsine oxide (PAO), which is also a known PTP inhibitor, on pp67 dephosphorylation. Furthermore, PAO blocks insulin's effect on glucose transport activation although it has no effect on insulin receptor auto- or substrate phosphorylation (Frost et al, 1987). Therefore, the ability of PAO to block activation of the PTP involved in pp67 dephosphorylation was examined over the concentration range already known to block activation of transport. The inhibition observed had an IC_{50} between 2-10 μ M PAO (Fig. 10). This corresponds quite well with the published value for inhibition of insulin-stimulated glucose transport ($IC_{50} \approx 7\mu$ M; Frost and Lane, 1985). Therefore, the PTP that is responsible for pp67 dephosphorylation and that is activated by insulin is sensitive to PAO over the same concentration range observed to inhibit the activation of glucose transport.

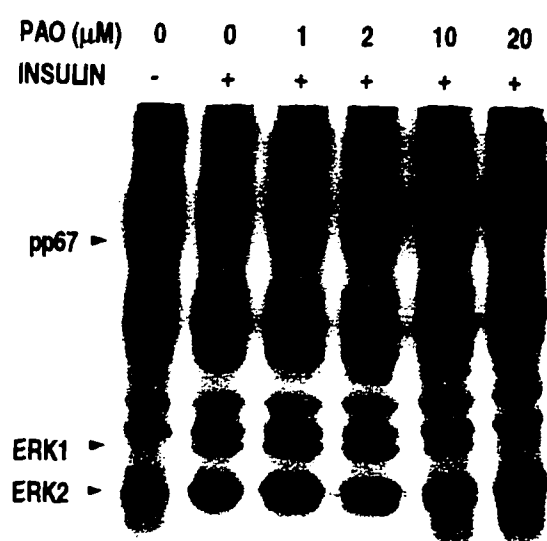


Figure 10. Effect of Phenylarsine Oxide on Insulin-Stimulated pp67 Dephosphorylation. 3T3-L1 adipocytes were serum-starved for 2 h, then exposed for 10 min to phenylarsine oxide (PAO) at the indicated concentrations, and subsequently challenged, or not, with 100 nM insulin for an additional 10 min. Cytosolic fractions were prepared from monolayers harvested in STV/10.5, the proteins resolved by SDS-PAGE, and phosphorylation on tyrosine detected by Western blot with anti-PTyr. The mobilities of phosphotyrosyl pp67 and ERK-1 (see Chapter 2) are marked.

2.4. Tests for Inhibition of pp67 Dephosphorylation by Wortmannin and Rapamycin.

The dose-response and time-course data for pp67 dephosphorylation, and the sensitivity to inhibition by PAO, are similar to those for insulin-stimulated glucose transport in these 3T3-L1 adipocytes. The results from the growth factors do not support, but also do not contradict, a linkage between pp67 dephosphorylation (or activation of its PTP) and the stimulation of glucose transport in these cells. Conclusive evidence to rule out a link would be found in the inhibition of pp67 dephosphorylation by an inhibitor that does *not* block the activation of glucose transport. On the other hand, failure of an inhibitor that blocks activation of glucose transport to also block pp67 dephosphorylation would not rule out a linkage, because activation of the PTP may be above the step inhibited in the signaling pathway, or it may be on a colinear and independent pathway. Specifically, in the former case, rapamycin does not inhibit glucose transport (Fingar et al., 1993) although it is a potent inhibitor for p70^{S6k} activation by insulin and many other factors (Chung et al., 1992). In the latter case, wortmannin *does* inhibit activation of glucose transport, through its ability to inhibit activation of PI3'-kinase at nM levels (Okada et al., 1994; Clarke et al., 1994). Based on these observations, a possible colinear pathway is one that is required for the activation of glucose transport but is not involved either upstream or downstream of PI3'-kinase.

We did not observe any significant inhibitory effect of 50 nM wortmannin on pp67 dephosphorylation (Fig. 11A). Furthermore, there was no effect of wortmannin on IRS-1 tyrosine phosphorylation (Fig. 11B). Since p70^{S6k} was demonstrated to be downstream of PI3'-kinase, as described in the Introduction, we tested for the effectiveness of wortmannin by assaying for inhibition of p70^{S6k} activation as reported by a growth factor-dependent

electrophoretic mobility shift (Chung *et al.*, 1994). Wortmannin was shown to produce partial inhibition of p70^{S6K} at 5 nM, but we did not find full inhibition even at 50 nM drug (Fig. 11C). The discrepancy between the reported IC₅₀ for wortmannin inhibition of p70^{S6K} (Chung *et al.*, 1994) and our results could be due to different cell types or effectiveness of the drug due to its preparation or storage. Therefore, to establish that our reagents were effective, these solutions of wortmannin were used to inhibit insulin-stimulated glucose transport (Fig. 11D). An IC₅₀ of 4 nM was observed for wortmannin inhibition of glucose transport. The observed near-complete inhibition of stimulated glucose transport at 50 nM demonstrated that the wortmannin retained its potency in our hands similar to that reported originally by Okada *et al.* (1994). In addition, *in vitro* titrations, using these same solutions of wortmannin, showed full potency in the inhibition of IRS-1-associated PI3'-kinase; an IC₅₀ of 4 nM and complete inhibition at 50 nM (data not shown). Therefore we are confident in concluding that wortmannin was without effect on pp67 dephosphorylation.

Because wortmannin did not have the expected inhibitory effect on p70^{S6K}, it is possible that the activation of this enzyme is not downstream of PI3'-kinase in 3T3-L1 adipocytes as it is in other cell types. Therefore, since insulin did activate p70^{S6K}, we tested for inhibition of pp67 dephosphorylation by rapamycin. There was no detectable inhibition by rapamycin (Fig. 12) although we could show that the drug was fully potent in blocking the activation of p70^{S6K}, as determined by its ability to block the insulin-induced electrophoretic mobility shift in p70^{S6K} (Figure 12). Thus, the lack of inhibition of pp67 dephosphorylation by wortmannin indicates that the PTP responsible is not under regulation by PI3'-kinase. Allowing for a colinear pathway in the activation of glucose transport, these

findings do not exclude the pp67 PTP from a role in glucose transport activation. Similarly, an inhibitory effect by rapamycin would have excluded pp67 (and/or its PTP) from this pathway, since rapamycin is not an inhibitor of glucose transport. Therefore, we needed to test for an effect of PAO on PI3'-kinase, to establish whether or not the pp67 PTP should be invoked as a candidate for a colinear pathway in the activation of glucose transport.

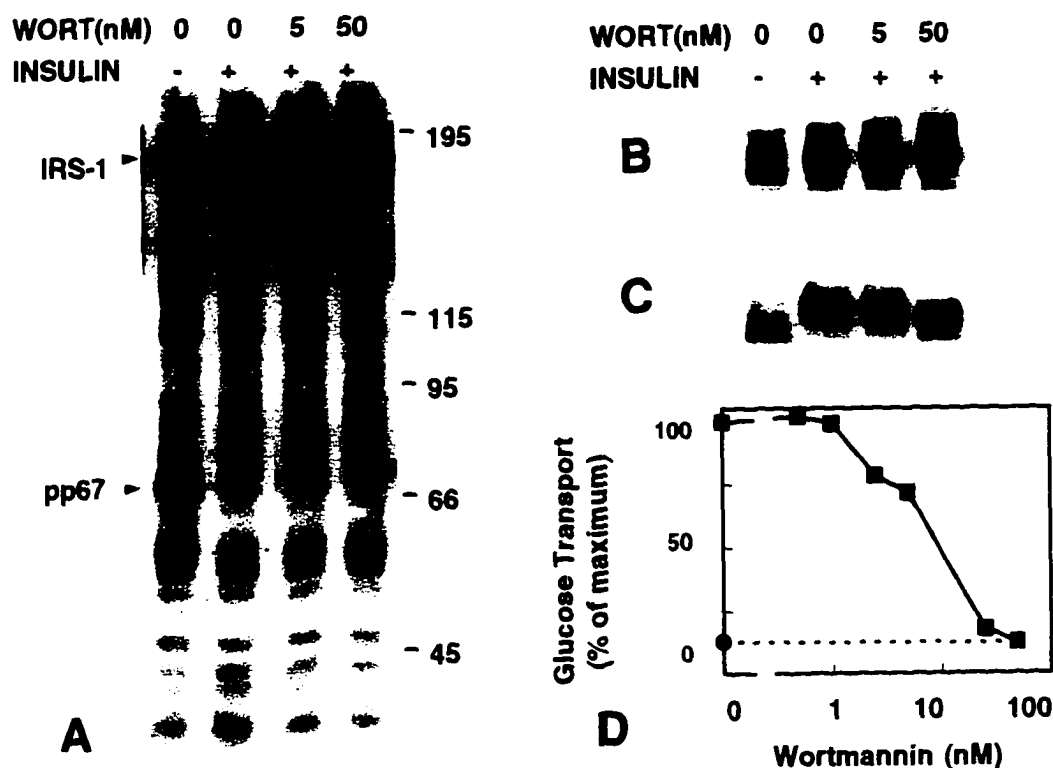


Figure 11. Test for an Effect of Wortmannin on Insulin-Stimulated pp67 Dephosphorylation. 3T3-L1 adipocytes were serum-starved for 2 h, then exposed for 10 min to wortmannin (*WOR*) at the indicated concentrations (nM), and subsequently challenged, or not, with 100nM insulin for an additional 10 min. Cytosolic fractions were prepared from monolayers harvested in STV/10.5, the proteins resolved by SDS-PAGE and electrotransferred to nitrocellulose for Western blots. **Panel A.** Western blot with anti-PTyr. The mobilities of phosphotyrosyl pp67 and IRS-1 are marked. **Panel B.** The region around IRS-1 from a lighter ECL exposure of the anti-PTyr Western blot shown in Panel A. **Panel C.** Western blot of insulin-dependent mobility shifts in p70^{S6k} determined by stripping the blot in Panel A and reprobing with anti-p70^{S6k}. **Panel D.** Inhibition of insulin-stimulated glucose transport by wortmannin. 3T3-L1 adipocytes were pre-treated for 10 min with wortmannin at the indicated concentrations, and then stimulated for 10 min with 1 μ M insulin, after which 2-deoxy-[¹⁴C]-glucose was added and transport permitted to occur for 5 min. Further details are given under Experimental Procedures. The data are averages of duplicate determinations, and are normalized to percent of the maximum transport rate. The basal transport rate is shown as a horizontal dashed line.



Figure 12. Test for Inhibition of Insulin-Dependent pp67 Dephosphorylation by Rapamycin. Insulin-stimulated dephosphorylation of pp67 was assayed by anti-phosphotyrosine Western blot, and the insulin-stimulated mobility shift in p70 S6 kinase was assayed with antibodies against that protein, as described in the previous Figure legend. Cytosolic fractions from control cells (without drug) are shown in the left-hand panels, *versus* cytosolic fractions from cells pretreated with rapamycin at 20ug/ml for 10 min before the addition of insulin, which are shown in the right-hand panels. The *upper panels* show Western blots with anti-phosphotyrosine, and the *lower panels* show Western blots with anti-p70^{S6K} antibodies. The mobilities of pp67, IRS-1, (upper panels) and of p70^{S6K} (lower panels) are indicated on the left.

3. PI3'-Kinase Activation and Glucose Transport.

3.1. Time Course of PI3'-Kinase Activation.

Data to be presented in the following sections will demonstrate a closer correlation between PI3'-kinase activation and glucose transport than is presently acknowledged in the literature. There are similar lag periods in the activation of glucose transport and the dephosphorylation of pp67, as described above. Therefore, we determined the time course of PI3'-kinase activation after the addition of insulin. To do this, we immunoprecipitated PI3'-kinase, either directly through antibodies against its regulatory subunit or by co-immunoprecipitation with antibodies against IRS-1. The level of PI3'-kinase activity present in the immunoprecipitates was assayed as described under Materials and Methods, and is shown in Fig. 13. Although the absolute activities are different in these two immunoprecipitates, the same 8- to 10-fold stimulation above basal was observed for each immunoprecipitation method. At one minute, PI3'-kinase activities were near the maximum observed, and such activation was stable up to the final measurement at 10 min. In addition to these activity assays, we also determined direct binding of PI3'-kinase to IRS-1 during this time course, as shown by the Western blot for phosphotyrosyl-IRS-1 co-immunoprecipitated with anti-p85 antibodies, where p85 is the regulatory subunit of PI3'-Kinase (Fig.13, inset panels). The control for net IRS-1 tyrosine phosphorylation is shown in Fig. 13, Panel A, and the association of phosphotyrosyl-IRS-1 with p85 is shown in Panel B. Consistent with the results of the PI3'-kinase assay, the association of PI3'-kinase with IRS-1 had the same time course following stimulation with insulin. Together, these results demonstrate that PI3-kinase activity reached its steady-state level of activation without a

detectable lag. Therefore, the activation of PI3'-kinase, *per se*, does not account for the lag in the activation of glucose transport.

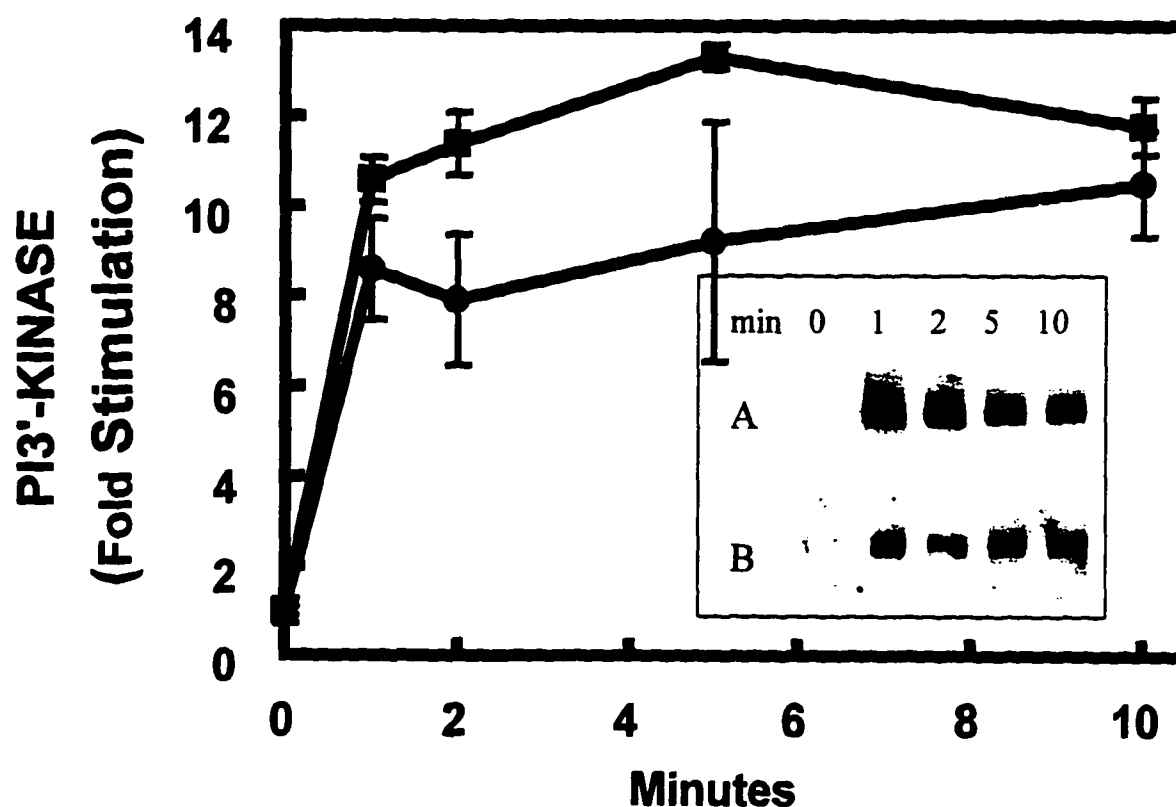


Figure 13. Time Course of PI3'-Kinase A ctivation. Insulin-stimulated PI3'-kinase activity was measured in α IRS-1 and in α -p85 immunoprecipitates. 3T3-L1 adipocytes were serum-starved for 2.5 h and then challenged with 100nM insulin. After the indicated times, the monolayers were lysed in STV/7.6 containing 1.2% Triton X-100 (w/v). Immunoprecipitations were done with either α IRS-1 (■) or α -p85 (●) rabbit polyclonal antibodies, and pI3'-kinase activities measured as described under Experimental Procedures. Results are the averages of duplicate determinations from duplicate immunoprecipitations, and are normalized to show fold-stimulation above each basal value; these were ~ 10:1 in the α IRS-1 versus the α -p85 immunoprecipitates. The inset shows time course of association PI3'-kinase with IRS-1: *Panel A*, tyrosine phosphorylation of IRS-1 was detected by α IRS-1 immunoprecipitates; *Panel B*, p85 subunit associated tyrosine phosphorylated IRS-1 was measured by immunoprecipitation of with α -p85 followed by Western blot with anti-PTyr.

