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The function of glycosyl phosphoinositides in hormone action

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The molecular events involved in the cellular actions of insulin remain unexplained. Some of the acute actions of the hormone may be due to the intracellular generation of a chemical substance which modulates certain enzyme activities. Such an enzyme-modulating substance has been identified as an inositol phosphate-glycan, produced by the insulin-sensitive hydrolysis of a glycosyl-phosphatidylinositol (glycosyl-PtdIns) precursor. This precursor glycolipid is structurally similar to the glycosyl–phosphoinositide membrane protein anchor. The exposure of fat, liver or muscle cells to insulin results in the hydrolysis of glycosyl-PtdIns, giving rise to the inositol phosphate glycan and diacylglycerol. This hydrolysis reaction is catalysed by a glycosyl-PtdIns-specific phospholipase C. This enzyme has been characterized and purified from a plasma membrane fraction of liver. This reaction also results in the acute release of certain glycosyl-PtdIns-anchored proteins from the cell surface. Elucidation of the functional role of glycosyl-phosphoinositides in the generation of second messengers or the release of proteins may provide further insights into the pleiotropic nature of insulin action.

Introduction

Although insulin is one of the most widely studied of the polypeptide hormones, our understanding of the molecular events that link the interaction of insulin with its receptor to the regulation of cellular metabolism remains incomplete. One explanation for this lack of progress lies in the pleiotropic nature of insulin action. The cellular effects of the hormone vary widely, including modulation of: (i) transport of molecules across the plasma membrane; (ii) levels of cyclic nucleotides; (iii) activities of key enzymes in intermediary metabolism; (iv) rates of protein synthesis; (v) DNA and RNA synthesis, including specific gene expression; and (vi) cellular growth and differentiation. The co-ordination of these distinct cellular processes by insulin differs with respect to cell type, dose–response and time course; this observation suggests that signal transduction in insulin action involves a network of biochemical signals or a bifurcation of an early signalling pathway.

There have been numerous proposals over the past twenty years to explain the molecular mechanisms of signal transduction in insulin action, including changes in cellular hyperpolarization, cyclic nucleotides, ion fluxes, polyphosphoinositide hydrolysis, generation of hydrogen peroxide and internalization of insulin or its proteolytic fragments (for review, see

Fain 1974; Czech 1977, 1985; Kahn *et al.* 1981; Jacobs & Cuatrecasas 1983; Rosen 1987; Goldfine 1987). As it became clear that none of these proposals could account for the primary transduction events in insulin action (Fain 1974; Czech 1977) many investigators focused on the role of protein phosphorylation. Early studies suggested that insulin promoted dephosphorylation reactions, presumably by activation of protein phosphatases or inhibition of kinases (Avruch *et al.* 1982; Denton 1986). The insulin-induced decrease in the phosphorylation of enzymes such as glycogen synthetase (Villar-Palasi & Larner 1960; Larner 1971), pyruvate dehydrogenase (Jungas 1971; Coore *et al.* 1971), hormone-sensitive lipase (Stralfors *et al.* 1984), pyruvate kinase (Claus *et al.* 1979), HMG-CoA reductase (Kennelly & Rodwell 1985), acetyl CoA carboxylase (Krakower & Kim 1981; Thampy & Wakil 1985; Jamil & Madsen 1987) and others (Denton 1986) is at least partly responsible for regulation of the activities of these enzymes. Later studies showed that insulin also stimulated the phosphorylation of certain proteins, including ribosomal S6 (Rosen *et al.* 1981), ATP citrate lyase (Alexander *et al.* 1979), acetyl CoA carboxylase (Witters 1981; Brownsey & Denton 1982) and several others (Denton 1986), although the functional consequences of enhanced phosphorylations are in most cases unknown.

Two hypotheses have emerged to explain the coupling of the insulin receptor to intracellular changes in protein phosphorylation: (i) a phosphorylation cascade, initiated by the tyrosine kinase activity of the receptor (Kasuga *et al.* 1982); and (ii) the generation of a novel second messenger, analogous to the cyclic nucleotides or inositol phosphates. These two pathways need not be mutually exclusive and in fact may operate synergistically to coordinate the series of cellular responses to insulin. Evaluation of the phosphorylation cascade hypothesis has centred on site-directed mutagenesis experiments, which indicate that the receptor tyrosine kinase is necessary for many of the actions of insulin (Chou *et al.* 1987; Ellis *et al.* 1986; Morgan *et al.* 1986; Morgan & Roth 1987). Although several proteins are phosphorylated on tyrosine in response to insulin (for review, see Rosen 1987; Goldfine 1987; Avruch *et al.* 1982; Denton 1986), the relevant substrates for the receptor kinase that might initiate an intracellular cascade have not been identified. The search for a second messenger has been underway since the early 1970s. Larner *et al.* (1979) first reported the detection of an insulin-sensitive substance in skeletal muscle that acutely modulated glycogen synthetase. Similar substances were later identified in a variety of cell types that regulated the activities of several insulin-sensitive enzymes (Jarrett & Kiechle 1984). Although these enzyme-modulating activities were detected in several laboratories in these early studies, there were some inconsistencies in their reported properties and definitive elucidation of structure was not achieved.

