Review

Signal transduction in insulin action

Alan R. Saltiel

Laboratory of Molecular Oncology, The Rockefeller University, New York, NY, USA

Introduction

Few hormones have received the attention accorded to insulin. It was one of the first peptide hormones to be purified, sequenced, and undergo structural analysis. The physiology of insulin action was evaluated in the 1950s.[1,2] Studies on the binding of insulin to its cell surface receptor and the subsequent purification and cloning of the receptor[3,4] served as a model of hormone-receptor interaction for other peptide hormones. Despite this intensive scrutiny, our understanding of the molecular events that link the insulin receptor to the regulation of cellular metabolism lags far behind that of other peptide hormones. One likely explanation for this slow progress may lie in the complicated nature of insulin action. The wide number and broad diversity of responses make it improbable that a single mechanism could account for all of the actions of insulin. Rather, it is more likely that insulin action involves a network of interrelated and independent pathways with differing levels of divergence regarding mechanisms of regulation.

As it became clear that the well-recognized mechanisms of signal transduction (i.e., cyclic nucleotides, ion channels) were not primarily responsible for explaining the actions of insulin, many investigators focused on the role of protein phosphorylation. Insulin simultaneously produces both the dephosphorylation of some proteins (i.e., glycogen synthetase, pyruvate dehydrogenase, hormone-sensitive lipase) along with the phosphorylation of other proteins (i.e., ribosomal S6, ATP citrate lyase). The dephosphorylation reactions induced by insulin result in the regulation of carbohydrate and lipid metabolism. Although it is not clear whether insulin-induced serine phosphorylation leads to changes in the catalytic activities of any enzymes, processes such as glucose transport and protein synthesis may require insulin-induced kinase reactions.[5] Interestingly, the specific protein phosphatase inhibitor okadaic acid prevented many of the acute anabolic effects of insulin, but potentiated the stimulation of glucose transport.[6] These and other observations have led to the emerging concept that two basic pathways, activation of protein phosphatases and kinases, may mediate many of insulin's actions.

Address reprint requests to Alan R. Saltiel, Laboratory of Molecular Oncology, The Rockefeller University, New York, NY 10021, USA.

Two major hypotheses have been proposed for coupling the insulin receptor to intracellular changes in protein phosphorylation: (1) a phosphorylation cascade, initiated by the tyrosine kinase activity of the receptor[7] and (2) the generation of a low molecular weight, diffusable second messenger. Evaluation of the phosphorylation cascade hypothesis has centered on site-directed mutagenesis and anti-receptor antibody experiments that suggest that the receptor tyrosine kinase is necessary for the full expression of insulin's activities.[8-10] The search for an insulin-dependent "second messenger has been underway since the early 1970s. An insulin-sensitive substance was first detected in skeletal muscle that could acutely modulate glycogen synthetase activity *in vitro*. Similar kinds of extracts or substances of elusive chemical identity were subsequently identified in a variety of cell types, reported capable of regulating the activities of several insulin-sensitive enzymes.[11] It is important to note that these two pathways need not be mutually exclusive, and, in fact, they may operate synergistically to coordinate a pattern of protein phosphorylation in response to insulin.

Biological characterization of a putative insulin second messenger

A low molecular weight substance was identified that was released from hepatic plasma membranes in response to insulin.[12] The purification of this enzyme-modulating activity relied mainly on ion exchange, molecular sizing, and phase partitioning procedures. The substance was negatively charged, insoluble in organic solvents, and not adsorbed to reversed phase columns, indicating a relatively high degree of polarity. The chemical properties inferred from chromatographic behavior and susceptibility to specific chemical modification suggested an oligosaccharide-phosphate structure.

Initial studies on the biological activities of this substance focused on the modification of the activity of the low Km cAMP phosphodiesterase in fat cell membranes. The activity of this cAMP phosphodiesterase was activated acutely by the modulator, as reflected by an increase in the Vmax of the enzyme, with no appreciable effect on its Km. The purified substance could also modify *in vitro* other insulin-sensitive enzymes assayed in subcellular fractions, including

adenylate cyclase, pyruvate dehydrogenase, phospholipid methyltransferase, cAMP dependent protein kinase, and acetyl CoA carboxylase.[13] Although the precise biochemical mechanism(s) by which this substance elicits its effects on these enzymes is unclear, the regulation of the activity of each of these enzymes might be explained by alterations in the state of phosphorylation of the enzyme or closely related regulatory factors. The specific regulation of protein phosphatase activities was, in fact, observed in lysates from fat, brain, and liver, although it is unknown whether the modulator produces the direct allosteric regulation of specific protein phosphatases.

Preliminary compositional analyses of the enzyme-modulating substance suggested the existence of inositol as a component. Several well-known inositol phosphate-containing compounds were evaluated, but none exhibited significant enzyme-modulating activity, and they did not share the chemical properties, chromatographic or electrophoretic behavior, or insulin sensitivity of the enzyme modulator. These results suggested that the enzyme modulator might be an unusual derivative containing inositol phosphate. A potential clue was identified when a novel glycosylated derivative of inositol was found in certain cell surface proteins. This novel molecular species was shown to result from a covalent bond between certain proteins and phosphatidylinositol (PI).[14] This unusual linkage at the carboxy terminus of these proteins serves as an anchor for attachment to the plasma membrane. The protein is coupled via an amide bond to ethanolamine, which is then attached through a phosphodiester linkage to an oligosaccharide that exhibits a terminal non-N-acetylated hexosamine glycosidically linked to the inositol ring of PI. The membrane-bound form of the protein can be converted to a water soluble form that contains a C− terminal glycosyl-inositol phosphate by digestion with a bacterial PI-specific phospholipase C (PLC), with the simultaneous liberation of diacylglycerol. (See *Figure 1*.)