3.2. Growth Factor-Stimulated Glucose Transport and PI3'-Kinase Activation.

There are two purposes for these experiments: *First*, we need to establish whether or not growth factor stimulated PI3'-kinase activities correlate with their potencies to stimulate glucose transport; PDGF has been reported to have a stimulatory effect and also to be without a stimulatory effect in these cells, as described in the Introduction. *Second*, we need to evaluate whether pp67 dephosphorylation was a candidate event needed to account for inhibition of glucose transport by PAO; i.e., whether or not any of the known steps leading to glucose transport were inhibited by PAO. Other laboratories have also measured the different effects of growth factors on glucose transport in this cell line, as cited above. We re-examined the abilities of the four growth factors to regulate glucose transport. We confirmed that EGF had little or no stimulatory effect, but surprisingly we found that PDGF stimulated glucose uptake about 4-fold, compared to approximately 10-fold for insulin and slightly more than 9-fold for IGF-1 (Fig. 14). In order to be certain that we had obtained maximal transport levels, we examined several concentrations of each growth factor. The near-maximal effect of IGF-1 at 10 nM hormone is consistent with its dissociation constant for its own receptor of ~ 1.1 nM, compared to its approximately 100-fold weaker affinity for the insulin receptor (Weiland et al., 1991). This further indicates that IGF-1 is acting through its own receptor and not through binding to the insulin receptor. Similarly, the stimulatory effect of PDGF was nearly saturated at 3 nM, as we observed here, consistent with the $K_d \approx 0.5$ nM of PDGF for its receptor.

Having observed PDGF-stimulated glucose transport, we then measured the levels of PI3'-kinase activity associated with each receptor, or with IRS-1. Cells that were

stimulated with 100 nM insulin or 100nM IGF-1 were lysed and the PI3'-kinase activities were co-immunoprecipitated with anti-IRS-1 antibodies. Cells challenged with 200 nM EGF or with 3 nM PDGF were lysed and the PI3'-kinase activities were immunoprecipitated with the appropriate anti-receptor antibodies; controls were done with each antibody using lysates from unstimulated cells. The levels of growth factor- stimulated PI3'-kinase activity in each case correlated well with the observed levels of glucose transport after maximally effective concentrations of each growth factor were used, as shown in Fig. 15.

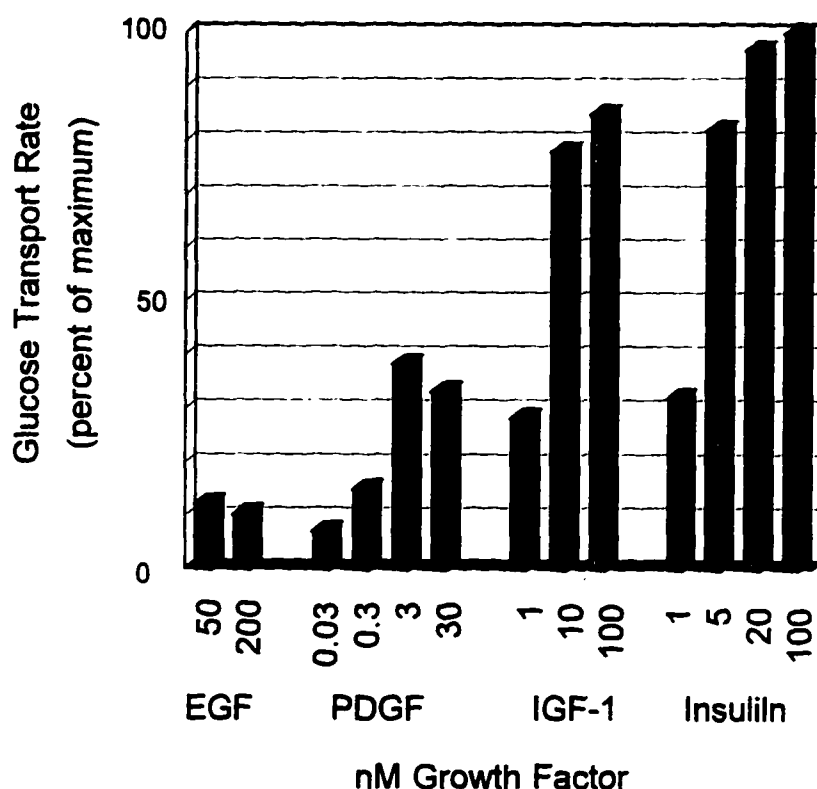


Figure 14. Titration of Growth Factor-Stimulated Glucose Transport Rates. Glucose transport rates were measured on 3.5-cm monolayers of 3T3-L1 adipocytes, after stimulation for 15 min with the indicated concentrations of each growth factor. The unstimulated rate was 10 % $\pm$ 3% of the maximum level observed with insulin. All data are the average of duplicate determinations and were corrected for nonspecific binding of 2-deoxy-[U- ^{14} C]-glucose, as described under Experimental Procedures. Results were normalized to the observed maximum transport rate, which was achieved at insulin $\geq$ 100 nM.

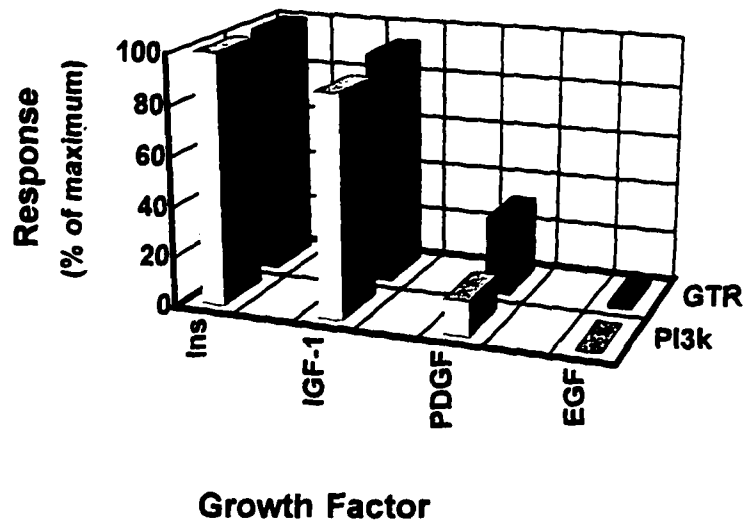


Figure 15. Growth Factor Activation of PI3'-Kinase and Glucose Transport. 3T3-L1 adipocytes were stimulated for 15 min with maximally effective concentrations of the indicated growth factors, and then lysed in STV/7.6 buffer containing 1 % Nonidet P-40. Co-immunoprecipitation of PI3' kinase activities from these lysates was assayed after affinity isolation with the following antibodies: α -IRS-1 after stimulation by 100 nM insulin or 100 nM IGF-1; α -PDGFR after stimulation with 3 nM PDGF; α -EGFR after stimulation with 200 nM EGF. Results of the PI3'-kinase assays (**PI3k**) are the average of duplicate immunoprecipitations, each assayed in duplicate; error bars are not shown for simplicity. Results are normalized to the observed maximum PI3'-kinase activity, which was detected in the α -IRS-1 immunoprecipitate after stimulation by 100 nM insulin. Data for the maximally activated glucose transport rates (**GTR**) are from Fig. 14.

3.3. Inhibition of PI3'-Kinase by Phenylarsine Oxide.

It was reported about ten years ago that phenylarsine oxide could effectively inhibit insulin-stimulated glucose transport in 3T3-L1 adipocytes (Frost and Lane, 1985). We attempted to address whether or not PI3'-kinase is a target for inhibition by PAO, because if it is, then we can account for the inhibitory effects of PAO on glucose transport without invoking a colinear pathway. 3T3-L1 adipocytes were pretreated with 20 μ M PAO before being challenged with insulin and then the PI3'-kinase activity co-immunoprecipitating with IRS-1 was assayed, as described previously. We found that PI3'-kinase activity was inhibited to near-basal levels by exposure of the cells to PAO (Fig. 16). This unexpected result differs from the inhibitory effect on PI3'-kinase by wortmannin, which is not preserved during the lysis and immunoprecipitation (Okada et al. 1994). However, wortmannin inhibits PI3'-kinase when added to the immunoprecipitated enzyme (Okada et al. 1994) because it is a reversible inhibitor. If PAO acts directly on the catalytic subunit of PI3'-kinase, then significant *in vivo* inhibition would be expected. Therefore we isolated IRS-1 associated PI3'-kinase from lysates prepared after insulin stimulation, and various concentrations of PAO were added *in vitro*. We did not find a statistically significant inhibitory effect over the concentration range examined, although later work (Kohanski) demonstrated that complete inhibition of PI3'-Kinase could be achieved *in vitro* (data not shown). In contrast, wortmannin added *in vitro* as a control was fully effective (Fig. 16B).

The discrepancy between *in vivo* and *in vitro* inhibition suggested that PAO might affect the association of PI3'-kinase with IRS-1 *in vivo*. Therefore we tested the effect of PAO on the association of PI3'-kinase (p85 subunit) with tyrosine-phosphorylated IRS-1.

Our results showed that PAO did not reduce recruitment of PI3'-kinase as measured by co-immunoprecipitation of the p85 subunit (Fig. 16C). Therefore it does not appear that the association between the regulatory subunit of PI3'-kinase and phosphotyrosyl-IRS-1 was affected by phenylarsine oxide.

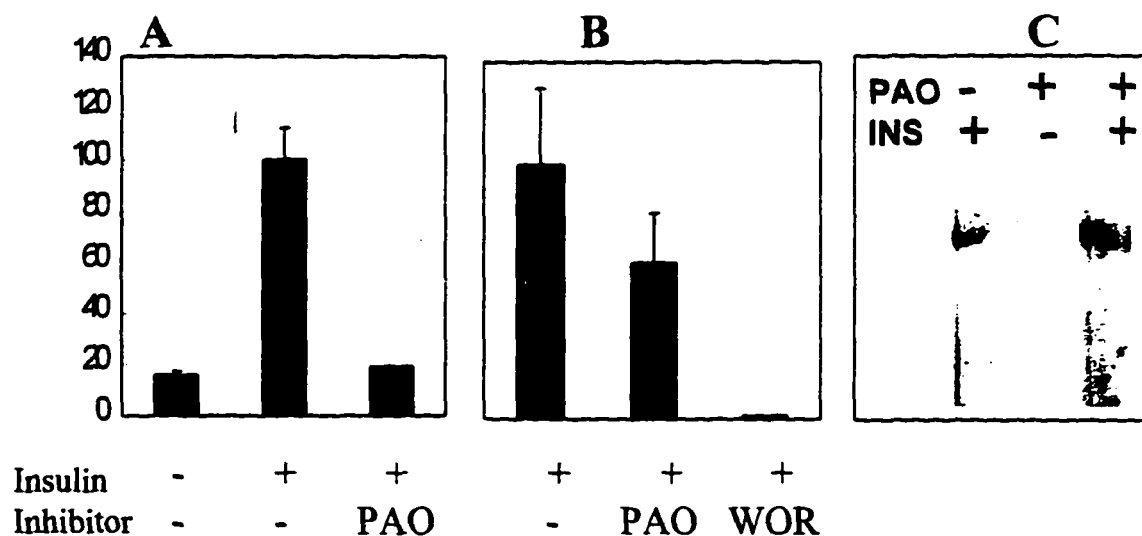


Figure 16 . Effects of Phenylarsine Oxide on IRS-1-Associated PI3'-Kinase Activity. PI3'-kinase activity was assayed in α IRS-1 immunoprecipitates prepared from Triton X-100 lysates of 3T3-L1 adipocytes. *Panel A.* *In vivo* effects of PAO were measured using *in vitro* assays of PI3'-kinase activity in α IRS-1 immunoprecipitates prepared from monolayers exposed to DMSO alone (control), to DMSO and then insulin for 15 min, or to PAO in DMSO for 10 min followed by insulin for 15 min. Neither carrier DMSO nor PAO were present when the monolayers were washed or lysed, nor during any subsequent step in the immunoprecipitation or assay. Results are the averages of triplicate determinations from duplicate immunoprecipitations, and represent one of two such experiments. *Panel B.* *In vitro* effects of 30 μ M phenylarsine oxide (PAO) and of 50nM wortmannin (WOR) were assayed in the α IRS-1 immunoprecipitate prepared from insulin-stimulated 3T3-L1 adipocytes. The control shows the level of PI3'-kinase activity in the presence of carrier DMSO at the same concentration used to present the drugs. *Panel C.* Co-immunoprecipitation of phosphotyrosyl-IRS-1 with PI3'-kinase. 3T3-L1 adipocytes were those treated as described in *Panel B*, but aliquots of the lysates were mixed with antibodies against the 85kDa subunit of PI3'-kinase before affinity adsorption on Protein A-agarose. Bound proteins were resolved by SDS-PAGE and the presence of phosphotyrosyl-IRS-1 was determined by Western blot using antibodies against phosphotyrosine.

DISCUSSION AND CONCLUSION

Regulation of glucose homeostasis is the major physiological function of insulin. In both growing and mature mammals. Regulation of circulating glucose levels is a tissue-specific and hormone-specific process: Muscle and fat are the primary targets for insulin action in dietary glucose uptake. There is also a muscle- and fat-specific glucose transporter, GLUT-4, which is found predominantly in these insulin-responsive tissues (Birnbaum et al., 1986; Charron et al., 1989; Fukumoto et al., 1989; Kaestner et al., 1989). Glucose uptake is regulated by the insulin-stimulated increase in GLUT-4 transporters in the plasma membrane which are recruited from an intracellular compartment; this is the generally accepted Cushman-Kono hypothesis (Cushman and Wardzala, 1980; Suzuki and Kono, 1980). Insulin is the primary physiological effector of this process, in contrast to other growth hormones such as epidermal growth factor or fibroblast growth factor. These growth factors may affect glucose uptake through the GLUT-1 transporter but are without effect on GLUT-4 compartmentalization (Robinson et al., 1993). Insulin-like growth factors, however, also can affect glucose uptake (Kohanski et al., 1986) but none of these hormones are clinically associated with diabetes, nor do they correlate physiologically with glucose homeostasis (Bell, 1990; Olefsky, 1990). The ability of PDGF to stimulate glucose uptake in fat or muscle is presently a matter of debate. Based on the evidence from two laboratories, PDGF has no stimulatory effect (Isakoff et al., 1995; Wiese et al., 1995), but there is also strong evidence that PDGF is effective in promoting GLUT-4 recruitment to the plasma membrane (Kamohara et al., 1995). The first two studies also made the important conclusion, that PI3'-

kinase was not sufficient to stimulate glucose transport, because PDGF stimulated PI3'-kinase but not transport; here we challenge both the observation and therefore the conclusion. The primary evidence that PI3'-kinase is necessary comes from inhibition of glucose transport by wortmannin at an IC_{50} sufficient to inhibit PI3'-kinase (Okada *et al.*, 1994). Two other observations are important for these studies: Phenylarsine oxide is an inhibitor of glucose transport (Frost and Lane, 1985), and this drug has since been shown to inhibit phosphotyrosine phosphatase activity (Liao *et al.*, 1991). Therefore a phosphotyrosine phosphatase might be expected to function in the insulin-stimulated signal transduction pathway leading to glucose transport. Such a phosphatase may or may not be independent of PI3'-kinase. Therefore we explored these possibilities through a series of anti-PTyr Western blots and PI3'-kinase assays in 3T3-L1 adipocytes.

Insulin-stimulated glucose transport has been characterized in cultured murine 3T3-L1 cells, which are propagated as fibroblasts and induced to differentiate in culture into adipocytes (Green and Kehinde, 1975; Green and Meuth, 1974; Green and Kehinde, 1974). This differentiation is accompanied by an acquired insulin-responsiveness of hexose transport (Rosen *et al.*, 1978; Rubin *et al.*, 1978), due to increased numbers of insulin receptors (Reed *et al.*, 1981; Reed and Lane, 1980) and to increased expression of GLUT-4 (Kaestner *et al.*, 1989; Tordjman *et al.*, 1989; Garcia de Herreros and Birnbaum, 1989). The ≈ 20 -fold increase in glucose transport rate by insulin (Frost and Lane, 1985) and its close correlation with insulin receptor autophosphorylation (Kohanski *et al.*, 1986) have also been documented.

1. A 67kDa Target for Phosphotyrosine Phosphatase Activity.

Most growth factors act through specific plasma membrane-associated receptors, each with an intracellular tyrosine kinase domain whose activity is stimulated following hormone binding. Through coordinated auto- and substrate phosphorylation, and through interactions mediated by Src-homology domain-containing proteins, intracellular processes are regulated everywhere in the cell, including the nucleus (Granner et al., 1983), ribosomes (Lin et al., 1994), cytoskeletal associations (Yang et al., 1994), the plasma membrane (Buss et al., 1989), and other subcellular compartments. In the glucose transport pathway, the expected key compartments would be the high and low density microsomal compartments, which include the GLUT-4 transporter, and the cytosolic compartment where some regulators of vesicle trafficking are found.

We found an insulin-stimulated phosphotyrosine-dephosphorylation of a 67kDa protein, pp67, occurring in the cytoplasmic fraction. Both the separation of cytosolic and membrane fractions and the increased pH for cell lysis improved the detection of pp67 dephosphorylation. In addition, it did not appear that pp67 is translocated to the membrane compartment. For reasons given in the Results section, it is not certain that this represents a dephosphorylation, although this is the simplest explanation for the loss of immunoreactivity on the anti-PTyr. Western blot. However two pieces of evidence support the explanation. First, pp67 dephosphorylation is a reversible and acute process; once insulin is removed pp67 recovers, where the first increase in anti-PTyr immunoreactivity is observable within minutes (data not shown). Second, PAO, the known phosphatase inhibitor can block pp67 dephosphorylation. Thus, the simplest model to explain these observations is that: 1. In the basal state, p67 is tyrosine phosphorylated by a constitutively activate PTK

since pp67 was found to be phosphorylated in the basal state. 2. Insulin and other growth factors stimulated a PTP which dephosphorylated pp67, and this putative PTP appeared to be sensitive to PAO. Removal of insulin led to deactivation of the receptor tyrosine kinase activity concomitant with recovery of pp67 through the putative PTK. Additional evidence obtained from two-dimensional gel electrophoresis during trial purifications supports this conclusion (Kohanski and Dao, unpublished observations). Although alternative explanations such as degradation are unlikely, we cannot exclude such a possibility at the present time. The final proof will require the purification of pp67 or at least generating antibodies that will allow the protein, and not only the phosphoprotein, to be monitored directly.