Biological characterization of the insulin-sensitive enzyme modulators

The search for an activity that could mediate some of the actions of insulin resulted in the isolation of two structurally similar substances that were released from hepatic plasma membranes in response to insulin (Saltiel & Cuatrecasas 1986). The purification of these enzyme-modulating activities relied mainly on ion exchange, molecular sizing and phase partitioning procedures. The two substances were resolved on ion-exchange columns or by high-voltage electrophoresis, owing to their distinct net negative charges. They were not soluble in organic solvents, nor were they adsorbed to reverse-phase columns; these observations

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indicate a relatively high degree of polarity. The chemical properties of these substances, inferred from their chromatographic behaviour and susceptibility to specific chemical modification, are summarized in table 1. The dissimilar net negative charges of the two substances were observed even at pH 2.0, suggesting phosphate as the major charged species. The substances were identical in all other chemical properties and enzyme-modulating

Table 1. Properties of the insulin-sensitive enzyme modulators

two activities, resolved by distinct net negative charges
acid-stable
alkaline-labile
relative molecular mass of 800–1000 on gel permeation
hydrophilic
inactivated by acetylation, methylation, periodate oxidation, nitrous acid deamination

activities. Their relative hydrophilicity, negative charges, apparent molecular mass of 800–1000 Da, and sensitivity to periodate and nitrous acid, suggested an oligosaccharide–phosphate structure.

Initial studies focused on the modulation of the low K_m cAMP phosphodiesterase in fat cell membranes (Saltiel & Cuatrecasas 1986). This enzyme was acutely activated by the enzyme modulator, reflected by an increase in the V_{max} of the enzyme with no appreciable effect on the K_m (Saltiel 1987). Interestingly, the insulin-independent low affinity form of the enzyme was not affected. In addition to the regulation of cAMP phosphodiesterase activity, the purified substances modified other insulin-sensitive enzymes assayed in subcellular fractions, including adenylate cyclase and pyruvate dehydrogenase (Saltiel 1987) and phospholipid methyltransferase (Kelly *et al.* 1986). In some cases, the modulation of enzyme activity was biphasic with respect to concentration, reminiscent of the paradoxical dose-responses observed for some of the metabolic activities of insulin (Saltiel 1987). Although the precise biochemical mechanism(s) by which this substance elicits its actions is unclear, the regulation of these enzymes might be explained by control of the state of phosphorylation of the enzyme or a regulatory factor (Saltiel 1987) due to the regulation of one or more specific protein phosphatases (A. R. Saltiel, unpublished data). The acute modulation of these rate-limiting enzymes and perhaps others suggests a role for the inositol glycans in the regulation of carbohydrate and lipid metabolism. Moreover, the inhibition of adenylate cyclase and the stimulation of cAMP phosphodiesterase indicates that these substances may play a part in reducing cAMP levels.

The enzyme modulators are inositol phosphate-glycans formed from a glycosylphosphatidylinositol precursor

Preliminary compositional analyses of the enzyme-modulating substances suggested inositol as a component. Several of the known inositol phosphate compounds were evaluated, yet none exhibited the enzyme-modulating activity or shared the chemical properties, chromatographic or electrophoretic behaviour or insulin-sensitivity of the enzyme modulators. These results suggested that the enzyme modulators might be inositol phosphate derivatives. A glycosylated derivative of inositol was recently identified as an anchor for certain proteins to the plasma

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membrane, in which the protein is coupled to phosphatidylinositol (PtdIns) (see Low & Saltiel (1988) for review). In this unusual linkage, the protein is covalently attached to ethanolamine via an amide bond. The ethanolamine is then bound through a phosphodiester linkage to an oligosaccharide that possesses a terminal non-*N*-acetylated hexosamine glycosidically linked to the inositol ring of PtdIns. The membrane bound form of the protein can be converted to a water soluble form that includes a C-terminal glycosylinositol phosphate by digestion with a bacterial PtdIns-specific phospholipase C (PI-PLC), with the simultaneous liberation of diacylglycerol (Low & Finean 1977; Low *et al.* 1986). Additionally, the membrane-bound form of the protein can also be hydrolysed by a specific phospholipase D found in plasma, resulting in the production of both a water-soluble protein containing glycosylinositol and phosphatidic acid (Davitz *et al.* 1987; Low & Prasad 1988).