To evaluate the possibility that the insulin-dependent enzyme modulator might arise from the phosphodiesteratic hydrolysis of a structurally similar glycolipid, the PI-specific bacterial phospholipase C was added to liver plasma membranes, and the release of the modulator into the medium was assayed. In this series of experiments, PI-PLC was found to reproduce the effect of insulin in facilitating the generation of the enzyme modulator. The PI-PLC digestions generated a substance that was chromatographically, electrophoretically, and chemically identical to that produced by insulin treatment, suggesting a basic inositol phosphate-glycan structure. Moreover, a potential precursor of the PI-PLC-generated substance could be extracted from liver membranes and chromatographically resolved from other known phosphoinositides. This precursor glycolipid was identified in a number of cell types and appeared to contain PI linked to a glycan through a non-N-substituted hexosamine. These experiments suggested that the enzyme modulator was produced as a result of a hormone-stimulated hydrolysis of this novel membrane-associated glycosyl-PI.

Considerable interest has been focused on the precise structure and biosynthesis of the glycosyl-PI precursor, and on its possible relationship to the glycosyl-PI protein anchor. Comparison of compositional analyses from several laboratories has indicated certain conserved and variant features. The precursor glycolipid appears to contain a basic core structure of PI-hexosamine. The T lymphocyte or BC_3H1 cell-derived glycolipid appears to contain diacylglycerol as the major glycerolipid moiety,[15-17] although in hepatoma cells a 1,2 alkylacylglycerol structure has been suggested.[18] More recent studies have indicated another possible structural variation in the liver-derived glycosyl-PI, the presence of significant but variable amounts of *chiro*inositol, which perhaps accounts for the apparent lack of [^{3}H]*myo*inositol labeling in hepatoma cells.[19,20] Another minor structural variation regards the presence of galactosamine in lieu of glucosamine.[20] Distal to the hexosamine there appear to be considerable differences in glycan composition reported by different laboratories. Whether these apparently different structures represent molecules with distinct cellular functions or subcellular localizations remains to be determined.

Time course studies in BC_3H1 cells suggest that the synthesis of the glycosyl-PI temporally follows that of PI.[21] Methodology has recently been developed to evaluate *in vitro* the biosynthesis of the structurally related precursor for the glycosyl-PI protein anchor in

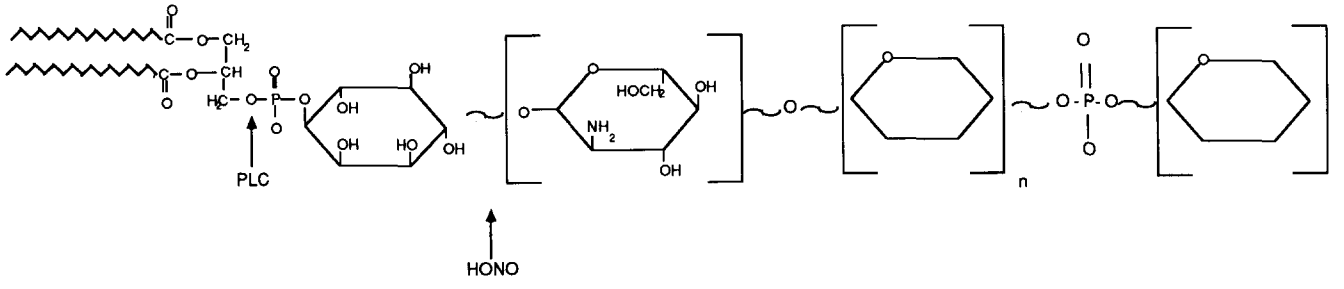

Figure 1 Partial structure of the insulin-sensitive glycosyl-PI. This glycophospholipid molecule consists of 1,2 diacylglycerol (or alkylacylglycerol) linked by a phosphodiester bond to inositol. The inositol is glycosidically linked to the C1 position of glucosamine. Glucosamine is then attached at the 4 or 6 position to additional monosaccharides. The terminal monosaccharide may be linked through a phosphodiester bond. The sites of hydrolysis for phospholipase C (PLC) and nitrous acid (HONO) are shown.

trypanosomes.[22] These studies suggest that the synthesis of the glycolipid results from the glycosylation of PI, rather than from the addition of a preformed inositol-containing oligosaccharide to diacylglycerol. This glycosylation appears to occur in a stepwise fashion, in which PI-glucosamine is first assembled by the transfer of an activated nucleotide N-acetylglucosamine, followed by further glycosylation.

The insulin-sensitive glycosyl-PI appears to exhibit considerable similarity to the glycosyl-PI protein anchor. The two types of glycolipid contain similar glycerolipid domains, sensitivity to PI-PLC and nitrous acid, the presence of inositol, nonacetylated glucosamine, and a variable glycan region. However, the insulin-sensitive glycosyl-PI apparently lacks two of the features commonly observed in the protein anchor, ethanolamine and amino acids. Additionally, the molecular size of the insulin sensitive glycosyl-PI is smaller than similar molecules bound to protein. Despite the similarities between the insulin-sensitive glycosyl-PI and the cell surface protein anchor, the topological distribution of the insulin-sensitive glycolipid in the plasma membrane is uncertain. Some studies have suggested a cytoplasmic orientation, since treatment of cells with PI–PLC (presumably exhaustive) did not block the insulin-induced intracellular accumulation of the inositol glycan. Gaulton and Pawlowski[23] have reported that fluorescently labeled monoclonal antibodies to the insulin-sensitive glycosyl-PI show cytoplasmic staining, indicting an intracellular localization. In contrast, Alvarez et al.[24] suggested an extracellular location for the lipid, since 85% of the total cellular glycosyl-PI was apparently surface amidinated with isethionyl acetimidate. Although the free release from cells of the inositol glycan in response to insulin has not been reported, the exogenous addition of PI–PLC to intact cells produces an insulin-like activity, exogenous addition of purified inositol glycan to intact cells mimics many of the effects of insulin, and insulin may lead to the release from the cell surface of certain glycosyl-PI anchored proteins (see below). Thus, the issue of topology of the relevant glycolipid must remain open until definitive evidence is available.