Three other growth factors were tested, and of these IGF-1 and EGF also stimulated pp67 dephosphorylation. The evidence presented here for PDGF did not demonstrate a clear effect on pp67 dephosphorylation (Fig. 3), although it does appear that PDGF is about as effective as EGF, when the background of increased tyrosine-phosphorylation is decreased (Kohanski and Dao, unpublished observations). Because we have demonstrated that PDGF also stimulated glucose transport (Fig. 14), a possible role for pp67 or its phosphatase in this pathway will be permitted only if PDGF can be shown conclusively to stimulate pp67 dephosphorylation. An alternative hypothesis that would permit a role for pp67 dephosphorylation, would be that insulin and PDGF use different pathways to activate transport. However, since insulin was the most potent growth factor for both pp67 dephosphorylation and for glucose transport, we further characterized the effects of insulin on pp67 dephosphorylation.

2. Insulin-Regulated Dephosphorylation of pp67.

It was shown previously that the E_{50} of insulin for glucose transport was slightly less than that for insulin receptor autophosphorylation (Kohanski *et al.*, 1986). Similarly, we have shown here that the E_{50} of insulin for pp67 dephosphorylation was slightly lower than that for stimulated autophosphorylation. The latter was measured by anti-PTyr Western blot, and agreed with the previously published findings obtained by incorporation of [^{32} P]orthophosphate into the receptor's β -subunit. This slightly greater potency of insulin for processes downstream of its receptor is probably due to signal amplification, whereas receptor autophosphorylation is directly proportional to occupancy (Kohanski and Lane, 1983).

The time course of pp67 dephosphorylation also fit very well into the time-frame for insulin-stimulated glucose transport (Kohanski *et al.*, 1986). The increase in receptor autophosphorylation should coincide with insulin binding and therefore there would be no delay in receptor autophosphorylation. Similarly, the phosphorylation of substrates might also be expected to occur without any lag if there is no hindrance in access to the kinase. As shown here, there was no lag in the phosphorylation of IRS-1 (Figs. 7 and 9), but as described later, there was a significant lag in the phosphorylation of Shc52, which is also a substrate for the insulin receptor (Pronk *et al.*, 1993). Although the occurrence of a lag phase in any two processes downstream of the insulin receptor would be consistent with a functional relationship between the two, this coincidence does not mean that a necessary relationship has been established. However, the time course of pp67 dephosphorylation *permits* it to be linked to the stimulation of glucose transport because the dephosphorylation does occur early enough to have a role in transporter recruitment. Finally, because of the very

close correspondence in time between these two processes, it is also possible that the activation of pp67 dephosphorylation is the event that depends upon the influx of glucose, placing the phosphatase downstream of transport activation. However, as discussed in the following section, this would make pp67 dephosphorylation sensitive to the same inhibitors that block the activation of glucose transport, and this is not always the case.

3. The Activation of PI3'-Kinase and Glucose Transport.

Phosphatidylinositol 3'-kinase (PI3-Kinase), composed of a regulatory 85kDa and a catalytic 110kDa subunit, has been implicated as a regulatory enzyme downstream from many growth factor receptors. There are reports that the 85kDa subunit can be phosphorylated by the insulin receptor (Hayashi et al., 1992; Endemann et al., 1990) and can be bound to the receptor itself (Van Horn et al., 1994; Levy-Toledano et al., 1994; Staubs et al., 1994) and/or to IRS-1 (Backer et al., 1992; Backer et al., 1993). However, we have not found evidence to support either the tyrosine-phosphorylation of p85 nor did we find it in association with the insulin receptor, although it was clearly bound in a complex with phosphotyrosyl-IRS-1 (data in this section and in the following studies).

When we examined the time course for PI3'-kinase activation (Fig. 13), there was no evidence of a latency period. This suggests that the association of the p85/p110 dimer with IRS-1 is about as rapid as the phosphorylation of IRS-1 itself. The results of the activity measurements and the Western blots were consistent with each other and with this conclusion. Furthermore, the molecular basis for a lag period in the activation of glucose transport is not explained by the timing of PI3'-kinase activation; an alternative explanation

is required. Obviously such a lag should include the time for translocation of GLUT4 from an intracellular pool to the plasma membrane.

The evidence that PI3'-kinase activation is necessary for glucose transport activation is based on the inhibition of both by wortmannin (Okada *et al.*, 1994). The evidence that it is not sufficient is based on the failure of PDGF to stimulate glucose transport even though it stimulates PI3'-kinase. However, we have found that PDGF did stimulate glucose transport and that the level of stimulation of glucose transport and the level of PI3'-kinase activation mirrored each other across the panel of growth factors tested (Fig. 15). Therefore, our findings are more consistent with the conclusion that growth factor-activation of PI3'-kinase will lead to activation of glucose transport. Never-the-less, until a constitutively activated PI3'-kinase is expressed in adipocytes, the issue of whether or not this enzyme is sufficient to produce transport activation will remain unanswered.

4. Inhibition of pp67 Dephosphorylation and PI3'-Kinase Activation.

The fact that **wortmannin** consistently inhibits both insulin-stimulated PI3-Kinase and glucose transport in rat adipocytes (Okada *et al.*, 1994) and in 3T3-L1 adipocytes (Clarke *et al.*, 1994) strongly supports the need for PI3-Kinase activation in GLUT-4 recruitment (Cheatham *et al.*, 1994). However, we showed that wortmannin had no effect on pp67 dephosphorylation. This demonstrated that the event of pp67 dephosphorylation is not downstream of PI3-Kinase activity. We also failed to detect any effect of rapamycin on pp67 dephosphorylation, which shows pp67 dephosphorylation is not downstream of p70^{S6K}. Although PI3'-kinase is essential for stimulation of GLUT4 translocation, the PI3'-kinase

initiated signal transduction pathway leading to p70S6K was shown to be irrelevant to stimulation of glucose transport. This conclusion was based on the lack of an effect of rapamycin on insulin-stimulated glucose transport in 3T3-L1 adipocytes (Fingar et al., 1993). Furthermore, since rapamycin inhibits other S6 kinase family members downstream of MAPK, such as p90^{S6K} (Kharbanda et al., 1994; Chen and Blenis, 1990), we can conclude that these other kinases are not essential for pp67 dephosphorylation.

Among the most significant studies to implicate phosphatase action in glucose transport activation are those with PAO. Before the phrase "signal transduction" had been coined, PAO was shown to inhibit insulin-stimulated glucose transport in 3T3-L1 adipocytes (Frost and Lane, 1985). It was then shown that PAO did not affect insulin receptor auto- or substrate phosphorylation (Frost et al., 1987); this demonstrated that PAO acted downstream of the receptor's tyrosine kinase. However, cellular phosphorylation events (on Ser and Thr) could be altered within the correct time-frame following stimulation by insulin (Frost et al., 1987). That time-frame had been established experimentally as the few minutes "downstream" of observable insulin receptor autophosphorylation and "upstream" of the increase in glucose transport rate (Kohanski et al., 1986). It was later shown in 3T3-L1 adipocytes, that PAO inhibits phosphotyrosine phosphatase activity. Apparently two discrete PTPs were affected in those studies, one of which was expressed in the adipocyte but not fibroblast stage (Liao et al., 1991).

We found that PAO inhibited pp67 dephosphorylation with a potency that matched its inhibition of insulin stimulated glucose transport. If we could have shown that PAO did not inhibit another major step in the activation pathway, then we would have had strong

evidence to support pp67 dephosphorylation as the “missing link” to explain both the lag-period and the inhibitor sensitivity. However, we found that PAO inhibited the activation of PI3'-kinase. This finding is sufficient to explain the inhibitory effect on stimulated glucose transport (Fig. 16). Further experimental work done after the defense of this thesis demonstrated that PI3'-kinase is a direct target for wortmannin.

Two laboratories used the failure of PDGF to stimulate glucose transport and the ability of PDGF to stimulate PI3'-kinase as evidence that this enzyme is insufficient to promote glucose transport, and an alternative or collinear signaling pathway may be activated. Based on the available data, we tested whether or not that alternative pathway could have been a PAO-sensitive phosphatase-dependent pathway. However, PI3'-kinase was shown to be PAO-sensitive. Since PDGF does stimulate glucose transport in 3T3-L1 adipocytes, the PI3'-kinase dependent pathway is not only necessary but it is also limiting, on the basis of the available evidence: There is a close correlation between growth factor potencies for PI3'-Kinase activation and glucose transport activation, and we observed inhibition of both PI3'-Kinase and glucose transport by wortmannin and phenylarsine oxide. Therefore, our results discourage us from thinking that a phosphotyrosine phosphatase, *per se*, is necessary for glucose transport activation based on PAO-sensitivity alone, and there is no other evidence to support the involvement of a PTP in this pathway. However, the stimulated dephosphorylation of pp67 represents the finding of a potentially novel PTP substrate. It is still an attractive candidate for phosphotyrosine phosphatase-mediated signal transduction, although its associated pathway remains obscure. Its identification will provide new clues and tools to explore the interplay between kinases and phosphatase in the

regulation of metabolism.

5. Conclusion

We presented evidence that three growth factors stimulated the decrease in tyrosine phosphorylation of a 67kDa phosphoprotein, pp67, which is present in the cytosol of 3T3-L1 adipocytes. Insulin was more potent than IGF-1 or EGF, and its effects were characterized in more detail. The dose-dependence on insulin and the progress curve of insulin-stimulated pp67 dephosphorylation were similar to those previously observed for activation of glucose transport in these cells. Furthermore, the dephosphorylation of pp67 was inhibited by phenylarsine oxide, but not by wortmannin. PAO is known to inhibit phosphotyrosine phosphatase activity, and is therefore supportive of the conclusion that these growth factors activated a PTP. Wortmannin is an inhibitor of phosphatidylinositol 3'-kinase and of glucose transport, and indicates that the pp67 PTP is not regulated by PI3'-kinase.

One well documented and physiologically important acute action of insulin in adipocytes is stimulation of glucose transport. The findings listed above could suggest that pp67 dephosphorylation, or its PTP, might have a role in insulin-stimulated glucose transport; *i.e.*, there was good correlative evidence, and we have no evidence to rule out a PTP in this pathway. The key to proposing that a PTP might be involved depended upon how one read the literature on the participation of PI3'-kinase in glucose transport. Two mutually exclusive conclusions about the importance of PI3'-Kinase have been made by others: PDGF has been reported to stimulate PI3'-kinase *but not* glucose transport in adipocytes, and PDGF has been reported to stimulate PI3'-kinase *and* glucose transport in adipocytes. The former observation, if true, would require that a collinear pathway participate in the activation of

glucose transport, since PI3'-kinase would not be sufficient, although it is well-known to be necessary. The latter observation, if true, would be consistent with - but not proof of - the sufficiency of PI3'-kinase activation for increased glucose transport. Similarly, EGF has been reported to have no effect on PI3'-kinase or transport in adipocytes, or it has been reported to stimulate both. Therefore, if we could confirm that PI3'-kinase activation by PDGF (or EGF) occurred without stimulation of glucose transport, then we would have a candidate for the putative collinear pathway in the observed insulin-stimulated pp67 dephosphorylation.

We found that PI3'-kinase activation is apparently a limiting step in glucose transport activation; PDGF stimulated both processes nearly equally, whereas EGF was without effect on either. Equally striking, we observed that phenylarsine oxide also inhibited activation of PI3'-kinase. Therefore we have demonstrated that a collinear pathway cannot be invoked from the evidence now available. In the future, it may be possible to show that a constitutively activated PI3'-kinase produces constitutively elevated glucose transport, which would indicate that this enzyme is also sufficient for activation. It may also be possible to determine if insulin-mimetic agents activate - or do not activate - PI3'-kinase without activating upstream events such as receptor autophosphorylation, or without stimulating independent events such as pp67 dephosphorylation. Finally, the identification of pp67 as a substrate for growth factor-regulated PTP activity is important, and may provide clues to its possible function in signal transduction.

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CHAPTER TWO

TRANSIENT ACTIVATION OF RAS DEPENDENT MAP KINASE IN ADIPOCYTES

ABSTRACT

In terminally differentiated 3T3-L1 adipocytes, the insulin-stimulated MAP kinase pathway is transiently activated, even under conditions of constant input signaling by the activated receptor tyrosine kinase. One basis for activation of the Ras-signaling pathway in non-terminally differentiated cells is the recruitment of Grb2 and SOS1 to the membrane compartment to form complexes with tyrosine-phosphorylated proteins. Here we show that insulin stimulation does not increase the net amount of SOS1 or IRS-1 in the membrane compartment, but it does promote the transient formation of a complex between phosphotyrosyl-IRS-1 and SOS1 prior to the observed increase in MAP kinase phosphorylation. Furthermore, the tyrosine-phosphorylation of IRS-1 in the membrane compartment reaches a maximum at 0.5-1 min, followed by a steady decline to near-basal levels by 20min. In contrast, tyrosine-phosphorylation of IRS-1 and complex formation with SOS1 in the cytosolic compartment also reach maxima at about 1min, but both remain at elevated steady-state levels in the continued presence of insulin. Finally, we have detected an additional growth-factor dependent modification of SOS1 that can be resolved electrophoretically, is not sensitive to phosphatase treatment, and is therefore distinguishable from hyperphosphorylation. This novel modification of SOS1 is found in both the cytosolic and membrane compartments. Hyperphosphorylation of SOS1, however, is found predominantly in the cytosolic compartment. Our evidence does not support a role for modification of SOS1 in the dissociation of the SOS1-IRS-1 complex.

INTRODUCTION

Ras-Dependent MAP Kinase Signal Transduction

Although adipocytes are terminally differentiated cells, the mitogenic pathways such as the Ras-dependent MAP kinase pathway, are dynamically responsive to growth factors. In the first chapter, metabolic functions of insulin have been reviewed. On the other hand, insulin is a growth factor. Like other growth factors, insulin stimulates mitogenic pathways which lead to a wide spectrum of gene expression. Phosphorylation of transcription factors is one of the major mechanisms to initiate transcription (reviewed by Hill, 1995). A key protein kinases involved in such phosphorylation of transcription factors is the MAP kinase (for mitogen activated protein kinase), also named ERK (for extracellular signal regulated kinase). MAP kinases are activated by phosphorylation at both a tyrosine and a threonine residue which are separated by one amino acid residue (Anderson et al, 1990; reviewed by Cobb and Goldsmith, 1995 and Cohen, 1993). This unusual phosphorylation motif somehow ensures specificity of this signaling. A dual specificity protein kinase, MAP kinase kinase (MAPKK), or MEK (for MAP or ERK kinase), rather than a receptor tyrosine kinase, is required for MAP kinase activation through phosphorylation of the tyrosine and threonine residues. Activation of MEK requires phosphorylation *per se*, and thus it was suggested that a MEK kinase should exist (Koller and Brownstein, 1987). Later it was shown that the MEK kinase is Raf (Howe *et al.*, 1992; Kyriakis *et al.*, 1992; Dent *et al.*, 1992).

The linkage of this kinase cascade to the receptor kinase have been dissected, showing that a serial protein-protein association is required to transmit the mitogenic signal

from one molecule to another, which ultimately leads to overall activation of the MAP kinase cascade. The first kinase of the cascade, Raf is activated through recruitment to plasma membrane region by a membrane anchored GTPase, p21Ras (reviewed by Avruch et al., 1994, and Howe, 1992). The actual mechanism for activation of Raf is very complicated; phosphorylation of the kinase was shown to be essential and many proteins may be involved (Dent et al, 1995). Ras is activated by binding to GTP. A guanine nucleotide exchange factor such as Sos (for son of sevenless gene product) promotes exchange of GDP and GTP on Ras. But how is this Ras activator, Sos, itself activated? Two lines of experiments point to the same protein: an adaptor protein containing one SH2 (src homology domain 2) and two SH3 domains (src homology domain 3), named Grb-2, for growth factor receptor binding protein-2. Grb-2 was identified by screening for proteins that could bind the tyrosine-phosphorylated carboxyl terminal fragment of the epidermal growth factor receptor (Skolnik *et al.*, 1991). On the other hand, genetic analysis indicated that *drk*, the *Drosophila* gene homolog of Grb-2, acted downstream of receptor tyrosine kinases and upstream of Ras during eye development (Simon *et al.*, 1993). The general outline of receptor-tyrosine kinase activation of the MAP kinase pathway is as follows: Growth factors stimulate the intrinsic tyrosine kinase of their receptors, which phosphorylate themselves and other protein substrates. Tyrosine phosphorylation generates binding sites to recruit Grb-2 through its SH2 domain, while the two SH3 domains of Grb-2, in turn, bind Sos (Lowenstein et al, 1992 and Li *et al.*, 1993). For insulin, tyrosine phosphorylated “docking” proteins like IRS-1, IRS-2, as well as SHC, instead of the receptor, were shown to recruit Grb-2 and Sos (Skolnik *et al.*, 1993 and Baltensperger et al, 1993). How the cytosolic signaling complex containing IRS-Grb2-Sos

migrates to the plasma membrane and activates Ras by promoting the GTP/GDP exchange is not fully understood. However, activated Ras appears to associate directly with Raf kinase (de Vries Sits *et al.*, 1992). Thus the signal from binding of a growth factor to its receptor is transmitted into the MAP kinase cascade.