The ability of the bacterial PI-PLC to release proteins has revealed that over 30 proteins are anchored in this manner to the plasma membrane (Low & Saltiel 1988). This list of proteins (table 2) is both evolutionarily and functionally diverse, including hydrolytic enzymes, complement regulatory proteins, adhesion molecules, coat proteins of parasites, a number of antigens with specific cellular distribution but unknown function, and additional proteins of unknown significance. The only property common to these proteins is their location at the cell surface.

Table 2. Cell surface proteins with a glycosyl-phosphatidylinositol membrane anchor

hydrolytic enzymes
- alkaline phosphatase
- 5′-nucleotidase
- acetylcholinesterase
- alkaline phosphodiesterase
- trehalase
- p63 protease (*Leishmania major*)
- renal dipeptidase
- merozoite protease (*Plasmodium falciparum*)
- lipoprotein lipase

mammalian antigens
- Thy-1
- RT-6 (rat lymphocytes)
- T-cell activating protein and other Ly-6 antigens
- Qa
- carcinoembryonic antigen
- Blast-1 (human lymphocytes)
- CD-14 (human monocytes)

cell adhesion
- neural cell adhesion molecule (N-CAM)
- heparan sulphate proteoglycan
- LFA-3 (human lymphocytes)
- contact site A (*Dictyostelium discoideum*)

protozoal coat proteins and antigens
- variant surface glycoprotein (*Trypanosoma brucei*)
- surface proteins (*Paramecium primaurelia*)
- Ssp-4 (*T. cruzi*)

miscellaneous
- tegument protein (*Schistosoma mansoni*)
- decay-accelerating factor
- 130 kDa hepatoma glycoprotein
- 34 kDa placental growth factor
- scrapie prion protein
- PH-20 protein (guinea pig sperm)
- GP-2 (pancreatic zymogen granule)

To evaluate the possibility that the enzyme modulators might arise from the phosphodiesteratic hydrolysis of a structurally similar, PtdIns-containing glycolipid, the specific bacterial phospholipase C was added to liver plasma membranes, and the release of the enzyme modulators was monitored. In a series of experiments, PI-PLC was found to reproduce the effect of insulin in facilitating the generation of the enzyme modulators (Saltiel & Cuatrecasas 1986; Saltiel 1987). The PI-PLC generated activities that were chromatographically, electrophoretically and chemically identical to those produced by insulin, suggesting an inositol phosphate-glycan structure for the enzyme modulator. Moreover, a precursor for the PI-PLC-generated substance was extracted with organic solvents from liver membranes and chromatographically resolved from other phosphoinositides (Saltiel & Cuatrecasas 1986).

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These preliminary experiments suggested that the enzyme modulators were produced as a result of the hormone-stimulated turnover of this novel glycosyl-PtdIns.

The generation of the inositol glycan enzyme modulator was also evaluated in a cultured myocyte cell line, BC_3H1, by following the incorporation of radioactive precursors (Saltiel *et al.* 1986, 1987). In preincubated cells, both of the enzyme-modulating substances were rapidly labelled with [^{3}H]*myo*-inositol in response to physiological concentrations of insulin. The presence of non-acetylated hexosamine in the molecule was suggested by the sensitivity of the enzyme-modulating activity to treatment with nitrous acid under conditions in which hexosamines that are not *N*-substituted undergo deamination with subsequent cleavage of adjacent glycosidic bonds. This specific reaction was used to probe the structure of the glycosyl-PtdIns protein anchor (Low & Saltiel 1988; Low *et al.* 1986). Exposure of cells to insulin also caused the incorporation of [^{3}H]glucosamine into the two peaks of enzyme-modulating activity in parallel to the incorporation of [^{3}H]inositol (Saltiel *et al.* 1986). The chromatographic, chemical and electrophoretic behaviour of the metabolically labelled compounds were identical to those of the biologically active enzyme modulators; this observation suggests a structure consisting of inositol phosphate glycosidically linked to the C1 position of glucosamine, which in turn is glycosidically linked to additional monosaccharides, the composition and orientation of which have not been precisely determined (figure 1). In recent studies (Osterman *et al.* 1988), the molecular size of the ^{3}H-labelled inositol phosphate glycan has been explored on gel permeation and HPLC columns. Comparison of its elution profiles on both a P-2 polyacrylamide column and an HPLC phase partition AX-10 column with those of radioactively labelled oligosaccharide standards suggests a total of 4–5 sugars. Additionally, evaluation of the hydrolysis products of the ^{3}H-labelled inositol phosphate glycan indicate the presence of a phosphodiester linking a monosaccharide to the inositol-containing core oligosaccharide. It appears that the original resolution of the two species of activity on ion exchange was due to the presence of a 1,2-cyclic inositol monophosphate versus a 1- or 2-monophosphate derivative (Saltiel *et al.* 1986), although the physiological significance of these distinct compounds remains uncertain.