The possibility that the protein-bound and free forms of glycosyl-PI are located on opposite sides of the plasma membrane leads to further uncertainty regarding their respective biosynthetic processing. One possibility to explain this apparent dilemma is that the early stages of glycosylation of PI occur on the cytoplasmic aspect of the endoplasmic reticulum. Upon attaining a certain level of glycosylation, a fraction of the glycolipid molecules ultimately destined for protein anchoring might be translocated from the cytoplasmic face of the endoplasmic reticulum membrane to the luminal side, in analogy to the translocation of the $(Man)_5(GlcNAc)_2$-lipid utilized for N-linked glycosylation of proteins. This translocation step may serve to segregate further biosynthetic modifications of the lipid molecules ultimately destined for protein attachment from those that will remain on the cytoplasmic face. Alternately, a final processing event, such as addition of a terminal sugar-phosphate, may serve to segregate those molecules not destined for translocation. In either case, the subsequent membrane trafficking to the cell surface might then result in a cytoplasmically oriented free glycolipid and a cell surface-oriented protein-anchored glycolipid. (See *Figure 2.*)

Regulation of glycosyl-PI hydrolysis

While insulin is known to cause increased labeling of several phospholipids, it has not been found to stimulate immediately the hydrolysis of PI or the polyphosphoinositides, and it does not induce calcium mobilization through the generation of inositol triphosphate. In contrast, insulin does stimulate hydrolysis of glycosyl-PI, with the simultaneous production of the [^{3}H]inositol glycan and diacylglycerol that contains predominantly saturated fatty acids, but little if any arachidonic acid. The rapid production of this specific species of labeled diacylglycerol is not observed with agonists known to stimulate the hydrolysis of polyphosphoinositides.[16] Thus, the unique species of diacylglycerol and the inositol glycan probably arise from the specific, insulin-sensitive hydrolysis of the free glycosyl-PI. Furthermore, these data suggest that the relevant phospholipase C might be highly selective for glycosyl-PI substrates.

These observations led to the search for a glycosyl-PI specific phospholipase C. Such an enzyme was isolated from a plasma membrane fraction of liver, using as an assay the liberation of diacylglycerol from the glycosyl-PI anchored variant surface glycoprotein from *Trypanosoma brucei*.[25] The catalytic activity appears to reside in a single polypeptide with an apparent molecular weight of about 52,000 daltons. The enzyme is calcium-independent, and is specific for glycosyl-PI; no hydrolysis of PI, PIP_2, or other phospholipids is observed under a variety of conditions. Although there is still no information available on the primary sequence or subcellular localization of the mammalian glycosyl-PI PLC, the cDNA for an enzyme with similar specificity was cloned and sequenced from *T. brucei*.[26] Interestingly, both immunohistochemical data and the predicted amino acid sequence indicate a cytoplasmic orientation for this parasitic enzyme.

How is the regulation of glycosyl-PI hydrolysis coupled to the activity of the insulin receptor? Although a phospholipase C capable of catalyzing this reaction has been purified, it has thus far been difficult to demonstrate directly the activation of this enzyme by insulin. Recent studies with anti-receptor antibodies or site-directed mutagenesis indicate that the tyrosine kinase activity of the receptor may be necessary for the expression of all of the biological actions of insulin.[3] In cells transfected with mutant insulin receptors that lack tyrosine kinase activity, glycosyl-PI hydrolysis is not observed in response to insulin, whereas cells transfected with wild type receptors respond normally.[27] This suggests that the activation of the glycosyl-PI–specific phospholipase C by the recep-

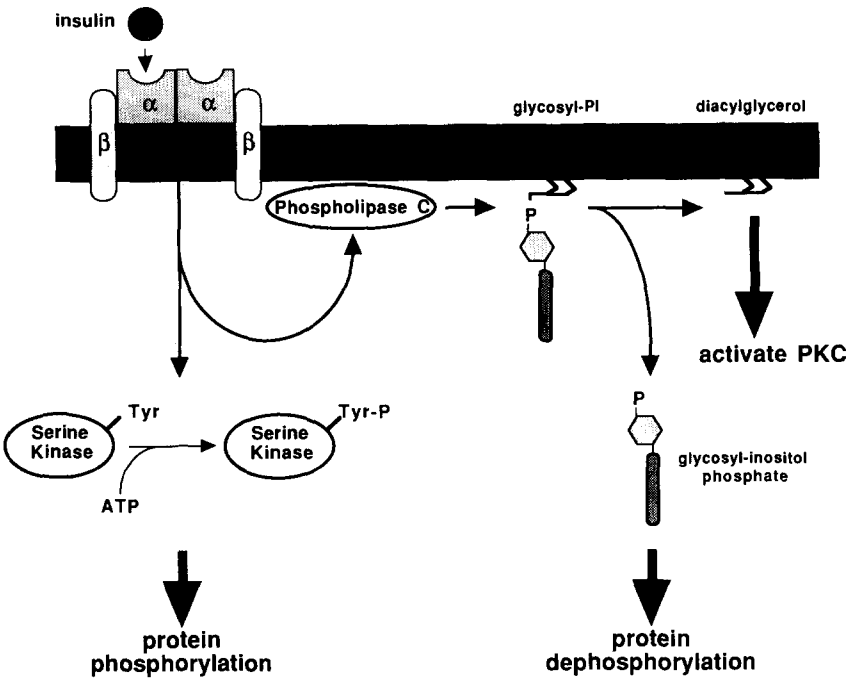

Figure 2 Regulation of protein phosphorylation by insulin. A hypothetical model is presented to explain the modulation of protein phosphorylation by insulin. Insulin coordinates a pattern of protein phosphorylation and dephosphorylation, perhaps through different mechanisms. The dephosphorylation of proteins such as hormone-sensitive lipase, pyruvate dehydrogenase or acetyl CoA carboxylase may result from the activation of specific protein phosphatases, due to the activity of the inositol glycan. This potential second messenger is generated by the coupling of the insulin receptor to a specific phospholipase C. The stimulation of protein phosphorylation by insulin may result from a combination of the selective activation of protein kinases C, as well as a receptor kinase induced cascade, in which tyrosyl phosphorylation of one or more serine kinases causes enzymatic activation. Thus, two separate but interrelated pathways may be involved in mediating many of the intracellular effects of insulin.