Insulin and growth factor stimulated mitogenesis can also be regulated at the protein translational level. A protein named PHAS-1 was found to associate with the initiation factor, eIF-4, in the basal state (Lin *et al.*, 1994). Insulin and other growth factors can stimulate phosphorylation of PHAS-1 at serine residues and cause dissociation of the PHAS-1·eIF-4 complex. The released eIF-4 can in turn form a pre-initiation complex with mRNA, and thus start translation. MAP kinase was originally suggested to be the kinase for PHAS-1 phosphorylation, since MAP kinase was the only insulin-stimulated PHAS-I kinase detected from fractionation of 3T3-L1 adipocytes. However the most recent report from the same group showed PHAS-1 kinase is not MAP kinase: the specific inhibitor, PD 098059 for MEK, the activator of MAP kinase, was ineffective in preventing the insulin-stimulated phosphorylation of PHAS-1 while MAP kinase activity was abolished. MAP kinase regulated phosphorylation of PHAS-1·eIF-4E complex is therefore not sufficient to promote dissociation *in vivo* (Lin *et al.*, 1995). Instead, rapamycin was found to attenuate the stimulation of PHAS-I phosphorylation by insulin and abolished the dissociation of PHAS-1·eIF-4E complex without decreasing MAP kinase activity. However it is still not firmly established that p70^{S6K} - which is a target for inhibition by rapamycin - is the PHAS-1 kinase.

Transient Activation of MAP Kinase.

Ras is anchored to the plasma membrane through its myristoylation and farnesylation. The Ras activator, such as Sos, is recruited to the plasma membrane region through its proline rich region with SH3 domain of Grb2. Because Grb2 binds directly to activated EGF and PDGF receptors, it is easy to understand the requirement for translocation of activators to the membrane. However IRS-1 and Shc are predominantly cytosolic proteins, in contrast to growth factor receptors. What is the basis, then, for recruitment of IRS1 (or Shc)-Grb2-Sos complexes to the plasma membrane region. There is a common protein domain called the pleckstrin homology domain (PH domain), which was originally identified from the major substrate for PKC, pleckstrin, and which has been found in many signaling molecules (Ingley et al, 1994 and Saraste, 1995). PH domains are approximately 110 amino acid residues in length. PH domain-containing proteins include, among others, IRS1, Shc, Sos, Akt (PKB, protein kinase B), G proteins, and GTPase activating protein (Datta et al, 1995; Inglese et al, 1995; Sun et, 1991; A role for these domains has been postulated for interaction with membrane components. Inositol phospholipids were found to bind to PH domains *in vitro* (Lemmon et al, 1995; Hyvonen et al, 1995; Ferguson et al, 1995). Even the tyrosine phosphorylation of IRS- was shown to depend on its PH domain (Voliovitch et al, 1995).

Despite the evidence described above that Grb2 functions to bring Sos to the plasma membrane region in cells, Grb2 is not always required for Ras activation. In fact, Ras can be activated as long as Sos is targeted to the membrane even by artificial means (Aronheim et al, 1994). Nevertheless, the mechanism for Sos to activate Ras is very

complicated. Expression of the catalytic domain of Sos1 in CHO cells is not sufficient to activate Ras unless the region of Sos1 containing its PH domain is present (McCollam *et al.*, 1995). However the region of Sos for binding Grb2 does not appear to be absolutely required for such activation. This raised the question whether other proteins are required to bring Sos to the membrane.

One unsolved mystery in the Ras-dependent MAP kinase pathway is the apparent requirement for a SH2 domain-containing PTP. Genetic analysis revealed that a *Drosophila* gene, *corkscrew*, is functionally downstream of a gene encoding the protein tyrosine kinase, *torso*, and plays a **positive** regulatory role in determination of cell fates during embryogenesis (Sprenger *et al.*, 1989). The biochemical identity of Csw was found to be a protein tyrosine phosphatase having two SH-2 domains (Perkins *et al.*, 1992; Shen *et al.*, 1991). The mammalian counterpart of Csw has been identified and named variously as SH-PTP2 (Ahmad *et al.*, 1993), PTP1D or PTP2C (Freeman *et al.*, 1992), SH-PTP3 (Adachi *et al.*, 1993) and Syp (Feng *et al.*, 1993). This protein tyrosine phosphatase, like many SH2 domain-containing proteins, can associate with tyrosine phosphorylated IRS-1 (Kuhne *et al.*, 1993), and PDGF or EGF receptors (Li *et al.*, 1994). SH-PTP2 seems not to be phosphorylated after insulin stimulation. In contrast, PDGF stimulates SH-PTP2 binding to the tyrosine phosphorylated receptor through the SH2 domain, and furthermore this phosphatase also shows tyrosine phosphorylation, apparently by the PDGF receptor. The tyrosine phosphorylated SH-PTP2 in turn recruits Grb-2/Sos, and thus may initiate an alternative Ras/MAP kinase pathway (Li *et al.*, 1994). SH-PTP2 was demonstrated to play a critical role in the Ras/MAP kinase signaling pathway: Expression of either a catalytically

inactive SH-PTP2 generated by changing the conserved catalytically important cysteine to serine, or expressing only the SH2 domain derived from the phosphatase, attenuated growth factor activation of the Ras/MAP kinase pathway (Yamauchi, 1995; Milarski, 1994). The exact mechanism of this inactivation is not known at the present time. However, expression of the dominant negative mutants seem not to interrupt receptor kinase "potency," since IRS-1, SHC and growth factor receptor were tyrosine phosphorylated normally. Also association of the GRB-2/mSOS complex to these phosphorylated RTK substrates or the growth factor receptors themselves were apparently not disrupted. However, in the presence of dominant negative SH-PTP2 mutants, Ras was not loaded with GTP, which is a critical step for the activation of Raf (Noguchi, 1994). SH-PTP2 was shown to be an authentic protein tyrosine phosphatase *in vitro*: An anti-IRS-1 immunoprecipitated complex could be used to dephosphorylate Raytide, whereas the anti-IRS-1 immunocomplex derived from cells expressing the inactive Cys-to-Ser mutant did not have this dephosphorylation activity (Noguchi, 1994), indicating that the co-immunoprecipitated SH-PTP2 was activated in the complex. Finally, recombinant SH-PTP2 can dephosphorylate p-nitrophenylphosphate and diverse phosphotyrosyl-peptides derived from EGF receptor and from IRS-1.

Because the catalytically important cysteine of SH-PTP2 is required for activation of the Ras/MAP kinase pathway, it immediately indicates that unidentified substrates for the PTPase may be important to mediate signals to Ras. Actual substrates for SH-PTP2 have yet to be identified. Alternatively, it is still possible that the catalytically inactive SH-PTP2 exerts its effect by binding and sequestering phosphotyrosyl-phosphoproteins. Indeed, it was found that in insulin stimulated 3T3-L1 adipocytes and CHO cells expressing the human

insulin receptor, a tyrosine phosphoprotein with apparent molecular of 115 kDa, called pp115, was associated with Syp (Yamauchi, K, *et al.* 1995). Although the authors suggested that pp115 may be the key and as-yet unidentified substrate for Syp, their data did not show decreasing tyrosine phosphorylation of pp115 following insulin stimulation. Secondly, pp115 was not shown to be tyrosine phosphorylated in the cells' basal state; instead, insulin **stimulated** tyrosine phosphorylation of pp115. If a PTP is activated, then dephosphorylation should result.

Another question in the Ras dependent activation of MAP kinase is its transient kinetics. Both GTP loading to Ras and MAP kinase activation were found to be a transient ways in present of growth factors (Waters et al, 1995a). Different mechanisms may be imposed for various growth factors. Despite the fact that Ras and MAP kinase are activated maximally at around 10 min by insulin, EGF and PDGF, the subsequent downregulation of EGF and PDGF receptors does not occur within a time-frame that is sufficient to explain such transient behavior of downstream events (Klarlund et al, 1995). However insulin may have its distinct mechanisms since the the receptor kinase is persistantly active during the time Ras and MAP kinase lose their activities. Following a few minutes of stimulation with insulin, Sos was gradually disassociated from Grb2 (Waters et al, 1995b). Furthermore, in examining this dissociation process, Sos changed its electrophoretic mobility, presumably through phosphorylation based on an observed sensitivity of electrophoretic mobility to alkaline phosphatase. Based on these observations, it was suggested that hyperphosphorylation of Sos lead to its disassociation from Grb2. It was originally suggested that MAP kinase is responsible for a “negative-feedback” phosphorylation of Sos (Waters

et al, 1995a). The the same group showed that MEK, instead of MAP kinase, is the likely kinase responsible for Sos phosphorylation: Expression of the dominant-interfering MEK mutant attenuated Sos phosphorylation and ERK activation. On the other hand, expression of the dual specificity phosphatase for MAP kinase (MKP-1) led to inactivation of ERK, suggesting that this phosphatase was the agent responsible for the transient activation of MAP kinase. However, overexpression of MKP-1 was without effect on insulin-stimulated Sos phosphorylation (Waters et al, 1995b).

OBJECTIVES

We found that insulin, and other growth factors, induced pp67 dephosphorylation in 3T3-L1 adipocytes. We also showed that pp67 dephosphorylation was not downstream of PI3-kinase. In attempting to answer whether pp67 dephosphorylation fits into the Ras/MAP kinase pathway, we needed to examine this signal transduction pathway in the adipocytes. Although the pathway has been largely established in recent years, there are still some missing links as reviewed above. Most of the evidences to establish the pathway come from fibroblasts overexpressing the protein(s) most likely to serve regulatory roles in the pathway. 3T3-L1 adipocytes are differentiated from the fibroblasts. Although the adipocytes lose the potential to enter the cell cycle, the Ras/MAP kinase pathway is dynamically activate, which may be responsible for gene expression in order to maintain the metabolic functions characteristic of adipocytes. The questions we want to address are: (1) Which signaling complexes for Ras activation are formed in the adipocytes, and whether they are different from those identified in other cells? (2) Whether other mechanisms are involved in the transient activation of MAP kinase? (3) Whether the distribution of specific signaling molecules in membrane compartment has a possible role in the signaling? (4) Finally, we want to re-examine the modification of Sos that results in its growth factor induced shift in electrophoretic mobility.

RESULTS

Tyrosine Phosphorylation of ERKs in 3T3-L1 Adipocytes.

MAP kinase can be activated by numerous mitogens including insulin, IGF-1, EGF, and PDGF. Tyrosine and threonine phosphorylation of ERK1 and ERK2 are known to be required for activation of these kinases (Anderson, et al.,1990). In 3T3-L1 adipocytes, insulin, EGF, PDGF and IGF-1 all stimulated tyrosine phosphorylation of two proteins with masses of 42 kDa and 44 kDa (Fig.1, marked as m & n) which comigrated with ERK1 and ERK2, respectively, as determined by Western blot with antiERK antibodies (not shown). The time course for insulin tyrosine-phosphorylation of ERK1 and ERK2 in the adipocytes was determined by Western blot with antiphosphotyrosine antibodies, and was taken as a measure of activation of these kinases (Fig.2). As can be seen in both Figures, there is a lag-period of about 4 min during which tyrosine-phosphorylation of ERKs was undetectable. This was followed by a rapid rise in tyrosine-phosphorylation of ERKs to maximum at 6 min and then it gradually dropped. Even in the continuous presence of insulin, the tyrosine-phosphorylation of ERKs returned almost to the basal state. In contrast, tyrosine phosphorylation of p52Shc reached a steady-state level at about 4 min and this level was maintain until 20 min. Despite the fact that both IRS-1 and Shc appear to be direct substrates for the insulin receptor, the kinetics of their respective phosphorylations are quite different. As described in the Chapter One, tyrosine phosphorylation of IRS-1 closely follows activation of the insulin receptor kinase without displaying any obvious lag period. The tyrosine-phosphorylation of Shc52 is more similar to the time course of pp67 dephosphorylation. Up to this point we have observed a lag-period in the insulin-stimulated

change in tyrosine-phosphorylation state of four different proteins, pp67, Shc52, ERK1 and ERK2. However, no delays were found in the tyrosine-phosphorylation of the insulin receptor or IRS-1. These kinetics of downstream tyrosine-phosphorylations indicate differently activated and maintained signaling pathways. It is reasonable to speculate that some events between the persistently activated receptor and MAP kinase must be delayed in onset and/or transient in duration.

Since IRS-1 and Shc are both substrates for the insulin receptor, and since both have potential roles in the regulation of the RAS/MAP kinase pathway - of which ERK tyrosine-phosphorylation is a marker - we examined further the formation of signaling complexes that might participate in this pathway. Even though the RAS/MAP kinase pathway has been shown to be separate from, and not coupled to, activation of glucose transport (Roberson, et al., 1993), it is possible that some common basis or principle may be discovered by exploring in greater detail these other lag-periods. In addition to the signaling complexes, we also examined the changes in electrophoretic mobility of several of these signaling molecules, especially of IRS-1 and Sos1, since there have been several reports that shifts in their electrophoretic mobility can be correlated with the decrease in MAP kinase activation (Cherniack et al., 1995 and Waters et al., 1995).



Figure 1. Growth Factor Stimulated Tyrosine Phosphorylation of 3T3-L1 Adipocyte Proteins Analyzed in Triton X-100 Lysates. 3T3-L1 adipocyte monolayers were serum-starved for 2.5 h and then challenged with the indicated growth factors for 20min. The monolayers were lysed in buffer containing 1.2% Triton X-100 (w/v), the insoluble material removed by centrifugation, and the solubilized proteins resolved by SDS-PAGE. Phosphotyrosyl proteins were detected by Western blot with a monoclonal antibody against phosphotyrosine, as described in detail under Experimental Procedures. The relative molecular masses are given on the right (kDa) and the relative mobilities of selected prominent phosphotyrosyl proteins are indicated by lower case letters on the left, and are discussed in the text. The concentrations of growth factors used were: Basal, no growth factor added (i.e., control); insulin, 100nM; EGF, epidermal growth factor at 200nM; PDGF, platelet-derived growth factor at 3nM; IGF-1, insulin-like growth factor-1 at 100nM. (the blot was adapted from a region of Figure 1 from chapter one; ERK1, ERK2 and the 52 kDa Shc are indicated)

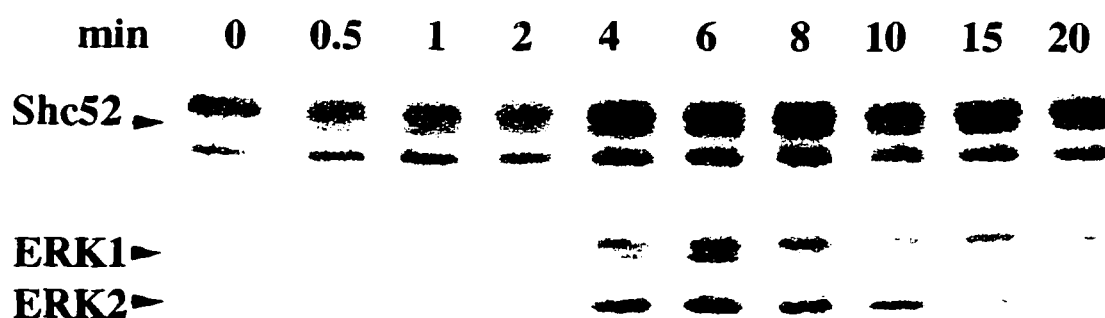


Figure 2. Time Course of Insulin-Stimulated Tyrosine phosphorylation of ERK1, ERK2 and Shc52. 3T3-L1 adipocytes were stimulated with insulin for times ranging from 0-20min, and then cytosolic fractions were prepared in STV/10.5. The proteins were resolved by SDS-PAGE and the presence of phosphotyrosyl proteins assessed by Western blot with antiphosphotyrosine antibodies. This blot was adapted from a region of Figure 7 from chapter one. ERK1, ERK2 and Shc52 were indicated. (Note: The identities of ERKs were confirmed by strip off the antibodies and reprobing with anti-ERK1 which cross-reacted with ERK2 (data not shown). Shc52 was identified by immunoprecipitation with anti-Shc and shown in Figure 5 and 6)

Activation of Insulin and Growth Factor Receptor Kinases.

Each growth factor activated autophosphorylation of its own receptor, as determined by immunoprecipitation with anti-receptor antibodies from cells stimulated with their respective growth factors (Fig. 3). However, immunoprecipitation of the IGF-1 receptor gave inconsistent results, apparently for several reasons: *First*, autophosphorylation of the IGF-1 receptor was not readily detected in whole lysates (Fig. 1 in Chapter One). *Second*, without antibodies that are monospecific for the IGF-1 receptor, we relied upon other evidence to show that this ligand acted through its own receptor and not that of insulin (see Discussion). We have already shown that both insulin and IGF-1 promote the tyrosine phosphorylation of IRS-1 (Figs. 1,3, and 5 in chapter one).The tyrosine-phosphorylated β -subunit of the insulin receptor could be immunoprecipitated with antibodies raised against its purified cytoplasmic kinase domain that had been expressed from a recombinant baculovirus in Sf9 cells (Villalba et al.). Here we show that the ~180 kDa phosphotyrosyl protein is immunoprecipitated by antibodies against IRS-1 from whole lysates of insulin-stimulated cells. It was suggested that IRS-1 and insulin receptor could form a complex using the presence of a third protein, PTP1D (SH-PTP2; Kharitonov et al, 1995). However, from anti-CKD immunoprecipitates we failed to observe the 185 kDa tyrosine phosphorylated IRS-1 and *vice versa*.The EGFR was immunoprecipitated after EGF-stimulation, with a sheep polyclonal antibody raised against the full-length purified receptor, and the PDGFR was immunoprecipitated with anti-PDGFR antibodies. The latter two antibodies were commercially obtained.