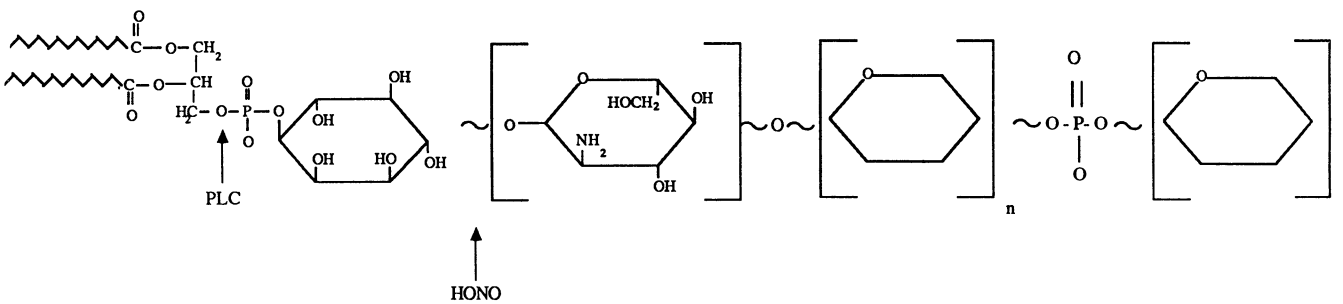

FIGURE 1. The metabolism of glycosyl-PtdIns in insulin action. A hypothetical model is presented, illustrating the hydrolysis of glycosyl-phosphoinositides in plasma membranes. The interaction of insulin with its receptor causes the activation of the receptor tyrosine kinase, probably the initial signal for receptor function. The activated receptor is then coupled by an unknown mechanism that may involve an intermediate G protein to the stimulation of one or more phospholipases C specific for glycosyl-PtdIns. This enzyme or group of enzymes might then catalyse the hydrolysis of a free glycosyl-PtdIns on the cytoplasmic side of the plasma membrane, resulting in the intracellular generation of the enzyme-modulating inositol phosphate glycan. Similarly, a glycosyl-PtdIns specific phospholipase C might also cause the release of anchored proteins like heparan sulphate proteoglycan, alkaline phosphatase or lipoprotein lipase. In both cases the hydrolysis also results in the production of diacylglycerol that may cause a selective activation of the protein kinases C.

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Considerable interest has focused on the structure and biosynthesis of the glycosyl-PtdIns precursor for the enzyme modulator, and its relationship to the glycosyl-PtdIns protein anchor. Some of the properties of these glycolipids are summarized in table 3. This or similar molecules have been metabolically labelled with inositol (Saltiel *et al.* 1986), glucosamine (Saltiel *et al.* 1986; Mato *et al.* 1987*a*); phosphate (Mato *et al.* 1987*b*; A. R. Saltiel, 1987, unpublished data) and saturated fatty acids (Saltiel *et al.* 1986, 1987; Osterman *et al.* 1988; Mato *et al.* 1987*a*) in a number of cell types, including BC_3H1 myocytes (Saltiel *et al.* 1986, 1987), H-4 hepatoma cells (Mato *et al.* 1987*a*), 3T3-L1 preadipocytes (A. R. Saltiel, unpublished data) and primary cultures of adrenocortical cells (Igareshi & Chambaz 1987). The glycolipid is rapidly turned over in response to insulin in BC_3H1, 3T3-L1 and hepatoma cells. A similar inositol-containing glycolipid that was metabolized in response to serum (Igareshi & Chambaz 1987) was identified in adrenocortical cells. Both the glycosyl-PtdIns precursor and the glycosyl-PtdIns protein anchor are also cleaved by the serum-derived, specific phospholipase D (D. G. Osterman *et al.*, unpublished data). The products of this reaction are phosphatidic acid and an inositol glycan lacking phosphate on the inositol ring. The net negative charge of this phospholipase D product further supports the presence of an additional phosphate in the glycolipid. Hydrolysis of the BC_3H1 cell-derived glycolipid with PI-PLC produced the inositol glycans and diacylglycerol. In contrast, Mato *et al.* (1987*a*) suggested a 1,2-alkylacylglycerol structure for the glycerolipid moiety in hepatoma cells. Another variation in the liver-derived glycosyl-PtdIns was indicated regarding the presence of significant but variable amounts of *chiro*-inositol, perhaps accounting for the apparent lack of [^{3}H]*myo*-inositol labelling in hepatoma cells (Mato *et al.* 1987*b*).