tor might occur as a consequence of a tyrosine kinase-induced cascade, possibly leading to changes in the state of phosphorylation of the enzyme or an associated regulatory factor. Alternatively, the autophosphorylation of the receptor on tyrosine residues could catalyze an intramolecular conformational change that initiates a membrane coupling event, involving noncovalent interactions with a regulatory factor. The latter possibility is supported by recent studies demonstrating that certain monoclonal antibodies to the insulin receptor that do not stimulate the receptor tyrosine kinase activity retain other insulin-mimetic properties regarding metabolic regulation.[28] Perhaps these antibodies induce a conformational change in the receptor similar to that caused by autophosphorylation, resulting in some intramembrane coupling event. The coupling factor might be a specific GTP-binding protein, which, in turn, could activate the PLC. The involvement of a G protein in insulin action has been suggested in studies demonstrating that pertussis toxin[29-31] or antibodies to the GTP-binding *ras* p21 protein[32] can block certain actions of insulin. Moreover, insulin inhibited the pertussis toxin–catalyzed ADP-ribosylation of a 41,000 Mr substrate in liver membranes.[33] Certain of the G proteins are relatively good substrates for the insulin receptor kinase *in vitro*.[34] Although the direct phosphorylation of a G protein on tyrosine residues in response to insulin has not been observed *in vivo*, these *in vitro* data suggest at least the possibility of a high affinity interaction between certain G proteins and the receptor.

Hormone-stimulated release of glycosyl-PI anchored proteins

Another result of the insulin-induced hydrolysis of glycosyl-PI might be the release of glycosyl-PI–anchored proteins. Insulin has been reported to cause reduced levels of cellular alkaline phosphatase or 5′-nucleotidase activities in different cell types.[35] Like PI–PLC, insulin caused the release of the glycosyl-PI anchored heparan sulfate proteoglycan from rat hepatocytes.[36] A form of lipoprotein lipase may be directly or indirectly anchored to the cell surface by a glycosyl-PI anchor, since enzyme activity or LPL protein labeled metabolically with [^{3}H]glucosamine or [^{32}P]orthophosphate can be specifically immunoprecipitated from the media of 3T3-L1 cells after treatment with PI–PLC. Additionally, membrane-associated lipoprotein lipase can be labeled at the cell surface with biotin and subsequently solubilized with PI–PLC. The kinetics of release of lipoprotein lipase activity from 3T3-L1 cells by insulin and PI–PLC are identical, indicating that the acute phase release by insulin may be due to activation of a glycosyl-PI–specific phospholipase.[37] The observation that lipoprotein lipase is itself anchored or is tightly coupled to a glycosyl-PI anchored protein is of special significance,

since this represents the first example of a protein anchored in this fashion that is known to be released from cells in response to hormones. Tissue or circulating levels of certain of the glycosyl-PI anchored proteins are altered in diabetic states, including alkaline phosphatase, 5′-nucleotidase, lipoprotein lipase, and heparan sulfate proteoglycan. However, it is unclear whether insulin will lead to the release of all accessible glycosyl-PI anchored proteins, or only a specific subset. Recent studies demonstrated that acute exposure of BC_3H1 cells to insulin or serum caused the selective loss of the glycosyl-PI anchored proteins (of Mr 35k and 130k) from the cell surface.[64] The exploration of this issue may help to resolve whether there are distinct hormonally sensitive and insensitive "structural" pools of glycosyl-PI, similar to what has been proposed for metabolic pooling of the inositol lipids. Although it is not yet known whether the insulin-induced release of these glycosyl-PI anchored proteins is due to a phospholipase C, the hydrolysis of glycolipid molecules on opposite sides of the membrane in response to insulin also raises questions concerning the polarity or orientation of the relevant hydrolytic enzymes. If the free glycosyl-PI is located on the cytoplasmic face of the plasma membrane, it may be necessary to invoke at least two separate enzymes, located on different sides of the membrane. One possibility is that the hormone-sensitive, cell surface–oriented enzyme is a specific phospholipase D.[38]

The role of diacylglycerol in insulin action

Another controversial issue in insulin action has been the possible involvement of the calcium- and phospholipid-dependent protein kinase C. Some studies have demonstrated that in intact cells, phorbol esters mimic some but inhibit other actions of insulin.[39] Much attention has focused on a possible role for kinase C in mediating the stimulation of glucose transport. Down regulation of kinase C by prolonged exposure to phorbol esters prevented the reactivation of glucose transport after a second challenge with phorbol esters, but did not alter the stimulation of transport by insulin in L6 muscle cells.[40] However, down regulation of kinase C did cause a 40–60% reduction in insulin-activated glucose transport in rat adipocytes. In addition, the potent kinase C inhibitor sphingosine completely blocked insulin-stimulated glucose transport in adipocytes.[41] The picture has been further complicated by studies examining the hormonal activation of kinase C. While phorbol esters induce the rapid loss of kinase C activity from cytosolic fractions of fat cells, insulin has no effect,[42,43] indicating that the hormone was ineffective in promoting the redistribution of the enzyme from a cytosolic to a membrane fraction thought to reflect kinase C activation. However, insulin has been reported to stimulate both a cytosolic and membrane-associated kinase C in BC_3H1 cells,[44] and a membrane-associated kinase C in rat diaphragm,[45] indicating a potential mechanism for the activation of the kinase that does not involve its translocation to the membrane.

It may be possible to accommodate these apparently conflicting observations by invoking the involvement of distinct chemical forms or metabolic pools of diacylglycerol produced in response to insulin. Most agonists that cause kinase C activation do so by stimulating the hydrolysis of the polyphosphoinositides, leading to the generation of inositol phosphates and diacylglycerol that contains arachidonate in the C2 position. The absence of phosphoinositide turnover in response to insulin, as well as the scarcity of arachidonate in the insulin-generated diacylglycerol suggests that this diacylglycerol is indeed different and may arise from an alternate source. Three potential alternate routes of diacylglycerol synthesis can be considered: (1) *de novo* synthesis from phosphatidic acid,[46] (2) hydrolysis of phosphatidylcholine,[46] and (3) hydrolysis of glycosyl-PI.[16] These distinct mechanisms can all lead to the production of a structurally distinct species of diacylglycerol without inositol trisphosphate–induced calcium mobilization. Thus, it is possible that insulin can cause a selective activation of kinase C depending upon the cell type, extent of activation, enzyme compartmentalization, substrate specificity, or susceptibility to proteolysis. Perhaps the most interesting possibility includes the selective activation of isoforms of kinase C by structurally distinct diacylglycerols. Multiple forms of the enzyme were predicted by the cloning of multiple cDNAs, and several isozymes have now been chromatographically resolved. Some evidence suggests that these isoforms may exhibit distinct regulatory properties, especially with regard to calcium and diacylglycerol sensitivity as well as substrate specificity. Moreover, these isoforms may exhibit different tissue and/or subcellular distributions, or may be differentially susceptible to down regulation or proteolysis. Thus, the selective activation of protein kinase C or a fraction of isozymes may explain the apparent discrepancies between the biological actions of phorbol esters and insulin.