Detection of Signaling Complexes by Co-immunoprecipitation.

Complexes with Sos1. Recruitment of Sos1 to the growth factor receptors is a likely early step in the activation of the RAS/MAP kinase pathway (Buday et al., 1993; Aronheim et al., 1994; Li, N. et al., 1993; Simon et al., 1993; Skolnik et al., 1993a and Skolnik et al., 1993b). Such recruitment is through the adaptor protein GRB2 by bridging between the tyrosyl proteins and Sos. Therefore we examined the Sos associated tyrosine phosphoproteins, since the formation of receptor-Sos1 complexes with the receptors would necessarily bring Sos1 to the membrane. The co-immunoprecipitation of the receptors was monitored by antiPTyr Western blot, rather than by anti-receptor antibodies for two reasons: *First*, the anti-receptor antibodies were of variable potencies in Western blots but the anti-PTyr antibody always gave the strongest response in ECL, and *second*, other phosphotyrosyl proteins might co-immunoprecipitate which would not be detected with anti-receptor antibodies.

Stimulation of serum-starved adipocytes with insulin produced Sos1-containing complexes that included phosphotyrosyl-IRS-1 and a second phosphotyrosyl protein of ~98kDa, designated pp98 (Fig.3). We show later that pp98, whose mobility is close to that of the insulin receptor's β -subunit, is a different protein. Stimulation with EGF and PDGF led to formation of a Sos1 complex including phosphotyrosyl EGFR and PDGFR respectively. However, pp98 was not found in association with these growth factor receptors. These results show that the major phosphotyrosyl protein in complex with Sos1 is IRS-1, EGFR, or PDGFR, following stimulation by insulin, EGF, or PDGF, respectively. In contrast, none of the three isoforms of Shc (with molecular weight of 46, 52 and 66 kD) was

found to bind Sos1, in spite of the facts that Shc proteins were phosphorylated and recruited Grb2 in response to insulin, EGF and PDGF in the adipocytes (shown below). It was suggested that for PDGF stimulation, Sos was recruited to the receptor through Sos/Grb2/SH-PTP2/PDGFR (Li, W. et al, 1994). However, we did not observe such a quaternary complex in the adipocytes although the ternary complex of Grb2/SH-PTP2/PDGFR was observed (see below). Instead we suggest that Sos is likely in direct association with Grb2/PDGFR complex.



Figure 3. Co-Immunoprecipitation of Phosphotyrosyl Proteins with Sos1. 3T3-L1 adipocytes were serum-starved for 2.5 h and then stimulated for 10 min with the indicated growth factors, or kept without growth factors (basal). Cells were then lysed in STV/7.6 containing 1.2 % Triton X-100 (w/v) and the lysates were clarified by centrifugation. Sos1 and associated proteins were immunoprecipitated with polyclonal antibodies directly specifically against Sos1. The associated tyrosine-phosphorylated proteins were detected by Western blot with anti-PTyr monoclonal antibody, and identified by comparison of their electrophoretic mobilities with known proteins. Concentrations of growth factors used here are 100 nM insulin, 100 nM EGF and 3 nM PDGF.

Complexes with Grb2. Grb2 was first identified as a protein that could associate with the carboxyl terminal fragment from autophosphorylated EGFR (Lowenstein et al., 1992), and was presumed to function in the activation of the RAS/MAP kinase pathway based upon homology with the *C.elegans* protein Sem-5 (Rozakis-Adock et al., 1992). Therefore, if all the Grb2-PTyr protein complexes are involved in Sos recruitment it would be expected that immunoprecipitation with anti-Grb2 would co-immunoprecipitate the same set of proteins as with anti-Sos1. We observed that Grb2 formed complexes with IRS-1, EGFR, and with PDGFR in cells that had been stimulated with insulin or IGF-1, with EGF, or with PDGF, respectively (Fig.4). In contrast to the failure to observe Shc-Sos1 complexes, Shc isoforms were found in association with Grb2, and the isoform pattern of phosphotyrosyl-Shc in complex with Grb2 is similar to the pattern of net tyrosine phosphorylation of Shc (shown below).

Interestingly, a 98 kDa tyrosine phosphorylation, pp98, was recruited by anti-Grb2 immunoprecipitation (Fig.4). Although weak, IGF1 also induced this association between pp98 and Grb2. This pp98 is likely the same protein as detected in anti-Sos immunoprecipitates. This association is apparently specific for insulin and IGF-1, since EGF and PDGF did not induce formation of a pp98-Grb2. Following stimulation with PDGF, it was not entirely clear if the 66-70 kDa phosphotyrosyl protein included both Shc66 and SHPTP2. However, the experiment described in the following section will show that SHPTP2 is found to associate with Grb2 only after PDGF stimulation. In summary, these results showed that diverse tyrosyl phosphoproteins formed complexes with Grb2 in these 3T3-L1 adipocytes while little Sos was present in these complexes. The failure of Shc to

association with Sos was not due to the absence or lower abundance of Shc-Grb2 since the levels of phosphotyrosyl-Shc and receptors appeared to be comparable.

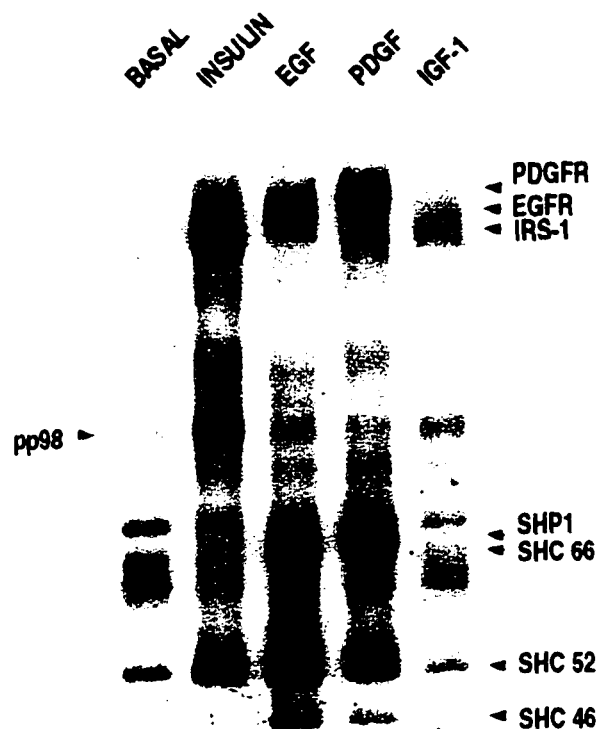


Figure 4. Co-Immunoprecipitation of Phosphotyrosyl Proteins with Grb2. 3T3-L1 adipocytes were serum-starved for 2.5 h and then stimulated for 10 min with the indicated growth factors. Cells were then lysed in STV/7.6 containing 1.2 % Triton X-100 (w/v) and the lysates clarified by centrifugation. Grb2 and associated proteins were immunoprecipitated with polyclonal antibodies directly specifically against Grb2. The associated tyrosine-phosphorylated proteins were detected by Western blot with anti-PTyr monoclonal antibody, and identified by comparison of their electrophoretic mobilities with known proteins, as shown in other Figures in this chapter.

Complexes with Shc. Since we observed the apparent co-immunoprecipitation of phosphotyrosyl Shc proteins in complexes with Grb2, we examined the patterns of phosphotyrosyl proteins that immunoprecipitated or co-immunoprecipitated with anti-Shc antibodies (Fig.5). In the basal state, a 52 kDa protein was detected to be slightly tyrosine phosphorylated; it does not appear to be phosphotyrosyl Shc52, since this PTyr-protein was still observed in anti-PTyr Western blots after efficient immunoprecipitation of Shc (not shown). However, from insulin stimulated cells there was increased immunoprecipitation of a 52kDa protein by anti-Shc antibodies. From EGF stimulated lysate, anti-Shc antibodies precipitated the three isoforms of Shc proteins. In addition, a protein comigrated with EGFR was also precipitated, which suggested the formation of a complex between Shc and the EGF receptor, although we could not distinguish which of the isoforms were actually in association with the EGFR. In contrast, PDGF did not promote association of its receptor with Shc despite the similar pattern of Shc tyrosyl phosphorylation. In addition, a 170 kDa tyrosyl protein, pp170, was also found from the immunocomplexes from insulin and PDGF stimulated cells. So the question become whether the pp170 is a Shc-associated protein or a Shc-homologous protein which cross-reacted reaction with anti-Shc antibodies. Western blots with anti-Shc antibodies showed immunoreactivity of the three isoform of Shc with the apparent molecular weights of 46, 52 and 66 kDa, but not the 170 kDa protein (Fig.6 and the region at 170 kDa which is not shown). Others have reported a high mass phosphotyrosyl protein, called pp145 that is found in anti-Shc immunoprecipitates, termed Shc-associated protein (Crowley et al., 1996). We did not further characterize the pp170, but conclude that it is a Shc-associated protein and was present by co-immunoprecipitation with Shc. We can

also conclude that this protein was not phosphotyrosyl-IRS-1 since it was found also in lysates from PDGF-stimulated cells and it does not have a mobility that matches that of IRS-1; we have already confirmed that PDGF does not promote tyrosine phosphorylation of IRS-1 (Figs. 1, 3, and 5 in Chapter One).

This set of samples showed a high “background” of phosphotyrosyl pp52, along with a second phosphotyrosyl protein. Since this signal was approximately equal to that observed from the anti-Shc immunoprecipitate from IGF-1 treated cells, we could not draw a firm conclusion regarding the ability of IGF-1 to stimulate Shc tyrosine-phosphorylation. In contrast, we observed strong tyrosine phosphorylation of Shc52 by the other three growth factors, and strong tyrosine-phosphorylation of Shc42 and Shc66 by EGF and PDGF, but weak tyrosine-phosphorylation in response to insulin. Since we typically observed lower backgrounds in anti-PTyr Western blots from cytosolic fractions than from whole cell lysates, Shc was immunoprecipitated from cytosolic fractions prepared at elevated pH and subsequently neutralized in order to improve the resolution of tyrosine-phosphorylated proteins (Fig. 5, panel B). Here it can be seen that IGF-1 has almost no effect on the tyrosine-phosphorylation of any Shc isoform.

To ensure capture of complexes formed between Grb2 and Shc, direct measurement of such complexes was pursued. The anti-PTyr Western blot (the same as shown in Fig. 5) was stripped and reprobed with a mixture of antibodies against Shc, Grb2, and SHPTP2 (Fig. 6). First, we observed that the amounts of Shc immunoprecipitated were fairly constant for all samples; the level of immunoreactive Shc was slightly higher in the sample from EGF-stimulated cells. Importantly, the amount of Shc immunoprecipitated from IGF-1

treated cells was about equal to that from insulin-stimulated cells, since insulin but not IGF1 stimulated Shc52 tyrosyl phosphorylation. The near absence of phosphotyrosyl-Shc after IGF-1 stimulation might still be accounted for by the generally lower level of tyrosine-phosphorylation in response to IGF-1 compared to that observed after insulin stimulation.

The reprobing with anti-Grb2 revealed that Grb2 actually co-immunoprecipitated with anti-Shc antibodies. It also revealed that only a very small fraction of the total Grb2 was associated Shc, compared to the amount of Grb2 from the direct anti-Grb2 immunoprecipitation (Fig.6). Similarly, very little Shc could be detected in the Grb2 immunoprecipitate. When analyzed on a single Western blot (Figures 5 and 6 are from the same blot but represent different exposure times during ECL development) the levels of phosphotyrosyl-Shc in the anti-Shc and anti-Grb2 immunoprecipitates are nearly equal for the pp52 and pp66 isoforms (summarized in Table 5). Together with the results of direct Western blots for the proteins (Fig.6), these observations indicate that only a small fraction of the total Shc becomes tyrosine-phosphorylated in response to any growth factor; according to the results here, this value is $\leq 1\%$ of the total Shc present in 3T3-L1 adipocytes.

Complexes with SH-PTP2. SH-PTP2 (Freeman et al., 1992), also named Syp (Feng et al., 1993) and PTP-1D (Vogel et al., 1993), contains two SH2 domains and a PTPase catalytic domain, and is ubiquitously expressed in many tissues including adipocytes (Kuhne et al., 1993). The presence of these SH2 domains suggested to many investigators that this PTPase would associate with tyrosine-phosphorylated molecules.

Although many tyrosine phosphoproteins are in the basal state, we did not detect any of them in stable association with SH-PTP2 (Fig.7). Stimulation with insulin or IGF-1 lead

to similar patterns but substantially weaker formation of complexes with SH-PTP2. From insulin stimulated cells' lysates, three additional phosphotyrosyl-proteins formed complexes with SH-PTP2. These proteins are designated here as pp120, pp105 and pp98. The following evidence, together with the results described above, indicate that pp98 is not the β -subunit of the insulin receptor. First, although pp98 was found from anti-IRS-1 immunoprecipitation, it has a different electrophoretic mobility than the autophosphorylated β -subunit of the insulin receptor (Figure 9). Furthermore, immunoprecipitation of the receptor through antibodies against its β -subunit did not co-immunoprecipitate phosphotyrosyl-IRS-1, which would have been consistent with a complex including the receptor, the phosphatase, and IRS-1.

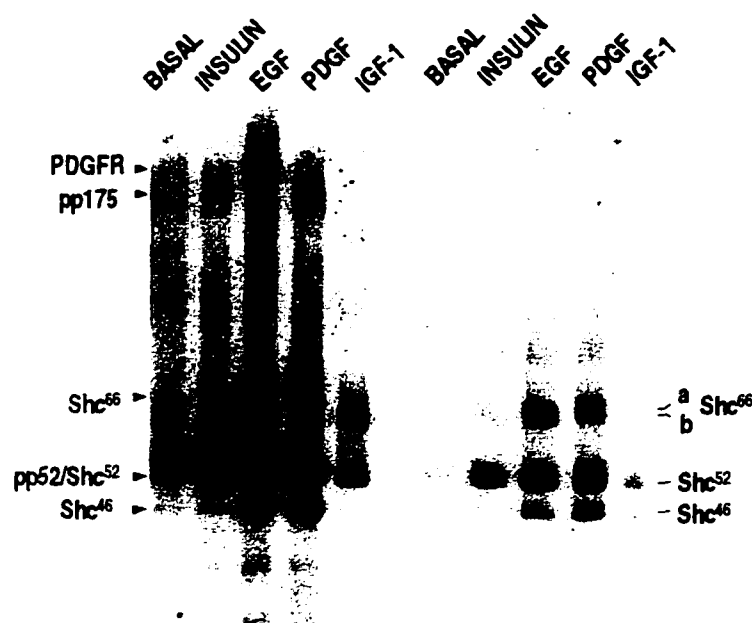


Figure 5. Variable Tyrosine Phosphorylation of Shc Isoforms and Co-Immunoprecipitation of Phosphotyrosyl Proteins with Shc. 3T3-L1 adipocytes were serum-starved for 2.5h and then stimulated for 10 min with the indicated growth factors. Cells were then lysed in STV/7.6 containing 1.2 % Triton X-100 (w/v) and the lysates were clarified by centrifugation, or cytosolic fractions were prepared from cells treated similarly, but lysed in STV/10.5 without detergent and then neutralized before addition of antibodies. The Shc isoforms and associated proteins were immunoprecipitated with polyclonal antibodies recognizing all three isoforms of Shc. Western blots with anti-PTyr revealed the immunoprecipitated phosphotyrosyl proteins from whole lysates (*Left Panel*), and from the cytosolic fractions (*Right Panel*). The associated tyrosine-phosphorylated proteins were identified by comparison of their electrophoretic mobilities with known proteins, and by complementary immunoprecipitations, as shown in other Figures in this section.



Figure 6. Co-Immunoprecipitation of Shc and Grb2. Complexes between Shc isoforms and Grb-2 were detected by Western blot with monoclonal antibodies against these proteins. The complexes were immunoprecipitated with rabbit polyclonal antibodies against Shc (left side) or Grb2 (right side) from whole lysates of basal or growth factor-stimulated cells, as indicated above each lane. Proteins in the immunoprecipitates were resolved by SDS-PAGE and electrotransferred to nitrocellulose. This membrane was first probed by Western blot with anti-Ptyr, and those results are shown in the left panel in Figure 5. The original membrane was then stripped and reprobed with a **mixture** of anti-Shc, anti-Grb2, and anti-SHP1 mouse monoclonal antibodies, shown here. Each protein is identified by its electrophoretic mobility on the left of the figure.

To be more certain, and to determine if there is a complex between the insulin receptor and SHPTP2 as reported by others (Kharitononkov *et al.*, 1995), we performed a Western blot with anti-SHPTP2 after first immunoprecipitating with anti-insulin receptor, anti-IRS-1, or anti-SHPTP2 from whole lysates of insulin-stimulated (and basal) adipocytes. Our results show that IRS-1 but not the β -subunit formed a complex with SH-PTP2. Furthermore, the complex with IRS-1 included only a small fraction of the total SH-PTP2 present in these cells, as determined by direct immunoprecipitation (Fig.8 and Table 4) and as reported previously by others (Kuhne *et al.*, 1993).

Interestingly, the SH-PTP2-containing complexes formed after stimulation with EGF did not include the EGFR, although in other cell types this complex has been reported (Feng *et al.*, 1993). Instead a phosphotyrosyl protein of ~ 115kDa was observed. Its identity remains unknown at this time, but it might be the same protein reported as pp115 and which was found in association with SH-PTP2 in 3T3-L1 adipocytes (Yamauchi *et al.*, 1995b). Finally, tyrosine-phosphorylated SH-PTP2 was found in association with the PDGFR and pp120, whose identity is also unknown. Western blot with antibodies to one candidate for pp120 (p120^{cas}) was inconclusive.