TABLE 3. PROPERTIES OF THE PLASMA MEMBRANE GLYCOSYL-PHOSPHATIDYLINOSITOLS

properties	protein-linked	free
biological function	protein anchoring	signal transduction
phospholipase C sensitivity	yes	yes
phospholipase D sensitivity	yes	yes
hydrophobic domain	1,2 diacylglycerol or 1,2 alkylacylglycerol	1,2 diacylglycerol or 1,2 alkylacylglycerol
nitrous acid cleavage product	phosphatidylinositol	phosphatidylinositol
glucosamine	yes	yes
ethanolamine	yes	no
approximate size of glycan (excluding inositol)	4–12 monosaccharides	2–4 monosaccharides
degradation stimulated by insulin	yes (in some cases)	yes
subcellular location	cell surface	intracellular

Kinetic studies in BC_3H1 cells suggest that the synthesis of the glycosyl-PtdIns temporally follows that of PtdIns, indicating that this lipid arises from the glycosylation of a pool of PtdIns (Farese *et al.* 1987, 1988). Methodology was recently developed to evaluate the *in vitro* biosynthesis of this glycolipid in a preparation of liver microsomes (Osterman *et al.* 1988). The synthesis of this molecule is dependent on the concentration of exogenously added CDP-diacylglycerol and inositol, and appears to follow the appearance of PtdIns. In addition, these studies indicate that, even within a single cell type, there may be multiple species of glycosyl-PtdIns, since different forms have been chromatographically resolved (Osterman *et al.* 1988).

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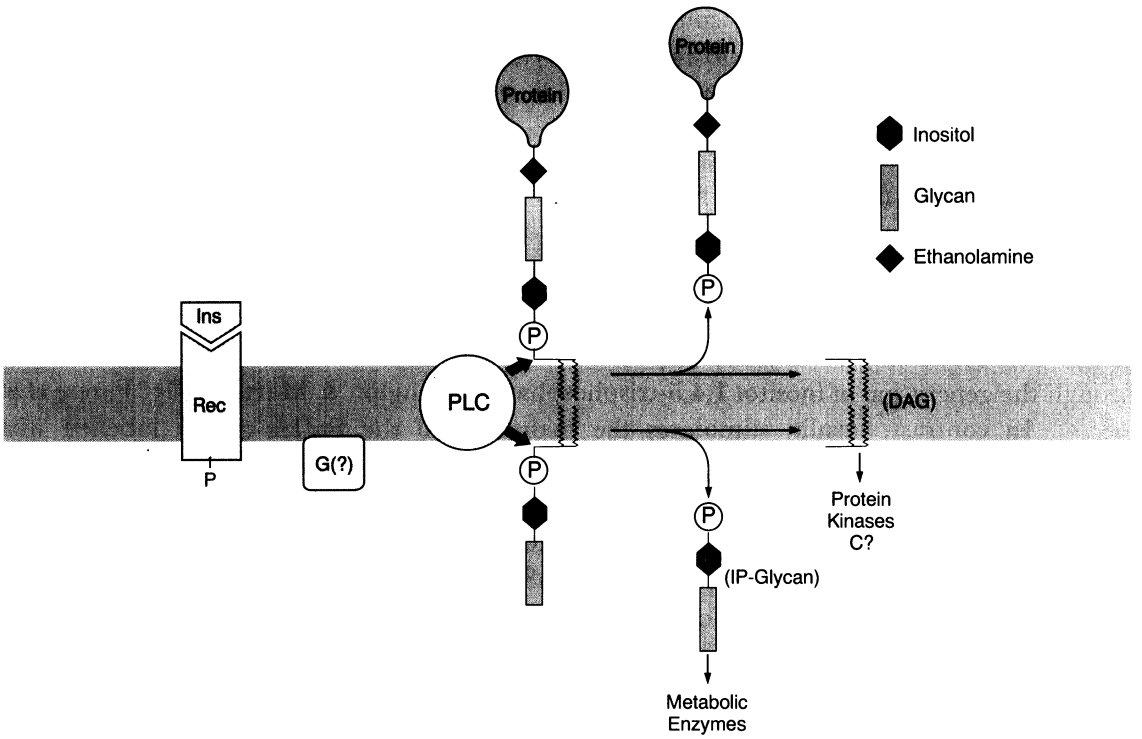

FIGURE 2. Structure of the inositol-glycan enzyme modulator. This molecule consists of phosphatidylinositol, glycosidically linked to the C1 position of glucosamine. Glucosamine is then attached at the 4- or 6-position to additional monosaccharides. The terminal monosaccharide may be linked through a phosphodiester bond. The sites of hydrolysis for phospholipase C (PLC) and nitrous acid (HONO) are shown.

Whether these distinct forms represent different stages in the biosynthesis of the glycolipid or perhaps molecules with distinct cellular functions remains to be determined.