Nerve growth factor stimulates glycosyl-PI hydrolysis

Although insulin and insulin-like growth factors are somewhat unique in their potent anabolic activities, there are a limited number of other peptides that share insulin-like biological activities. Among those is nerve growth factor (NGF). NGF, like insulin, promotes neurite outgrowth and enhances survival of certain neurons.[47] Additionally, insulin and NGF share many long- and short-term effects, including stimulation of amino acid and glucose transport, proteoglycan production, induction of certain proteins, and proto-oncogenes. Like insulin, reports have suggested an involvement of protein kinase C in NGF action, although there appears to be no effect of the hormone on polyphosphoinositide hydrolysis. Because of these

similarities, the effect of NGF on glycosyl-PI metabolism was explored in the pheochromocytoma clonal line, PC12.[48] NGF was found to stimulate the production of a [^{3}H]myristate-labeled species of diacylglycerol, with no effect on arachidonyl-labeled diacylglycerol. This was accompanied by the simultaneous NGF-stimulated hydrolysis of glycosyl-PI, along with the intracellular production of the inositol glycan. The specificity of this action was confirmed by the failure of NGF to stimulate diacylglycerol or inositol glycan production in a receptor-negative genetic variant of the PC12 line.[48]

In addition to explaining, in part, some of the anabolic effects of NGF, hydrolysis of glycosyl-PI might also provide a mechanism for activation of protein kinase C without PIP_2 hydrolysis or calcium mobilization. This possibility was examined by evaluating the effects of a kinase C inhibitor on the NGF-dependent induction of the *c-fos* gene. The specific kinase C inhibitor staurosporin attenuated this effect of NGF in a dose-dependent manner, indicating that active protein kinase C is necessary for the induction of certain protooncogenes by NGF.[48]

Despite the similarities in these biological actions of insulin and NGF, there are significant differences in the basic structures of their receptors. Molecular cloning of the high affinity NGF receptor revealed a sequence that predicted a monomeric structure containing a single transmembrane domain.[49] Moreover, there was no evidence for tyrosine kinase activity in the cytoplasmic domain of the receptor. This predicted structure is unlike the basic form thought to be required for receptors that couple to G proteins (seven membrane spanning domains), and also unlike many other growth factor receptors and proto-oncogene products that contain a tyrosine kinase activity in the cytoplasmic domain that catalyzes the autophosphorylation of the receptor. In addition to NGF, human growth hormone exhibits certain insulin-like effects,[50] although the receptor for this hormone is similar to the NGF receptor, in that its sequence predicts a single transmembrane spanning domain with no apparent tyrosine kinase in the cytoplasmic domain.[51] Thus, it will be important to determine the precise biochemical events distal to receptor binding at which the actions of insulin (tyrosine kinase) and NGF (nontyrosine kinase) receptors converge to elicit similar biological responses. This information may help to elucidate the molecular events in transmembrane signaling for these hormones.

Inositol glycan as a second messenger of insulin action

Despite the progress made in identifying the structure and biogenesis of the inositol glycan, it is still premature to regard this compound as a second messenger for any of the actions of insulin. The apparent insulin-dependency, rapidity, and extent of the generation of the inositol-glycan are consistent with the properties expected for second messengers. However, many questions remain concerning precise chemical structure as well as the nature of the insulin-mimetic properties. Thus far, the actions of the inositol glycan have been explored mainly in subcellular assays, so that the extent to which this molecule reproduces the actions of insulin in intact cells remains unclear. In recent studies, these issues have been addressed by evaluating the effects of the inositol glycan in fat and liver cells. Purified preparations of these compounds mimic the lipogenic[52] and anti-lipolytic[53] actions of insulin, as well as the regulation of phosphorylase a,[54] pyruvate kinase,[54] cAMP levels,[54] and pyruvate dehydrogenase.[55] Moreover, they produce specific protein phosphorylation patterns similar to those produced by insulin in intact fat or liver cells.[56] Interestingly, preparations of the inositol glycan also caused the direct inhibition of insulin release in isolated rat islet cells.[57] However, the compound does not appear to modulate glucose transport activity or ribosomal S6 kinase. An oligosaccharide with chemical and chromatographic properties similar to the inositol glycan has recently been isolated from conditioned media of Reuber hepatoma cells.[58] This substance stimulates both [^{3}H]thymidine uptake and activation of acetyl CoA carboxylase in a manner kinetically indistinguishable from and not additive with insulin. In contrast, this glycan does not stimulate amino acid uptake or tyrosine aminotransferase induction in hepatoma cells nor glucose transport in 3T3-L1 cells. It is especially interesting to note that the insulin-mimetic actions of the inositol glycan appear to be limited to those anabolic activities that are (1) more or less insulin-specific (i.e., are generally not reproduced by other growth factors like EGF or PDGF) and (2) are probably mediated by enhanced dephosphorylation (i.e., stimulation of protein phosphatase). Thus, the selective ability of the inositol glycan to mimic only a subset of the actions of insulin provides further evidence for diverse pathways of signal transduction in the actions of the hormone.