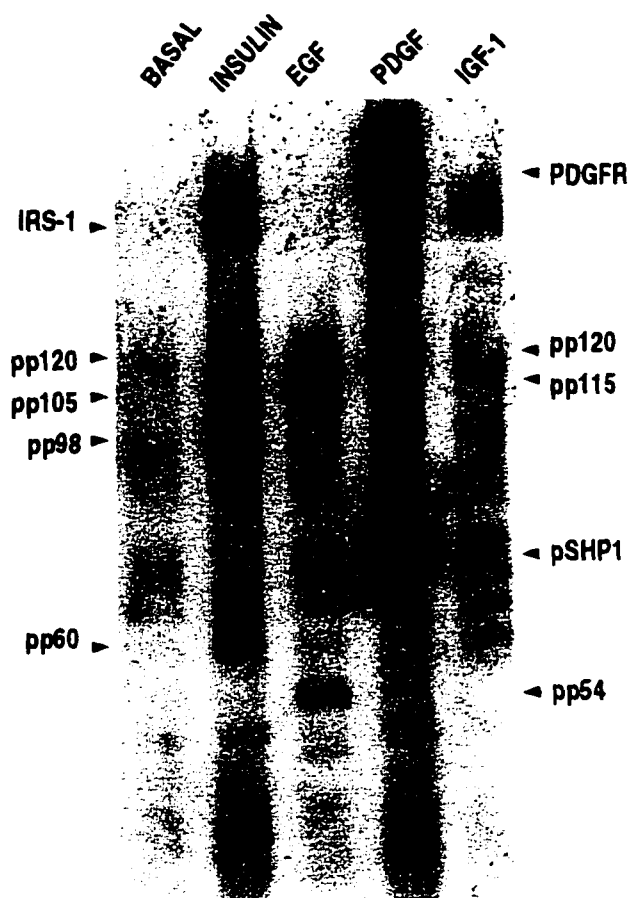


Figure 7. Variable Tyrosine Phosphorylation of SHP1 and Co-Immunoprecipitation of Phosphotyrosyl Proteins with SHP1. 3T3-L1 adipocytes were serum-starved for 2.5 h and then stimulated for 10 min with the indicated growth factors, or maintained without growth factors (basal). Cells were then lysed in STV/7.6 containing 1.2% Triton X-100 (w/v) and the lysates were clarified by centrifugation. SHP1 (tyrosine-phosphorylated after PDGF stimulation) and SHP1-associated proteins were immunoprecipitated with a polyclonal antibody recognizing SHP1. The tyrosine-phosphorylated proteins were detected by Western blot with anti-PTyr monoclonal antibody, and identified by comparison of their electrophoretic mobilities with known proteins, as shown in other Figures in this section.

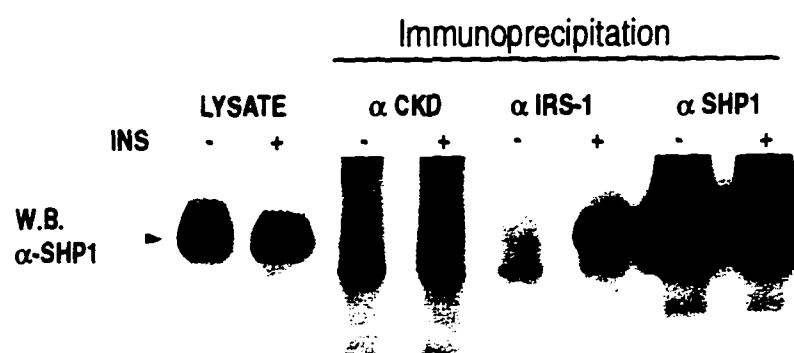


Figure 8. Co-Immunoprecipitation of SHP1 with IRS-1 but not with the Insulin Receptor. 3T3-L1 adipocytes were serum-starved for 2.5 h and then incubated for 10 min with or without 100 nM insulin, as indicated above each lane. Cells were then lysed in STV/7.6 containing 1.2 % Triton X-100 (w/v) and the lysates were clarified by centrifugation. Proteins in whole lysates (lane 1, 2), and immunoprecipitates from lysates with anti- β -subunit of the insulin receptor (lane 3, 4), with anti-IRS-1 (lane 5 and 6), or with anti-SHP1 (lane 7 and 8), were resolved by SDS-PAGE. The presence of SHP1 in these samples was then determined by Western blot with anti-SHP1 antibody. The same rabbit polyclonal antibody against SHP1 was used for both its immunoprecipitation and for the Western blot; further details are given under Experimental Procedures.

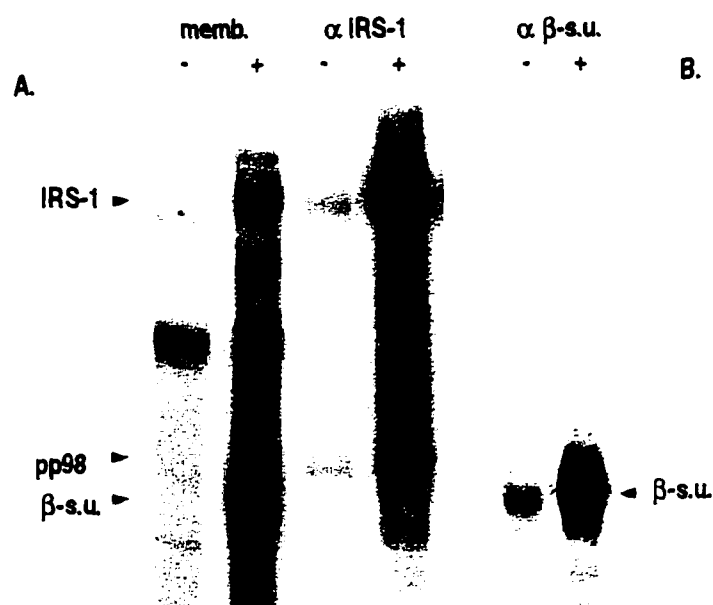


Figure 9. Electrophoretic Mobilities of Phosphoproteins pp98 and the β -Subunit of the Insulin Receptor. 3T3-L1 adipocytes were serum-starved for 2.5 h and then incubated for 10 min with or without 100 nM insulin, as indicated above each lane. Cells were then lysed in STV/7.6 with or without 1.2 % Triton X-100; the membrane fraction was prepared from lysates without detergent and then solubilized in 1.2 % Triton X-100 and also were clarified by centrifugation (lane 1 and 2). Immunoprecipitation from whole lysates was done with anti-IRS-1 (lane 3 and 4) or with anti- β -subunit of the insulin receptor (lane 5 and 6), and the immunoprecipitated proteins resolved by SDS-PAGE. The tyrosine-phosphorylated proteins were detected by Western blot with anti-PTyr monoclonal antibody; the phosphotyrosyl proteins IRS-1, pp98 and β -subunit (β) are marked.

Summary of Signaling Complexes. The preceding results indicate that the insulin receptor does *not* form complexes with the other signaling proteins examined in this study. The alternative explanation for our observations is that these complexes do form but are not as stable as those formed by other autophosphorylated growth factor receptors with those same signaling molecules, all present in the same adipocytes. The primary phosphotyrosyl proteins of adipocytes that form signaling complexes after exposure of these cells to insulin are IRS-1 and Shc52. The complex of Shc52 with Grb2 seems to be low in abundance (Fig. 6) but appears to involve most of the phosphotyrosyl-Shc present (Table 5). Similarly, the Grb2•IRS-1 complex involves about half of the phosphotyrosyl-IRS-1 (Table 4). Phosphotyrosyl-IRS-1 seems to be involved in complexes with Sos1 and SHPTP2, although neither of these includes a large fraction of the net phosphotyrosyl-IRS-1. Although both IRS-1 and Shc52 were bound to Grb2, only IRS-1 was found to recruit SOS in the adipocytes. The unidentified pp98 may be a candidate for the Ras activation pathway controlled by insulin (also may be for IGF-1). It has all the same patterns as IRS-1 to form signal complex: pp98 was found in complexes with Grb2, Sos1, and SH-PTP2 (Fig. 3, 4 and 7). Furthermore pp98 can be immunoprecipitated by anti-IRS-1 polyclonal antibodies, which suggested it either has a similar epitope as IRS-1 or it is associated with IRS-1 (Fig.9).

The EGFR forms complexes with Grb2 and with Shc - mostly Shc52 - that are of nearly equal abundance. Since the fraction of phosphotyrosyl-EGFR present in both adds up to more than 100% of the phosphotyrosyl-EGFR, it is likely that a ternary complex has been formed, as suggested by others (Rozakis-Adcock et al., 1992). Very small amount of Sos1 was recruited to EGFR even at the initiation of stimulation however much more EGFR-Grb2

existed in the cells. Also in different from other cell lines, SH-PTP2 was not found in association with the EGFR in the adipocytes. Instead, pp115 was found to associate with the PTP.

In distinct contrast to these findings, PDGF induced different complexes with its tyrosine phosphorylated receptor. Although SH-PTP2 is recruited to PDGF receptor (Fig.7) and even Grb2 was recruited to SH-PTP2 (Fig.8), SH-PTP2 is not likely to function as adaptor to directly activate Ras in the adipocytes, since Sos was not recruited to tyrosine phosphorylated SH-PTP2 (Fig.3). It was reported that PDGF receptor associated with SH-PTP2 recruited Sos, and this was suggested to be an alternative pathway to the activation of Ras (Li, W. et al, 1994). It is unlikely that pp170 would be involved in Ras activation since it neither recruited Grb2 nor bound Sos (Fig.3, 4 and 5). In summary, with respect to the activation of RAS through complex formation with Sos1, it is apparent that only IRS-1 or the receptors for EGF or PDGF, but not Shc and other tyrosine phosphoproteins, mediate this entry point, since only these formed detectable complexes with Sos1.

Electrophoretic Mobility of Sos1 and its Possible Relation to ERK Activation

In 3T3-L1 adipocytes, we have observed three patterns of time-dependent changes in the tyrosine-phosphorylation states of signal transduction molecules under control of insulin: 1. A rapid rise to steady-state, as shown by the insulin receptor and IRS-1. 2. A lag-period followed by a steady-state, during which there is little or no detectable change in tyrosine-phosphorylation (such as pp67), or during which there is a slow change followed by a faster change before the steady-state is reached (such as Shc52). 3. A transient, involving a maximum change in tyrosine phosphorylation, followed by a return to basal

levels even while other signaling molecules remain in a steady-state of tyrosine phosphorylation, such as was shown for ERK1 and ERK2. In addition to monitoring changes in phosphotyrosine levels, many laboratories have reported changes in electrophoretic mobilities of proteins caused by phosphorylation on serine or threonine, as in the case of p70^{S6K} (Chung et al., 1992). The general approach taken by others, and applied here, is to use a Western blot with anti-protein antibody to assess changes in electrophoretic mobility, and to test the relationship between this phenomenon and serine/threonine- *versus* tyrosine-phosphorylation by using phosphoserine- *versus* phosphotyrosine-phosphatases to reverse the mobility shifts. Because it has been reported that “hyper-phosphorylation” of Sos1 on serine may be correlated with the transient nature of RAS/MAP kinase activation (Cherniack et al., 1995; Waters et al., 1995), we have re-examined this phenomenon in 3T3-L1 adipocytes.

Survey of Electrophoretic Mobility Shifts.

We have shown that IRS-1 undergoes an electrophoretic mobility shift after stimulation of adipocytes by insulin or by IGF-1 (Fig.5 of Chapter One). By Western blot with antibodies against each protein, whole lysates were probed for Sos1, Shc isoforms, ERKs, and p70^{S6K} (Fig.10). Using a modified ratio of acrylamide to bisacrylamide in the gel shown in panel A, we were able to resolve three discrete forms of Sos1, designated as Sos1-a, -b, and -c in order of their electrophoretic mobilities (Fig.10, panel A). In contrast, in this experiment we observed a more pronounced effect by insulin, rather than by PDGF, and no differences in mobilities of Shc52 or Shc46. Whether or not these differences were based on serine/threonine- rather than tyrosine-phosphorylation, or if they have any special

significance in signaling in these cells was not pursued because of the irregularity in our observations, as just described. We also observed a slight change in the electrophoretic mobilities of ERK1 and ERK2, and again the effect was strongest after insulin stimulation. It is probable that this mobility shift is not correlated with tyrosine-phosphorylation because all four growth factors promoted similar extent of tyrosine-phosphorylation of ERKs (Fig.1). The change in electrophoretic mobility of p70^{S6K} was strongest, due to insulin and to IGF-1, very slight after PDGF stimulation, but not detectable after EGF stimulation. This might correlate with the relative potencies of these hormones to activate PI3'-kinase in these cells as shown in chapter one. These results agreed with the notion that PI3'-kinase activation is required for this effect on p70^{S6K} (Chung et al., 1994).

The change of Sos1 electrophoretic mobilities may reflect the possible discrete modification by phosphorylation of the proteins, we tested for serine/threonine phosphorylation as a possible basis for these findings. In addition, because Sos1 complexes should be migrated to close the plasma membrane in order to activate the membrane anchored RAS, we have separated the membrane and cytosolic fractions and examined the Sos1-mobility shifts in each.

Phosphatase-Sensitivity of Sos1 Electrophoretic Mobility.

A panel of three protein serine/threonine phosphatase (PP1A, PP2A and PP2B) and one broad-spectrum phosphatase, alkaline phosphatase were used to test for PPase sensitivity of Sos1 electrophoretic mobility changes induced by each growth factor (Fig.11). In the basal state, Sos1 exist mainly as a-form with minor b-form (Fig. 11, lane 1). The electrophoretic mobilities of the a- and b-forms were unchanged followign treament with the four

phosphatases (Fig.11, lane 2-5). After insulin activation for 10 min, most of the Sos1 was found in the c-form with a minor amount in the b-form (Fig.10, panel A and Fig.11, lane 6). Treatment with phosphatase caused the recovery of the Sos1-b form but not the Sos1-a form (Fig.11, lanes 7-10). Ppase 2A was the most effective and was used in subsequent experiments. Stimulation of cells with EGF, PDGF or IGF-1 produced variable proportions of the Sos1 forms (Fig.10, panel A and Fig.11, lane 11, 13 and 15 respectively). Similarly, treatment with Ppase 2A left the relative amount of Sos1-a unchanged, removed completely the Sos1-c form, and caused a corresponding increase in Sos1-b. Together, these results consistently show that Sos1-c is a PPase-sensitive form that probably arises by serine- and/or threonine-phosphorylation of Sos1 (neither we nor others have shown tyrosine-phosphorylation of Sos1). Also, the Sos1-b and Sos1-a forms are not sensitive to PPases. These results further suggest that Sos1-c and Sos1-b seem to be differently modified. The growth factor induced b-form of Sos1 is probably not due to phosphorylation, since Sos1-c is convertible to Sos1-b by Ppase treatment, but neither are convertible by Ppase treatment to Sos1-a. To our knowledge, this is the first report of an additional modification of Sos1 that is PPase-insensitive. However, we have no information on what that modification might be, or whether it occurs with the -a or -b forms in a defined order, or if either -a or -b forms can be modified. That is, we cannot conclude that the modifications follow a defined order, such as Sos1-a -> Sos1-b -> Sos1-c.

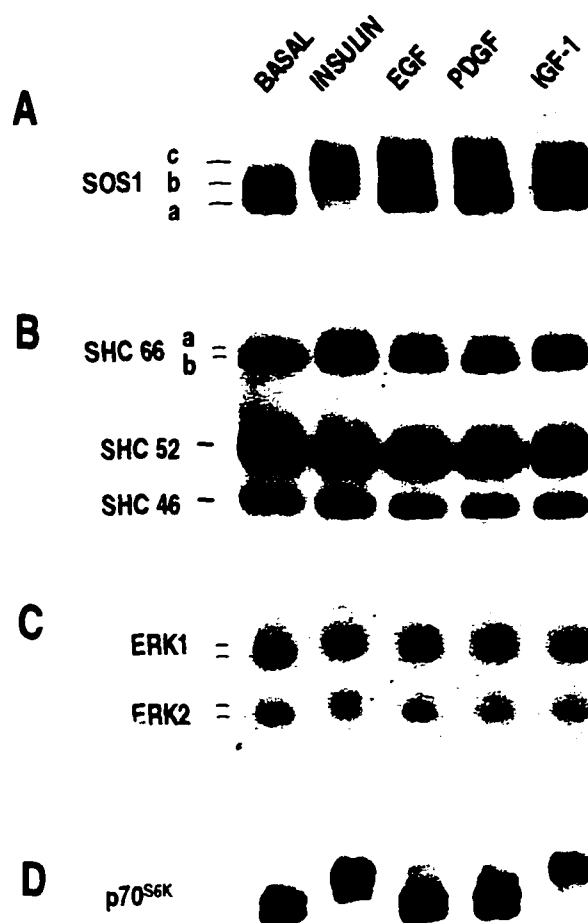


Figure 10. Growth Factor Dependent Changes in Electrophoretic Mobilities of Cytosolic Signaling Proteins. 3T3-L1 adipocytes were serum-starved for 2.5 h and then incubated for 10 min with the indicated growth factors, or maintained without growth factor (basal). Cells were then lysed in STV/10.5 and cytosolic fractions prepared, and the proteins resolved by SDS-PAGE. Specific proteins were detected by separate Western blots with antibodies anti-Sos1 (Panel A), anti-Shc (Panel B), one antibody recognizing both ERK-1 and ERK-2 (Panel C), or against p70S6k (Panel D). The multiple forms of Sos1 and Shc-66 are marked as "a,b, or c" as described in detail in the text.