The insulin-sensitive glycosyl-PtdIns exhibits considerable homology to the glycosyl-PtdIns protein anchor (table 3). The two types of glycolipid contain similar glycerolipid domains, inositol, non-*N*-acetylated glucosamine and a variable glycan region. Both lipids are sensitive to PI-PLC, glycosyl-PtdIns-specific phospholipases C and D, and nitrous acid. However, the insulin-sensitive glycosyl-PtdIns apparently lacks two of the features commonly observed in the protein anchor: ethanolamine and amino acids. Additionally, approximation of the molecular size of the insulin sensitive glycosyl-PtdIns suggests that the glycan moiety is smaller than similar molecules bound to protein (Osterman *et al.* 1988). Although the topological distribution of the insulin-sensitive glycolipid in the plasma membrane is uncertain, preliminary studies suggest a cytoplasmic orientation; pretreatment of cells with PI-PLC does not prevent the insulin-induced intracellular accumulation of the inositol phosphate glycans (A. R. Saltiel, unpublished data). The assumption that the protein-bound and free forms of glycosyl-PtdIns are located on opposite sides of the plasma membrane leads to further uncertainty about their biosynthetic processes. One possibility is that the early stages of glycosylation of PtdIns occur on the cytoplasmic aspect of the endoplasmic reticulum. On attaining a certain level of glycosylation, a fraction of molecules ultimately destined for protein anchoring might be translocated across the membrane, in analogy to the translocation of the $(Man)_5$ $(GlcNAc)_2$-lipid utilized for N-linked glycosylation of proteins in the endoplasmic reticulum (Hirschberg & Snider 1987). Thereafter, those molecules situated in the lumen of the endoplasmic

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reticulum might be processed for condensation with protein, and the subsequent membrane trafficking to the cell surface would result in a cytoplasmically oriented free glycolipid and a cell-surface oriented protein-anchored glycolipid.

The inositol glycans are produced by an insulin-sensitive, glycosyl-PtdIns-specific phospholipase C

Although insulin is known to cause increased labelling of several phospholipids (Manchester 1963; De Torrentegui & Berthet 1966; Stein & Hale 1974) it has not been found to stimulate the hydrolysis of PtdIns or the polyphosphoinositides and does not induce calcium mobilization through the generation of inositol 1,4,5-trisphosphate (Penington & Martin 1985; Farese *et al.* 1985*a*). In contrast, insulin stimulates the hydrolysis of the PtdIns-glycan labelled with [^{3}H]inositol or [^{3}H]myristate, with the simultaneous production of the [^{3}H]inositol phosphate glycan and [^{3}H]myristate-labelled diacylglycerol. The rapid production of this specifically labelled diacylglycerol was not observed with agonists known to stimulate the hydrolysis of the polyphosphoinositides (Saltiel *et al.* 1987), indicating that both the specifically labelled diacylglycerol and the inositol phosphate glycans arise from the specific, insulin-sensitive hydrolysis of the glycosyl-PtdIns. It also suggested that the relevant phospholipase C might be selective for this class of glycophospholipids.

These observations led to the search for a glycosyl-PtdIns specific phospholipase C. Such an enzyme was isolated from a plasma membrane fraction of liver, assayed by measuring the liberation of diacylglycerol from the glycosyl-PtdIns-anchored variant surface glycoprotein from *T. brucei* or the inositol phosphate glycan from the BC_3H1 cell-derived glycosyl-PtdIns (Fox *et al.* 1987). The catalytic activity appears to reside in a single polypeptide with an apparent molecular mass of 52 kDa. The enzyme is calcium-independent and specific for glycosyl-PtdIns; no hydrolysis of PtdIns, PtdIns(4,5)P_2 or other phospholipids was observed under a variety of conditions. Although its orientation in the membrane is unknown, retention of the enzyme on immobilized wheatgerm agglutinin suggests a transmembrane glycoprotein.

One of the important questions to be addressed concerns the molecular interactions leading to the regulation of glycosyl-PtdIns hydrolysis by the insulin receptor. Although a phospholipase C capable of catalysing this reaction has been purified, it has thus far been difficult to demonstrate an activation of the enzyme by insulin in plasma membranes that is comparable to that observed in intact cells (A. R. Saltiel, unpublished observations). This apparent inconsistency may be due to the activation of the enzyme upon homogenization, as observed for the glycosyl-PtdIns-specific enzyme in *T. brucei*. Such a constitutively activated phospholipase might also account for the high degree of spontaneously released enzyme-modulating activity (Saltiel & Cuatrecasas 1986). Nevertheless, elucidation of the mechanism(s) whereby the insulin receptor is coupled to the stimulation of the phospholipase activity remains one of the important links in establishing the involvement of this reaction in insulin action.