The role of serine kinases in insulin action

The observation that the inositol glycan plays a limited role in insulin action gives further support to the dual phosphatase/kinase model, and suggests that certain actions of insulin might be mediated by a tyrosine kinase-induced phosphorylation cascade involving the activation of one or more serine kinases.[59] Although the functional consequences of serine phosphorylation are unknown, it may play some role in the metabolism or subcellular distribution of certain proteins. The likelihood of an important role for serine phosphorylation is supported by (1) numerous reports of enhanced phosphorylations on serine in response to insulin that are not second messenger-mediated[59]; (2) observations that receptors for growth factors (i.e., EGF, PDGF) and protooncogene products (i.e., src) that contain tyrosine kinase activities also provoke increases in serine kinase activities, although these other ligands do not provide the full spectrum of insulin-like activi-

ties; and (3) the ribosomal protein S6 kinase derived from a number of cell types appears to be acutely activated by phosphorylation and inactivated by dephosphorylation. This activation apparently results from serine phosphorylation, indicating that S6 kinase is not the immediate substrate for the insulin receptor. The implication is, therefore, that an intermediate insulin-induced serine kinase may be responsible.

A number of insulin-sensitive serine kinase activities in both cytosol and membrane fractions have been identified[59]; however, there has been no evidence to directly link any of these kinases to the insulin receptor itself. Recently, a novel serine kinase has been identified that is apparently activated by tyrosine phosphorylation in response to insulin. This kinase, which catalyzes the phosphorylation of microtubule associated protein (MAP) 2 and possibly the insulin-sensitive S6 kinase *in vitro*, has been detected in 3T3-L1 cells and human fibroblasts. This MAP kinase is activated by insulin in a manner that appears to require tyrosine phosphorylation. The enzyme activity derived from 3T3-L1 cells has been partially characterized regarding molecular weight, behavior on some columns and substrate specificity.[60,61] In recent studies, a serine kinase with identical characteristics has been identified in H-35 hepatoma cells,[62] rat adipocytes,[62] and PC-12 pheochromocytoma cells.[63] Interestingly, the PC-12 derived MAP kinase is acutely activated by NGF in a manner similar to insulin.[63] Although there is still insufficient information to determine the importance of this kinase, it is clearly a promising candidate for mediating the actions of insulin and other growth factors that require protein phosphorylation.

Signal transduction in insulin action

Because of the pleiotropic nature of insulin action, the search for a single biochemical pathway to explain all of the actions of the hormone is an oversimplistic expectation. As mentioned above, insulin action probably requires the activation of both serine kinases and phosphatases, most likely through different mechanisms. Moreover, it is still uncertain whether other actions of insulin, such as enhanced translocation of membrane proteins, regulation of gene expression, and regulation of protein synthesis or degradation require any changes in protein phosphorylation.

Although the inositol glycan appears to be a promising candidate for a second messenger of insulin action, a number of issues remain to be resolved. The ultimate proof of a role for this compound as a second messenger will critically depend, among other things, on the determination of its precise structure. This may be complicated by the chromatographic resolution of multiple species of these molecules, perhaps reflecting the existence of distinct forms with different enzyme-modulating functions or subcellular distributions. It will also be important to produce these compounds in large quantity and homogeneous form, perhaps by organic synthesis, in order to reevaluate each of their biological activities in detail. Moreover, the biosynthetic route, mode of production and degradation, relationship to the insulin receptor, and the precise biochemical actions of these molecules need further exploration. It will be especially important to determine the topological distribution of the glycosyl-PI precursor in the plasma membrane. More detailed molecular characterization of the glycosyl-PI–specific phospholipase C will be necessary. Development of inhibitors or neutralizing antisera to this enzyme, and eventually site-directed mutagenesis studies, should help to define the functional role of this reaction in the pleiotropic actions of insulin.

References

1 Cahill, Jr. G. F. (1971). Physiology of insulin in man. *Diabetes* **20,** 785–797

2 Levine, R. (1982). Insulin: the effects and mode of action of the hormone. *Vit. Horm.* **39,** 145–173

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

4 Goldfine, I. D. (1987). The insulin receptor. *Endo. Rev.* **8,** 235–255

5 Denton, R. M. (1986). Early events in insulin action. Adv. Cyc. Nuc. and Protein Phos. Res., vol 26. P Greengard, G A Robinson, 293–341

6 Haystead, T. A. J., Sim, A. T. R., Carling, D., Honnor, R. C., Tsukitani, Y., Cohen, P., Hardie, D. G. (1989). Effects of the tumour promoter okadaic acid on intracellular protein phosphorylation and metabolism. *Nature* **337,** 78–81

7 Kasuga, M., Karlsson, F. A., Kahn, C. R. (1982). Insulin stimulates the phosphorylation of the 95,000 dalton subunit of its own receptor. *Science* **215,** 185–187

8 Chou, C. D., Cull, T. J., Russel, O. S., Cherzi, R., Lebwohl, D., Ullrich, A., Rosen, O. M. (1987). Human insulin receptors mutated at the ATP binding site lack protein kinase activity and fail to mediate postreceptor effects of insulin. *J. Biol. Chem.* **262,** 1842–1847

9 Ellis, L., Clauser, E., Morgan, D. O., Edery, M., Roth, R. A., Rutter, W. J. (1986). Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. *Cell* **45,** 721–729

10 Morgan, D. O., Roth, R. A. (1987). Acute insulin action requires insulin receptor kinase activity: Introduction of an inhibitory monoclonal antibody into mammalian cells blocks the rapid effects of insulin. *Proc. Natl. Acad. Sci. USA* **84,** 41–45

11 Jarett, L., Kiechle, P. L. (1984). Intracellular mediators of insulin action. *Vit. Horm.* **41,** 51–78

12 Saltiel, A. R., Cuatrecasas, P. (1986). Insulin stimulates the generation from hepatic plasma membranes of modulators derived from an inositol glycolipid. *Proc. Natl. Acad. Sci. USA* **83,** 5793–5797

13 Saltiel, A. R., Cuatrecasas, P. (1988). In search of a second messenger for insulin. *Am. J. Physiol.* **255,** C1–C11

14 Low, M. G., Saltiel, A. R. (1988). Structural and functional roles of glycosyl-phosphatidylinositol in membranes. *Science* **239,** 268–275

15 Saltiel, A. R., Fox, J. A., Sherline, P., Cuatrecasas, P. (1986). Insulin stimulated hydrolysis of a novel glycolipid generates modulators of cAMP phosphodiesterase. *Science* **233,** 967–972