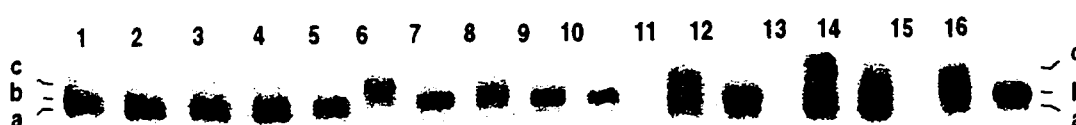


Figure 11. Phosphatase Sensitivity of Growth Factor-Regulated Mobility Forms of Sos1. The cytosolic fractions derived from growth-factor stimulated 3T3-L1 adipocytes were treated with different phosphoserine/threonine phosphatase prior to SDS-PAGE. After SDS-PAGE, the three electrophoretic forms of Sos1 were detected by Western blot with anti-Sos1. Adipocytes were kept without growth factors (basal, *lanes 1-5*), stimulated for 10min with 100nM insulin (*lanes 6-10*), with 200 nM EGF (*lanes 11 & 12*), with 3 nM PDGF (*lanes 13 & 14*), or with 100 nM IGF-1 (*lanes 15 & 16*) before lysis in STV/10.5. Samples were kept without phosphatase (*lanes 1, 6, 11, 13, & 15*), or incubated with CIP (*lanes 2 and 7*), PP1A (*lanes 3, 8, 12, 14 and 16*), with PP2A (*lane 9*), and with PP2B (*lane 10*).

Time-Dependence for Sos1 Electrophoretic Mobility Shifts: Comparison of changes in the cytosolic versus membrane fractions.

The currently accepted hypothesis for activation of the RAS/MAP kinase pathway by Sos1 states that Sos1 is recruited to the membrane by association with a PTyr-protein. Therefore, we examined in more detail the time-dependence and cytosolic *versus* membrane distributions of the Sos1 electrophoretic mobility forms, after stimulation by insulin. In addition, we monitored the distribution of IRS-1 and Grb2 in these compartments.

The changes in Sos1 electrophoretic mobility was monitored at timed intervals after the addition of insulin by Western blots with anti-Sos1. Identical aliquots from each sample were also analyzed for IRS-1 and Grb2 by a Western blots with anti-IRS-1 and anti-Grb2 polyclonal antibodies. Apparently, there was a rapid decrease in the immunoreactivity of IRS-1 to its antibodies from the cytosolic fractions following the addition of insulin (Fig.12, panel A). It is not clear whether this reflects the loss of IRS-1 protein from the cytosolic pool or an actual change of immunoreactivity due to modification; however there was no corresponding increase of IRS-1 in the membrane fraction. The electrophoretic mobility shift of IRS-1 in the **cytosolic** pool was not observed until 4 min after stimulation with insulin. In contrast, IRS-1 in the **membrane** fractions was shown to shift immediately within 0.5 min after addition of insulin (Fig.13, panel A). It is noted that a small amount IRS-1 is present in the membrane fraction in the basal state. Instead of an increase in the amount of IRS-1 in the membrane fractions IRS-1 gradually decreased in the membrane pool. We have described in Chapter One that tyrosine phosphorylation of IRS-1 rapidly reaches the maximum without any measurable lag (Fig.7 in Chapter One). However, we did not observe an electrophoretic

mobility shift of IRS-1 until 4 min in the cytosolic fractions. Therefore, the mobility shift of IRS-1 could not come from tyrosine phosphorylation. Thus, from these results it is suggested that serine or threonine phosphorylation are likely causing the IRS-1 mobility shift.

Cytosolic Sos1 exists mostly as the a-form with minor amounts of the b-form in the basal state (Fig.12, panel B). At 4 min, there appeared to be an increase in Sos1-b and/or Sos1-c, although it was difficult to resolve these two quantitatively. By 8 min, it was clear that the abundance of Sos1-a had decreased, and it appears to be maintained at this low level up to 20 min; i.e., a steady-state may have been reached. The Sos1-c form, however, seemed to reach a maximum in abundance at about 8 min and then declined slightly over the next twelve minutes.

Results in the membrane fractions were significantly different: the Sos1-c was not observed in the membrane fractions. However, both a-form and b-forms of Sos1 were present in the membrane compartment (Fig.12, panel B). Insulin treatment induced a decrease of Sos1-a from 4 min, which was in parallel with an increase of Sos1-b. At 8 min, most of the Sos1-a was apparently converted into the b-form, and this apparent steady state held at least until 20 min. The presence of the a- and b-forms in the membrane but the absence of Sos1-c may be due to a higher affinity of the a- and b-forms for membrane components, and phosphorylation (the c-form) therefore reduce its affinity. There also appeared to be a slight but steady decrease in the total amount of Sos1 in the membrane fraction, as well as an apparent decrease in the amount of IRS-1. On the other hand, other proteins in the membrane fractions such as Shc52 and Shc46 did not show such a decrease in their respective distributions between membrane and cytosolic compartments in response to insulin (Fig.13,

panel A).

Using the same samples from these analyses of Sos1 and IRS-1, we also measured the distribution of Grb2 before and after insulin stimulation. First, Grb2 was distributed in both cytosolic and membrane compartments in the basal state (Fig. 11 and 12). Compared with Sos1 and IRS-1, a higher fraction of total Grb2 was found in the membrane fractions. Also, in contrast to Sos1 and IRS-1, the distribution of Grb2 apparently was not altered.

We have the following evidence that the distributions of these signaling molecules in the membrane fractions did not come from contamination from the cytosolic pool or from unbroken cells in the particulate fractions. Two cytosolic marker proteins, Shc66 and p70 S6 kinase were mostly, if not exclusively, found in cytosolic pools and not in the membrane fractions (Fig. 13). More importantly, a substantial ratio of Shc52 and Shc46 were found in membrane fractions.

There are three basic observations. *First*, in these membrane fractions there was no clearly resolved Sos1-c. This may have resulted from the stringency of preparation of these samples, or it might reflect the real distribution of these proteins. Even in less-stringently prepared samples, however, we have found only very low levels of Sos1-c in the membrane compared to the other two forms (not shown), and in that case Sos1-c might have been present from cytosolic contamination. *Second*, it is important that we observed a substantial fraction of Sos1 in the membrane *prior* to stimulation by insulin and growth factors. It has been suggested that membrane targeting of SOS is sufficient to activate Ras (Aronheim et al., 1994). However we did not observe any net increase of Sos in the membrane compartment in response to insulin stimulation. *Third*, the burst of tyrosine phosphorylation

of membrane associated IRS-1 observed here might suggest that complex formation within the membrane-associated pools of IRS-1 and Sos1 could be sufficient for the activation of the RAS/MAP kinase pathway, and that recruitment of either or both proteins from a cytosolic pool is less important.

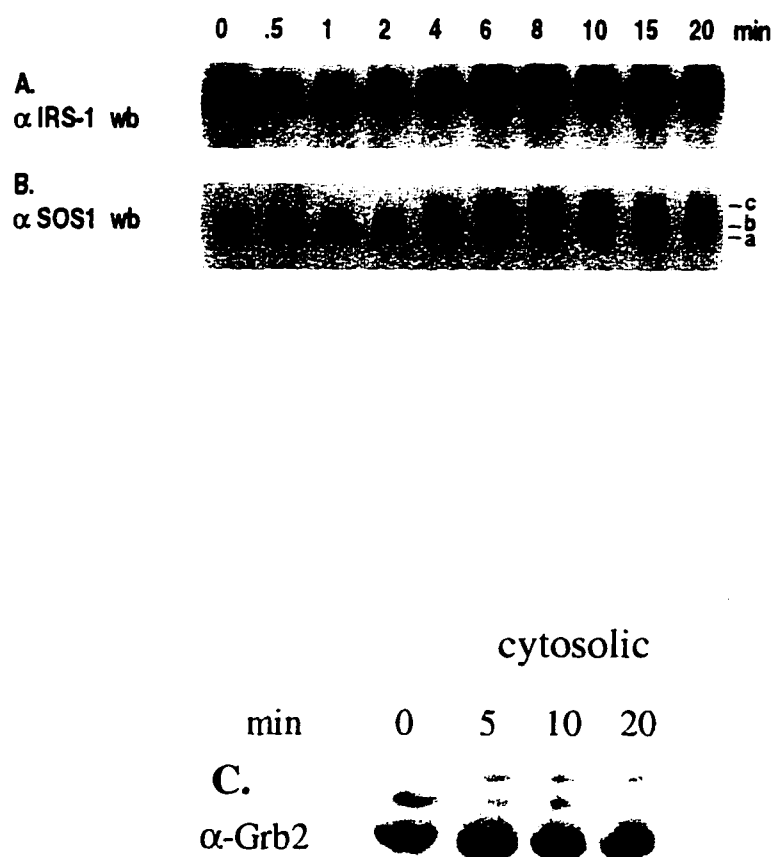


Figure 12. Time Course of Electrophoretic Mobility Shifts in Cytosolic IRS-1, Sos1 and Grb2. Cytosolic fractions were prepared from 3T3-L1 adipocytes stimulated with 100 nM insulin for the indicated time. The electrophoretic mobilities of IRS-1 (*panel A*) or of Sos1 (*panel B*) and of Grb2 (*panel C*) were determined by separate Western blots with antibodies against each protein. The three electrophoretic forms of Sos1 are marked.

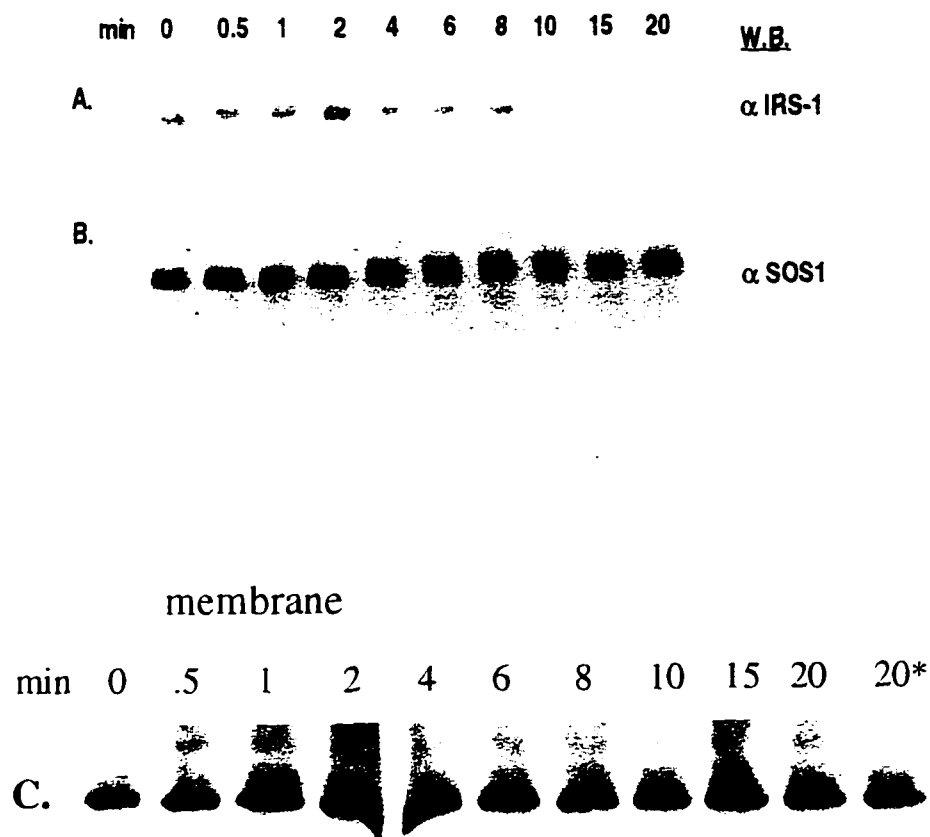


Figure 13. Time Course of Electrophoretic Mobility Shifts in Membrane-Associated IRS-1, Sos1 and Grb2. Membrane fractions were prepared from the same 3T3-L1 adipocytes stimulated with 100 nM insulin for the indicated times as shown in the preceding figure. The electrophoretic mobilities of IRS-1 (*panel A*) or of Sos1 (*panel B*) and Grb2 (*panel C*) were determined by separate Western blots with antibodies against each protein. The two electrophoretic forms of Sos1 that were detected are marked on the right.

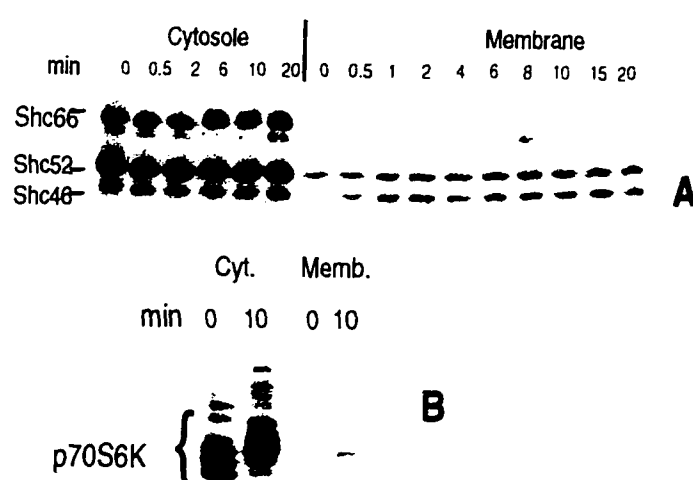


Figure 14. Distribution of Shc and p70 S6 kinase in membrane versus in cytosolic compartments. The cytosolic and membrane fractions were the same as described in Figure 11 and Figure 12. Shc proteins were detected by Western blot with polyclonal antibodies for Shc (panel A). p70 S6 kinase in membrane and cytosolic fractions was determined by Western blot with anti-p70 S6 kinase polyclonal antibodies (panel B). In this case, samples were from insulin stimulation for 10 min and basal state.

CONCLUSIONS AND DISCUSSION

Receptor and IRS-1 Tyrosine Phosphorylation. Insulin and IGF-1 not only have similarity between their structures (49% sequence similarity between IGF-1 and insulin), but their receptors have the same overall architecture (both are α - β - β - α heterotetramers) and have the most closely related kinase domains among receptor tyrosine kinases (84% identity in amino acid sequences; Ullrich et al., 1986). It would be not surprising to find shared signal transduction pathways between insulin and IGF-1. Actually, both hormones have some overlapping cellular functions, such as the activation of glucose transport (Kohanski *et al.*, 1986). However, the physiological functions for insulin and IGF-1 are as often different as they are overlapping: Insulin mainly regulates metabolic actions while IGF-1 may be more important for mitogenesis and differentiation. Secretion of these hormones are regulated differently, which may be largely related to the differences in their respective functions.

Even though structurally similar, insulin and IGF-1 each uses its own receptor in initiating signal transduction, although cross reaction may occur at high concentrations. Unfortunately, the highly specific antibodies to distinguish the insulin receptor and IGF-1 receptor were not available in this study. However there is sufficient evidence to conclude that each ligand functioned through its own receptor: *First*, the affinities of insulin and IGF-1 for each others' receptors is low (Weiland et al, 1991). In all the experiments the concentration of IGF-1 was used at 100 nM, which is sufficient to yield maximal biological function as determinate by activation of glucose transport. *Second*, however at 100 nM IGF-1, IRS-1 tyrosine phosphorylation was observed. Furthermore, the electrophoretic mobility shift in IRS-1 was equivalent to that promoted by insulin. *Third*, there was no significant

tyrosine phosphorylation of Shc52 above background - unlike other cell types - but there was tyrosine phosphorylation of Shc52 in response to insulin. In summary, all these results show IGF-1 acts through its own receptor and not through the insulin receptor.

The autophosphorylation of the EGFR was not readily detected in anti-PTyr Western blots from whole lysates, and the antibodies available to us did not provide Western blots with sufficiently low backgrounds or strong enough signals to identify the EGFR directly. However, the sheep polyclonal antibody was effective in the immunoprecipitation of the EGFR, which was then readily detected by antiPTyr Western blot. The PDGFR autophosphorylation was very strong, even in direct Western blots with antiPTyr were clearly seen. However, the identification of the ~200 kDa tyrosine-phosphorylated protein as the PDGFR seems entirely consistent with the primary evidence: it was strongly phosphorylated in response to PDGF stimulation and not to stimulation by other growth factors, and it was immunoprecipitated with anti-PDGFR antibodies under lysis conditions that severely reduced the stability of complexes, and therefore the chance that this protein co-immunoprecipitated with the receptor. In conclusion, at the concentration we applied here (100 nM for insulin and IGF-1, 100 nM EGF and 3 nM PDGF), each of these growth factors acts specifically on its own receptor tyrosine kinase.

Shc phosphorylation and MAP kinase activation. There have been many reports of the potential role of Shc in transmitting mitogenic signals in response to numerous stimuli, including insulin and other growth factors (reviewed in introduction). Shc proteins were suggested to function as adaptor proteins to recruit Grb2 and Sos and lead to activation of Ras and MAP kinase. However, we have found no evidence to support such a mechanism

in 3T3-L1 adipocytes, since we did not observe Sos1 in a complex with Shc following stimulation with any of the four growth factors used in this study. In addition, the ability of IGF-1 to promote ERKs tyrosine phosphorylation but the failure to promote Shc phosphorylation is contrary to a role for Shc in the activation RAS/MAPK pathway. Although Shc did not recruit Sos1, substantial Grb2 was found to be associated with Shc (mainly Shc52). The reason why Shc-Grb2 did not recruit Sos is not clear, but this is not likely due to an insufficient pool of Sos. In fact, most of the signaling molecules such as IRS-1, Grb2, Sos, and Shc do not form complexes in response to growth factor stimulation, as summarized in Table 4. In addition, we observed a 170 kDa tyrosine phosphoprotein from Shc immunoprecipitation. It is likely a Shc-associated protein rather than one with an epitope recognized by anti-Shc antibodies. The pp170 was not detected in anti-Sos1 immunoprecipitated complexes. In conclusion, our results suggest that Shc may not be important for activation of Ras/MAP kinase pathway in 3T3-L1 adipocytes.