Recent studies with anti-receptor antibodies (Morgan *et al.* 1986; Morgan & Roth 1987) or site-directed mutagenesis (Chou *et al.* 1987; Ellis *et al.* 1986) indicate that the tyrosine kinase activity of the receptor is probably necessary for all of the biological actions of insulin. This suggests that the activation of the glycosyl-PtdIns-specific phospholipase C by the receptor might occur as a consequence of a tyrosine kinase-induced cascade, leading to changes in the

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phosphorylation state of the enzyme. Alternatively, the autophosphorylation of the receptor on tyrosine residues could constitute the activation signal that allows the initiation of a membrane-coupling event, perhaps including an intermediate factor, such as a G-protein. The involvement of a G-protein in insulin action has been suggested in studies demonstrating that pertussis toxin (Goren *et al.* 1985, Elks *et al.* 1987) or antibodies to the GTP-binding p21*ras* protein (Korn *et al.* 1987; Deshpande & Kung 1987) can block certain actions of insulin. Additionally, certain of these transducing proteins are relatively good substrates for the insulin receptor kinase *in vitro* (Zick *et al.* 1986; Kamata *et al.* 1987; O'Brien *et al.* 1987) suggesting a possible high-affinity interaction between a G-protein and the receptor that may lead to regulation of the specific phospholipase C. Along these lines, a recent report has suggested that the insulin-dependent production of myristate-labelled diacylglycerol can be blocked by pretreatment of cells with pertussis toxin (Luttrell & Rogol 1987).

Another result of the insulin-induced activation of a glycosyl-PtdIns phospholipase C might be the release of glycosyl-PtdIns-anchored proteins. Insulin caused reduced levels of cellular alkaline phosphatase in the rat osteogenic sarcoma line ROS 17/2.8 (Levy *et al.* 1986), perhaps as a result of a phospholipase-catalysed cleavage of the glycosyl-PtdIns anchor for this enzyme. Like PI-PLC, insulin has been reported to acutely promote the release of the glycosyl-PtdIns-anchored heparan sulphate proteoglycan from rat hepatocytes (Ishihara *et al.* 1987), perhaps by activation of a glycosyl-PtdIns-specific phospholipase C. Interestingly, the released proteoglycan with terminal glycosylinositol phosphate appeared to behave as an autocrine growth regulator in these cells, owing to its specific internalization at sites which recognize the inositol phosphate moiety. Lipoprotein lipase is acutely released from 3T3-LI adipocytes in response to insulin (Spooner *et al.* 1979). Recent studies indicate that a form of this enzyme may be anchored to the cell surface by glycosyl-PtdIns, since both enzyme activity and immunoprecipitable protein can be released with PI-PLC (Chan *et al.* 1988). The similarities in the kinetics of lipoprotein lipase release by insulin and PI-PLC indicate that the acute phase release by insulin may be due to activation of the glycosyl-PtdIns-specific phospholipase C (Chan *et al.* 1988). The possibility of an insulin-dependent phospholipase that cleaves glycolipid molecules on opposite sides of the membrane also raises questions concerning the orientation of the relevant hydrolytic enzymes, and suggests that the kinetics of the insulin-induced hydrolysis of these forms of glycosyl-PtdIns may differ, or that separate enzymes might be involved on different sides of the membrane.

The role of diacylglycerol in insulin action

Another controversial issue in insulin action has been the involvement of the Ca^{2+}- and phospholipid-dependent protein kinase C. Some studies have demonstrated that phorbol esters (the tumour-promoting activators of protein kinase C which substitute for diacylglycerol), mimic the actions of insulin on glucose transport (Farese *et al.* 1988), lipogenesis (Van de Werve *et al.* 1985), glucose oxidation (Honeyman *et al.* 1983), pyruvate dehydrogenase (Farese *et al.* 1985*b*) and the phosphorylation of several proteins (Trevillyan *et al.* 1985; Graves & McDonald 1985). On the other hand, phorbol esters inhibit insulin-stimulated lipogenesis (Van de Werve *et al.* 1985) and antagonize insulin action on glycogen synthesis and glycogenolysis (Ahmad *et al.* 1984). Some of the insulin-mimetic actions of phorbol esters might operate by mechanisms different from those of insulin, especially regarding glucose transport

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(Gibbs *et al.* 1986; Joost *et al.* 1987; Klip & Ramlal 1987). Down-regulation of kinase C in L6 muscle cells 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 (Klip & Ramlal 1987). However, down-regulation of kinase C did cause a 60% reduction in insulin-activated glucose transport in rat adipocytes (Chergui *et al.* 1987) and a 40% loss of insulin-activated lipogenesis (Smal & DeMeyts 1987). In addition, the potent kinase C inhibitor sphingosine completely blocked the insulin-stimulated glucose transport in rat adipocytes (Robertson *et al.* 1988). The picture has been further complicated by studies examining the hormonal activation of kinase C. Several investigators have reported that phorbol esters induced the rapid loss of kinase C activity from cytosolic fractions, but insulin was ineffective in promoting such a subcellular redistribution of the enzyme (Glynn *et al.* 1986; Vaartges *et al.* 1986). However, insulin stimulated both a cytosolic and membrane-associated kinase C in BC_3H1 cells (Cooper *et al.* 1987) and a membrane kinase C in rat diaphragm (Walaas *et al.* 1987) indicating a potential mechanism for the activation of the kinase that does not involve translocation to the membrane.