16 Saltiel, A. R., Sherline, P., Fox, J. A. (1987). Insulin-stimulated diacylglycerol production results from the hydrolysis of a novel phosphatidylinositol glycan. *J. Biol. Chem.* **262,** 1116–1121

17 Gaulton, G. N., Kelly, K. L., Pawlowski, J., Mato, J. M., Jarett, J. (1988). Regulation and function of an insulin-sensitive glycosyl-phosphatidylinositol during T lymphocyte activation. *Cell* **53,** 963–970

18 Mato, J. M., Kelly, K. L., Abler, A., Jarret, L. (1987).

Identification of a novel insulin-sensitive glycophospholipid from H35 hepatoma cells. *J. Biol. Chem.* **262,** 2131–2137
19 Mato, J. M., Kelly, K. L., Abler, A., Jarett, L., Corkey, B. E., Cashel, J. A., Zopf, D. (1987). Partial structure of an insulin-sensitive glycophospholipid. *Biochem. Biophys. Res. Comm.* **146,** 764–772
20 Larner, J., Huang, L. C., Schwartz, C. F. W., Oswald, A. S., Shen, T-Y., Kinter, M., Tang, G., Zeller, K. (1988). Rat liver insulin mediator which stimulates pyruvate dehydrogenase phosphatase contains galactosamine and D-chiroinositol. *Biochem. Biophys. Res. Comm.* **151,** 1416–1426
21 Masterson, W. J., Doering, T. C., Hart, G. W., Englund, P. T. (1989). A novel pathway for glycan assembly: biosynthesis of the glycosyl-phosphatidylinositol anchor of the trypanosome variant surface glycoprotein. *Cell* **56,** 793–800
22 Farese, R. V., Cooper, D. R., Konda, G., Standaert, M. L., Davis, J. S., Pollet, R. J. (1988). Insulin provokes co-ordinated increases in the synthesis of phosphatidylinositol, phosphatidylinositol phosphates and the phosphatidylinositol-glycan in BC3H-1 myocytes. *Biochem. J.* **256,** 175–184.
23 Gaulton, G. N., Pawlowski, J. (1988). Monoclonal antibodies define the regulation and location of glycosyl-phosphatidylinositol on T lymphocytes. *Joslin Diabetes Symposium*
24 Alvarez, J. F., Varela, I., Ruiz-Albusac, J. M., Mato, J. M. (1988). Localisation of the insulin-sensitive phosphatidylinositol glycan at the outer surface of the cell membrane. *Biochem. Biophys. Res. Comm.* **152,** 1455–1462
25 Fox, J. A., Soliz, N. M., Saltiel, A. R. (1987). Purification of a phosphatidylinositol-glycan specific phospholipase C from liver plasma membranes; a possible target of insulin action. *Proc. Natl. Acad. Sci. USA* **84,** 2663–2667
26 Hereld, D., Hart, G. W., Englund, P. T. (1988). cDNA encoding VSG lipase, the glycosyl-phosphatidylinositol-specific phospholipase C. *Proc. Natl. Acad. Sci. USA* **85,** 8914–8918
27 Rosen, O. M. Personal communication
28 Hawley, D. M., Maddux, B. A., Patel, R. G., Wong, K-Y., Mamula, P. W. Firestone, G. L., Burnetti, A., Verspohl, E., Goldfine, I. D. (1989). Insulin receptor monoclonal antibodies that mimic insulin action without activating tyrosine kinase. *J. Biol. Chem.* **264,** 2438–2444
29 Elks, M. L., Jackson, M., Manganiello, V. C., Vaughan, M. (1987). Effect of NG-(2-2-phenylisopropyl) adenosine and insulin on cAMP metabolism in 3T3-L1 adipocytes. *Am. J. Physiol.* **252,** C342–C348
30 Goren, J. J., Northrup, J. K., Hollenberg, M. D. (1985). Action of insulin modulated by pertussis toxin in rat adipocytes. *Can. J. Physiol. Pharmacol.* **63,** 1017–1022
31 Luttrell, L. M., Hewlett, E. L., Romero, G., Rogol, A. K. (1988). Bordetella pertussis toxin treatment attenuates some of the effects of insulin in BC_3H-1 murine myocytes. *J. Biol. Chem.* **263,** 6134–6141
32 Korn, L. J., Siebel, C. W., McCormick, F., Roth, R. A. (1987). *Ras* p21 as a potential mediator of insulin action in *Xenopus* oocytes. *Science* **236,** 840–843
33 Rothenberg, P. L., Kahn, C. R. (1988). Insulin inhibits pertussis toxin-catalyzed ADP-ribosylation of G-proteins. *J. Biol. Chem.* **263,** 15546–15552
34 Zick, Y., Sagi-Eisenberg, R., Pines, M., Gierschik, P., Spiegel, A. M. (1986). Multisite phosphorylation of the α subunit of transducin by the insulin receptor kinase and protein kinase C. *Proc. Natl. Acad. Sci. USA* **83,** 9294–9247
35 Romero, G., Luttrell, L., Rogol, A., Zeller, K., Hewlett, E. Larner, J. (1988). Phosphatidylinositol-glycan anchors of membrane proteins: potential precursors of insulin mediators. *Science* **240,** 509–511
36 Ishihara, M., Fedarko, N. S., Conrad, H. E. (1987). Involvement of phosphatidylinositol and insulin in the coordinate regulation of proteoheparan sulfate metabolism and hepatocyte growth. *J. Biol. Chem.* **262,** 4708–4716
37 Chan, B. L., Lisanti, M. R., Rodriguez-Boulan, E., Saltiel, A. R. (1988). Lipoprotein lipase is anchored to the cell surface by glycosyl-phosphatidylinositol: regulation of release by insulin. *Science* **241,** 1670–1672
38 Low, M. G., Prasad, A. R. S. (1988). A phospholipase D specific for the phosphatidylinositol anchor of cell surface proteins is abundant in plasma. *Proc. Natl. Acad. Sci. USA* **85,** 980–984
39 Standaert, M. L., Pollet, R. J. (1988). Insulin-glycerolipid mediators and gene expression. *FASEB J.* **2,** 2453–2461
40 Klip, A., Ramlal, T. (1987). Protein kinase C is not required for insulin stimulation of hexose uptake in muscle cells in culture. *Biochem. J.* **242,** 131–136
41 Smal, J., DeMeyts, P. (1987). Role of kinase C in the insulin-like effects of human growth hormone in rat adipocytes. *Biochem. Biophys. Res. Comm.* **147,** 1232–1240
42 Glynn, B. P., Calliton, J. W., McDermott, J. M., Witters, L. A. (1986). Phorbol esters, but not insulin, promote depletion of cytosolic protein kinase C in rat adipocytes. *Biochem. Biophys. Res. Comm.* **135,** 1119–1125
43 Vaartges, W. J., deHaas, C. G. M., Van den Bergh, S. G. (1986). Phorbol esters, but not epidermal growth factor or insulin, rapidly decrease soluble protein kinase C activity in rat hepatocytes. *Biochem. Biophys. Res. Comm.* **130,** 1328–1333.
44 Cooper, D. R., Konda, T. S., Standaert, M. L., Davis, J. S., Pollet, R. J., Farese, R. V. (1987). Insulin increases membrane and cytosolic protein kinase C activity in BC3H-1 myocytes. *J. Biol. Chem.* **262,** 3633–3639
45 Walaas, S. I., Horn, R. S., Adler, A., Albert, K. A., Walaas, O. (1987). Insulin increases membrane protein kinase C activity in rat diaphragm. *FEBS Let.* **220,** 311–318
46 Farese, R. V., Konda, T. S., Davis, J. S., Standaert, M. L., Pollet, R. J., Cooper, D. R. (1987). Insulin rapidly increases diacylglycerol by activating *de novo* phosphatidic acid synthesis. *Science* **238,** 586–589
47 Recio-Pinto, E., Land, F. F., and Ishii, D. N. (1984). Insulin and insulin-like growth factor II permit nerve growth factor binding and the neurite formation response in cultured human neuroblastoma cells. *Proc. Natl. Acad. Sci. USA* **81,** 2562–2566
48 Chan, B. L., Chao, M. V., and Saltiel, A. (1989). Nerve growth factor stimulates the hydrolysis of glycosyl-phosphatidylinositol in PC-12 cells: A mechanism of protein kinase C regulation. *Proc. Natl. Acad. Sci. USA* **86,** 1756–1760
49 Chao, M. V., Bothwell, M. A., Ross, A. H., Koprowski, H., Lanahan, A. A., Buck C. R., and Schgal, A. (1986). Gene transfer and molecular cloning of the human NGF receptor. *Science* **232,** 518–521
50 Isaksson, O. G. P., Eden, S., and Jansson, J. (1985). Mode of action of pituitary growth hormone on target cells. *Ann. Rev. Physiol.* **47,** 483
51 Leung, D. W., Spencer, S. A., Cachianes, G., Hammonds, R. G., Collins, C., Henzel, W. J., Barnard, R., Waters, M. J., and Wood, W. I. (1987). Growth hormone receptor and serum binding protein: purification, cloning and expression. *Nature* **330,** 537
52 Saltiel, A. R., Sorbara-Cazan, L. R. (1987). Inositol glycan mimics the action of insulin on glucose utilization in rat adipocytes. *Biochem. Biophys. Res. Comm.* **149,** 1084–1092
53 Kelly, K. L., Mato, J. M., Merida, I., Jarett, L. (1987). Glucose transport and antilipolysis are differentially regulated by the polar head group of an insulin sensitive glycophospholipid. *Proc. Natl. Acad. Sci. USA* **84,** 6404–6407
54 Alvarez, J. F., Cabello, M. A., Feliu, J. E., Mato, J. M. (1987). A phospho-oligosaccharide mimics insulin action on glycogen phosphorylase and pyruvate kinase activities in isolated rat hepatocytes. *Biochem. Biophys. Res. Comm.* **147,** 765–771
55 Gottschalk, W. K., Jarett, L. (1988). The insulinomimetic effect of the polar head group of an insulin-sensitive glycophospholipid on pyruvate dehydrogenase in both subcellular and whole cell assays. *Arch. Biochem. Biophys.* **261,** 175–185
56 Alemany, S., Mato, J. M., Stralfors, P. (1987). Phosphodephospho control by insulin is mimicked by a phospho-oligosaccharide in adipocytes. *Nature* **330,** 77–79