Signaling Complexes Including SH-PTP2. There are definite differences among the signaling complexes involving SH-PTP2. SH-PTP2 was reported in association with tyrosine phosphorylated EGF receptor in other cells (Li, W. et al, 1994). However, SH-PTP2 did not co-immunoprecipitate phosphotyrosyl-EGFR from EGF stimulated 3T3-L1 adipocytes and therefore is apparently not in complex with this receptor. Instead, two tyrosine phosphoproteins with apparent molecular weights of 110 kDa and 53 kDa (pp110 and pp53) were found to be recruited to SH-PTP2 in response to EGF (Fig.7). The reason that SH-PTP2 was not recruited to EGF receptor is not clear. It is possible that the tyrosine site in EGF receptor essential for SH-PTP2 recruitment was not phosphorylated in these adipocytes, or

that the affinity between this receptor and SHPTP2 is too low to preserve the complex during immunoprecipitation. As described in Chapter One, EGF stimulation also did not result in EGFR recruiting the SH2 domain-containing p85-subunit of PI-3' kinase. This has been observed in other cell types, despite the fact that PI3'-K was cloned *via* its binding to the autophosphorylated carboxyl-terminus of the EGFR (Skolnik et al,1991). However, the EGFR was autophosphorylated and capable of recruiting the SH2 domain-containing proteins Grb2 and Shc.

In contrast, the insulin stimulated co-immunoprecipitation of phosphotyrosyl-IRS-1 and several unidentified phosphotyrosyl proteins with SH-PTP2, pp120, pp105 and pp98, which were not found in association with SH-PTP2 from EGF stimulation. Of these, only pp120 is a candidate for complex formation with SH-PTP2 following PDGF stimulation. PDGF promotes a different panel of complexes between its receptor and other unidentified proteins with SH-PTP2. Of the SH-PTP2 associated tyrosine phosphoproteins the most heavily phosphorylated in addition to the receptor is SH-PTP2. This is an exclusive event only for PDGF stimulation.

Insulin and IGF-1 stimulation generated the same complexes between phosphotyrosyl proteins and SH-PTP2. We have not been able to demonstrate a consistent picture for IGF-1 receptor autophosphorylation in these cells, but it is clear that the response to IGF-1 was not due to engagement of the insulin receptor, as discussed above. It was suggested that SH-PTP2 may function as an adaptor to link the insulin receptor to IRS-1 (Kharitononkov et al, 1995). If this is the case in 3T3-L1 adipocytes, we would expect "co-immunoprecipitation" between the insulin receptor and IRS-1. Since we observed a 98 kDa tyrosine phosphoprotein

that could be immunoprecipitated by anti-IRS-1, we asked whether it is the β -subunit of the insulin receptor. First, we have analyzed carefully the relative mobility of pp98 in comparison with the β -subunit, which showed they have distinct mobilities on SDS-PAGE (Fig.9). Furthermore, immunoprecipitation of the insulin receptor did not co-immunoprecipitate either SH-PTP2 (Fig.8) or IRS-1 (Fig.7). This unidentified, insulin induced pp98 may function in activation of the Ras/MAP kinase pathway because it recruited Sos1, Grb2 and SH-PTP2 (Fig.3, 4 and 7). In these respects the pp98 works like IRS-1. However, unlike IRS-1, pp98 was not found from anti-PI3'-kinase immunoprecipitate (Chapter One).

These observations of the complexes formed in response to growth factor stimulation suggest that in 3T3-L1 adipocytes, the specific signaling complexes formed in response to insulin are most similar to those formed in response to IGF-1, whose receptors are within the same family. But the exception noted for Shc phosphorylation demonstrates that even when the receptors are so similar, not every biological response will be the same. For receptors that are structurally as different as those of insulin, PDGF, and EGF, there may be more similarities in the signaling complexes formed between any two of them.

Could SH-PTP2 be the PTPase Responsible for pp67 Dephosphorylation? It has been reported that SH-PTP2 and its homolog in *Drosophila*, the *crws* gene product, plays a positive role in receptor tyrosine kinase-mediated signal transduction leading to activated MAP kinase. It was shown that a catalytically active SH-PTP2 was required, because mutation of the conserved catalytic-site cysteine prevented transmission of the kinase-dependent signal to the MAP kinase pathway (reviewed in the Introduction). Neither the

exact mechanism nor the immediate substrate of SH-PTP2 are known at this time. One possible explanation for the requirement of PTPase catalytic activity is that SH-PTP2 must dephosphorylate its substrate which could have some inhibitory function in its phosphorylated state but not in its apo-state. Recruitment of SH-PTP2 to tyrosine phosphoproteins was suggested to activate SH-PTP2 (Pluskey, 1995). Thus, one hypothesis for the essential catalytic activity would be that the "free" SH-PTP2 in basal state cells may exist as an inactive or less active form. By analogy with the activation of PI-3' kinase through recruitment to phosphotyrosyl-IRS-1 or PDGFR, recruitment of SH-PTP2 to tyrosine phosphoproteins would stimulate its catalytic activity. If such a hypothesis is correct, it is expected that insulin and other growth factors would stimulate dephosphorylation of a tyrosine phosphoprotein in these adipocytes. A possible second criterion might be that any growth factor that promotes the recruitment of SH-PTP2 would promote the dephosphorylation of the same protein. Two lines of evidence support such a prediction: (1) SH-PTP2 was functionally required for a panel of growth factors to activate Ras; (2) Although SH-PTP2 was recruited to diverse phosphoproteins, the effect of SH-PTP2 converges on the pathway before Ras, since mutants of the PTP failed to activate Ras for a wide spectrum of growth factors. However, this criterion would clearly mean that the dephosphorylation mediated by SH-PTP2 need not be specific to insulin. It is also possible that the substrate would be tyrosine-phosphorylated in the absence of growth factor stimulation, in keeping with the possibility that the phosphoprotein might serve as an inhibitor of a pathway. According to these several criteria, pp67 could be a potential substrate for activated SH-PTP2. However, to test this hypothesis directly, it will be

necessary to have either a purified or at least highly enriched preparation of pp67 that does not display endogenous PTPase activity. Then pp67 dephosphorylation could be examined in SH-PTP2 immunoprecipitates from basal-state or from growth factor-stimulated cells.

SOS modification and transient ERK phosphorylation. The association of Grb2 with Sos1 is mediated through binding of the amino-terminal SH3 domain of Grb2 and the proline-rich motif in Sos (Mayer and Baltimore, 1993). It was suggested that Grb2 is necessary for the activation of p21^{ras} by targeting Sos1 to the plasma membrane (Aronheim et al. 1994). However, preventing the interaction between Grb2 and Sos1 by deleting the Grb2-binding region did not attenuate Sos1 to activate p21^{ras}. In contrast, deletion of either the Pleckstrin or/and Dbl homology-domains from Sos rendered it unable to activate p21^{ras}. Thus the authors suggested that the presence of unidentified cellular elements which interact with the Pleckstrin and/or Dbl homology-domains participate in the activation of p21^{ras}.

The PH domain is homologous to regions in membrane phospholipid binding proteins; indeed the PH domain from Sos was shown to bind phosphatidylinositol-4,5-bisphosphate *in vitro* (Harlan, 1994). This indicated that the PH domains may be important for membrane localization through interaction with phospholipids. Therefore, the cytosolic-to-membrane translocation of these PH domain-containing proteins may be governed by changes that alter the affinity of a PH domain for phospholipid. Therefore we examined the membrane distribution of the PH-domain containing proteins IRS-1, Sos, Shc and the one without a PH-domain, Grb2.

We resolved Sos1 into three different electrophoretic forms. Of the two retarded forms induced by insulin and growth factors, only the c-form is sensitive to phosphatase

treatment. However the b-form is insensitive to all kinds of phosphatase employed. These results imply that the b-form of Sos1 is likely modified by non-phosphorylation. However at this time we can not absolutely exclude that a special conformation of the b-form may lead to resistance to phosphatase treatment. Electrophoretic mobility shifts of Sos have been reported, however no reports showed three discreet bands and therefore they concluded that phosphorylation caused the mobility shift (Waters et al. 1995 and Cherniack et al,1995). Their failure to find the phosphatase insensitive form may derive from the resolution of gel. The hyperphosphorylation of Sos was suggested to lead to dissociation from Grb2. The function of the c-form of Sos is not known, however preliminary experiments showed both a- and b-forms of Sos1 were potentially bound to Grb2 (Kohanski, not shown). Since Ras is a membrane anchored protein, the translocation of the activator, Sos to the membrane may be essential. Therefore we analyzed the distribution of Sos as well as other signal molecules to the membrane.

Here we found that signaling molecules are selectively associated with the membrane compartment. IRS-1, Sos1, Grb2, and Shc52 and Shc46 - but not p70 S6 kinase nor Shc66 - were found present in the membrane compartment at significant levels, even in the basal state. However, Ras presumably is not active in the basal. These observations suggested that the simple presence of Sos and other molecules in the membrane is not sufficient to activate Ras. On the other hand, insulin does not increase recruitment of Sos, Grb2 and IRS-1 to the membrane compartment. However one event in the membrane shows a dramatic change by insulin stimulation, and that is the transient tyrosine phosphorylation of IRS-1 (Chapter One). In addition, formation of an IRS-1•Grb2•Sos1 complex is also transient (Fig.13). This

transient formation of a signal complex in the membrane is sufficient to explain the transient kinetics of ERK tyrosine-phosphorylation.

In fact, the rapid disassociation of SOS from IRS-1 in the membrane compartment may be the most significant transient event upstream of the RAS/MAP kinase pathway, since it is the triggering event; if the ternary complex is persistent, then some other event or complex between this complex and ERK phosphorylation must be transient, but since this complex forms only transiently in the membrane compartment, it is sufficient to explain the transient of ERK phosphorylation. Of course there must a mechanism for deactivation, or in this case dephosphorylation, or the activated/phosphorylated state would persist until the protein was degraded.

There are several potential mechanisms that might explain the transient formation of an IRS-1•Grb2•Sos1 complex. It is possible that the increase in phosphotyrosyl-Shc52 binds Grb2 and thereby “pulls” it away from the IRS-1•Grb2•Sos1 complex, causing it to dissociate if Grb2 availability is limiting. This would be consistent with the hypothesis that there is a competition between phosphotyrosyl-IRS-1 and phosphotyrosyl-Shc52 for Grb2. However, the fraction of Grb2 associated with Shc is so small that this seems unlikely to be responsible for the transient of ERK phosphorylation observed in this system.

The disassociation of Sos1 from Grb2, and therefore from phosphotyrosyl-IRS-1, has been proposed as a possible mechanism to explain the transient activation of Ras. It was further suggested that such dissociation was caused by hyper-phosphorylation of Sos1 through a MEK-dependent feedback pathway. Since we have been able to resolve three forms of Sos1, where previous reports have shown only two (or fewer), it would be

important to know which forms bind Grb2. In addition, we have shown that only the Sos1-c form is sensitive to PPASE action, and therefore it would also be important to know what the modification is that gives rise to the PPase-insensitive Sos1-b form; this assumes that Sos1-a is the “basal” unmodified form of Sos1.

In summary, our results do not contradict the proposal that Sos hyperphosphorylation and dissociation from Grb2 is one possible mechanism to regulate complex formation in the cytoplasm. However, the abundance of this signaling complex in the membrane might be regulated more by the level of phosphotyrosyl-IRS-1. However, there may be a change in the ability of Sos1 to form a complex with Grb2 and phosphotyrosyl-proteins, and this change in function might be reported by changes in Sos1 electrophoretic mobility, as discussed above. Because of the observed transient of IRS-1 tyrosine-phosphorylation in the membrane but not in the cytosolic pool, changes in Sos1 may not be important for the transient nature of ERK tyrosine-phosphorylation. However, they may be necessary to maintain a desensitized state of the RAS/MAP kinase pathway.

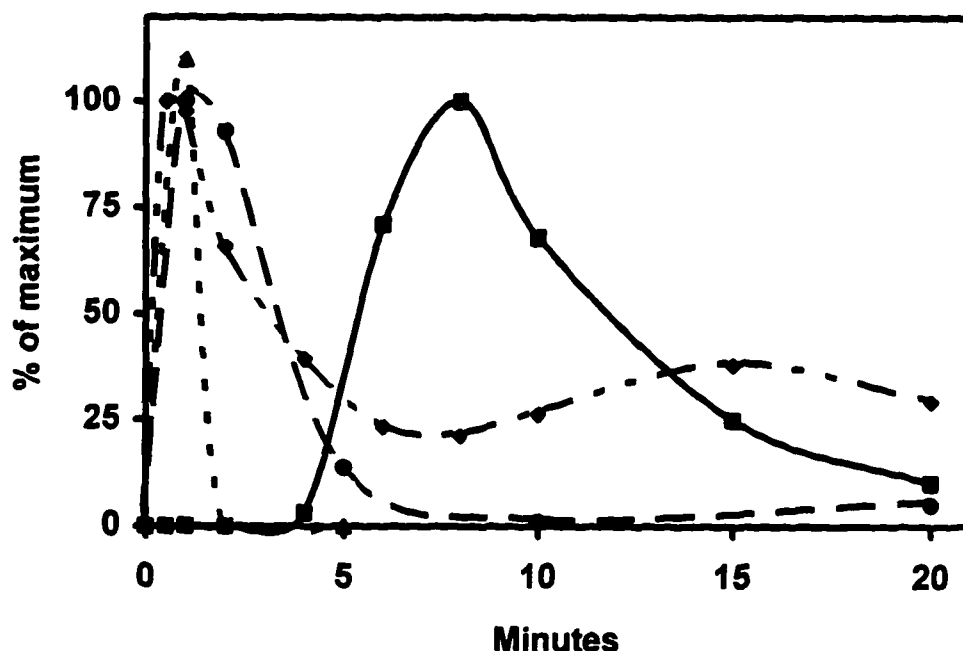


Figure 15. Comparison of Time-Dependent Changes in Phosphotyrosyl-IRS-1 Sos1•IRS-1 and Grb2•IRS-1 Complexes with ERK2 Tyrosine Phosphorylation. Over the time course of insulin-stimulation shown in Fig.2, the levels of phosphotyrosyl phosphorylation of ERK2 (■) and of membrane-associated phosphotyrosyl-IRS-1 (◆) were determined. In separate experiments, the amounts of phosphotyrosyl-IRS-1 in complexes with Sos1 (▲) and with Grb2 (●) were determined by anti-PTyr Western blots from anti-Sos1 and anti-Grb2 immunoprecipitates, respectively, prepared from the detergent-solubilized membrane fractions of cells stimulated with insulin for the indicated times (data are taken from Han, Dao, & Kohanski; manuscript in preparation). The levels are normalized to the observed maxima; for phosphotyrosyl-IRS-1 in complex with Sos1, the maximum was set to “120” so that this point could be seen in the plot.

Table 4

Relative Abundances of IRS-1 or Receptors in Signaling Complexes

Signaling complexes induced by insulin and involving phosphotyrosyl-IRS-1, or those induced by EGF or by PDGF and involving their respective tyrosine-phosphorylated receptors, were detected in anti-phosphotyrosine Western blots after immunoprecipitation from whole lysates. Quantification was done by densitometry, as described in the text. Direct immunoprecipitation of each listed phosphoprotein provided the “net” phosphotyrosine content of the phosphoproteins IRS-1, EGF receptor (EGFR) or PDGF receptor (PDGFR), as shown for example in Fig. A; these are set to a level of “100” for each phosphoprotein. Co-immunoprecipitation of these phosphoproteins with antibodies against the “signaling molecules” Sos1, Grb2, Shc, or SHP1 (e.g., Figs. 19-21) yielded the relative abundance of each complex, which varied between “zero” - for no complex formed - and “100” - indicating that all of that phosphoprotein was in a complex including that signaling molecule, but not exclusive of other signaling molecules occurring in the same complex. Further details are given in the text.

Phosphoprotein	Signaling Molecule			
	Sos1	Grb2	Shc	SHP1
IRS-1	≤5	50	«1	5
EGFR	≤1	70	70	≤1
PDGFR	≤1	10	«1	80

Table 5

Ratios of PTyr-Shc•Grb2 Complexes to Total PTyr-Shc

Signaling complexes formed between Grb2 and phosphotyrosyl-Shc isoforms, induced by growth factors, were detected in anti-PTyr Western blots after immunoprecipitation from whole lysates. The complex was detected after immunoprecipitation with anti-Grb2, and net PTyr-Shc was detected after immunoprecipitation with anti-Shc. Quantification was done by densitometry, as described in the text.

Growth Factor	Ratio of PTyr-Shc•Grb2 to PTyr-Shc		
	Shc46	Shc52	Shc66
Insulin	na	0.5-1	na
EGF	≤0.2	0.5-1	1
PDGF	≤0.1	0.5-1	U

na- not applicable because not phosphotyrosine phosphorylated

U - not determined because of interference from PTyr-SHPT1

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