It may be possible to accommodate these apparently incongruous observations by considering the source of diacylglycerol produced in response to insulin. Most agonists that cause kinase C activation do so by stimulating the hydrolysis of phosphoinositides, 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 observation that the insulin-generated diacylglycerol contains little arachidonate, suggests that this diacylglycerol must arise from an alternative source. Three potential pathways have been considered: (i) *de novo* synthesis from phosphatidic acid (Farese *et al.* 1988); (ii) hydrolysis of phosphatidylcholine (Farese *et al.* 1988); and (iii) hydrolysis of glycosyl-PtdIns (Saltiel *et al.* 1987). These distinct mechanisms can all lead to the production of structurally distinct species of diacylglycerol, without inositol 1,4,5-trisphosphate-induced calcium mobilization. Thus, it is possible that insulin causes the selective activation of kinase C with regard to 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 (Coussens *et al.* 1986; Knopf *et al.* 1986), and several isozymes have been chromatographically resolved (Huang *et al.* 1986). Some evidence suggests that these isoforms may exhibit distinct regulatory properties, especially with regard to calcium and diacylglycerol sensitivity and substrate specificity (Pelosin *et al.* 1987; Kikkawa *et al.*, this symposium). Moreover, these isoforms may exhibit different subcellular distribution or may be differentially sensitive to down-regulation by phorbol esters. Thus, the selective activation of protein kinase C or a fraction of isozymes may explain the apparent discrepancy between the biological actions of phorbol esters and insulin.

Inositol phosphate glycans as second messengers of insulin action

Despite the progress made in identifying the structure and biogenesis of the inositol phosphate glycans, it is still premature to regard these compounds as second messengers for any of the actions of insulin. Although the apparent insulin-dependency, rapidity and extent of the generation of the inositol phosphate glycans are suggestive of properties of second messengers,

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questions remain concerning their insulin-mimetic properties. Thus far, the actions and properties of the inositol phosphate glycans have been explored mainly in subcellular assays, so that the extent to which these molecules reproduce the actions of insulin in intact cells remains unclear. In recent studies, these issues have been addressed by evaluating the effects of the inositol phosphate glycan in fat cells. Purified preparations of these compounds mimicked the lipogenic (Saltiel & Sorbara-Cazan 1988) and anti-lipolytic (Kelly *et al.* 1987) actions of insulin, as well as the regulation of specific protein phosphorylation (Alemany *et al.* 1987) but did not appear to modulate glucose transport (Kelly *et al.* 1987). Interestingly, at millimolar concentrations, inositol monophosphate blocked the lipogenic effect of the inositol phosphate glycan in intact adipocytes, but was ineffective in attenuating the stimulation of cAMP phosphodiesterase activity in adipocyte membranes (Saltiel & Sorbara-Cazan 1988). These observations may be related to reports of the cellular uptake of the phospholipase C-released heparan sulphate proteoglycan that was blocked with inositol phosphates (Ishihara *et al.* 1987) and suggests the possibility of a specific transport system that recognizes the inositol phosphate portion of the glycan. However, there is no evidence that the inositol phosphate-glycans are released from cells upon hormone stimulation, and the significance of a putative cellular uptake for phospholipase C-released proteins or glycans requires further investigation.

Although the inositol phosphate 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 these compounds as second messengers will rely on the determination of precise structure. This may be complicated by the chromatographic resolution of multiple species of these molecules, perhaps indicating forms with different enzyme-modulating functions. Following elucidation of structure, it will be important to synthesize these compounds organically and re-evaluate each of their biological activities. Moreover, the biochemical actions of these molecules need further exploration, especially regarding mechanism. Another approach to evaluating the role of the inositol phosphate glycans in insulin action will include the molecular characterization of the glycosyl-PtdIns-specific phospholipase C. 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. Although there is no reason to expect that these molecules will answer all of the questions regarding the mechanisms of signal transduction in insulin action, the evaluation of the metabolism of glycosyl-PtdIns in pathological states of insulin resistance may lead to the identification of critical post-receptor defects in diabetes.

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