57 Malaisse, W. J., Albor, A., Blachier, F., Vaverde, I., Sener, A., Mato, J. M. (1989). Effect of a phospho-oligosaccharidic putative insulin messenger on insulin release in rats. *Diabetologia* **32,** 295

58 Witters, L. A., Watts, T. D. (1988). An autocrine factor from Reuber hepatoma cells that stimulates DNA synthesis and acetyl CoA carboxylase: Characterization of biologic activity and evidence for a glycan structure. *J. Biol. Chem.* **263,** 8027–8036

59 Czech, M. P., Klarlund, J. K., Yagdoff, K. A., Bradford, A. P., Lamis, R. E. (1988). Insulin receptor signaling. *J. Biol. Chem.* **263,** 11017–11020

60 Ray, L. B., Sturgill, T. W. (1988). Insulin-stimulated microtubule-associated protein kinase is phosphorylated on tyrosine and threonine *in vivo*. *Proc. Natl. Acad. Sci. USA* **85,** 3753–3757

61 Sturgill, T. W., Ray, L. B., Erikson, E., Maller, J. L. (1988). Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. *Nature* **334,** 715–718

62 Miyasaka, T. and Saltiel, A. R. (unpublished results)

63 Miyasaka, T., Chao, M. V., Sherline, P. and Saltiel, A. R. Nerve growth factor stimulates a protein kinase in PC-12 cells that phosphorylates microtubule associated protein-2. *J. Biol. Chem.* (in press)

64 Lisanti, M. P., Darnell, J. C., Chan, B. L., Rodriguez-Boulan, E., Saltiel, A. R. (1989). The distribution of glycosylphosphatidylinositol anchored proteins is differentially regulated by serum and insulin. *Biochem. Biophys. Res. Commun.* **164,** 824–832