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MINI REVIEW

Is there evidence for phospho-oligosaccharides as insulin mediators?

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Introduction

Insulin belongs to the disulfide-rich subclass of group 3 small α/β cytokines, which includes the insulin-like growth factors (IGF) 1 and 2, relaxin and bombyxin, and contains a conserved set of three disulfide bonds linking three short α-helices (Nicola, 1994). All insulin class cytokines/hormones bind to class II covalent tyrosine kinase receptors (Figure 1), and while the IGFs can also bind to the mannose-6-phosphate receptor, the biological relevance of this interaction is unclear at the present time. Insulin has a number of potent biological effects on metabolic processes including many aspects of glucose and lipid primary metabolism.

The established downstream mode of signal transduction for insulin is somewhat unusual. Engagement of the insulin receptor by insulin results in activation of tyrosine kinase activity in the receptor's β-chain. This activity is able to autophosphorylate multiple tyrosine residues on the companion β-chain in the heterotetramer receptor complex, but unlike many other tyrosine kinase receptors, this phosphorylation does not result in the direct creation of SH2 or SH3-domain binding sites. Instead the activated insulin receptor is capable of phosphorylating two related polypeptides, insulin receptor substrates 1 and 2 (IRS-1 and 2), on multiple sites. IRS-1 and IRS-2 are able to interact with a large number of SH2 and SH3-domain-containing downstream effectors including Grb2 and phosphatidyl inositol(PI)-3 kinase (see White and Kahn 1994; Waters and Pessin, 1996, and references therein) and provides a link into the ras/MAP kinase pathway. Interestingly both IRS-1 and IRS-2 contain pleckstrin homology domains at their N-termini, potentially allowing these molecules to interact with PI (Myers *et al.*, 1995). IRS-1 and IRS-2 are also substrates for several members of the Janus kinase family which themselves are associated with signal transduction pathways from members of the interleukin (IL-4 and 9), interferon (IFN-α and IFN-γ), and growth hormone families thereby providing enormous potential for flexibility in signal propagation.

Not all of the great many biological effects of insulin have been definitively shown to progress through IRS-1 or -2, but the majority of insulin actions have been tied in, to greater or lesser extent, with one or more of the established signal transduction pathways. Obviously most of the gaps in our understanding of these pathways are just incomplete data, but the concept of a novel mediator for insulin action, that is, a molecule that can act as an insulinomimetic and is in some manner uniquely distinct from conventional signaling components, is quite old and predates molecular understanding of receptor-type tyrosine kinase pathways (Larner, 1972). Mediators with many different biochemical properties have been proposed, ranging from peptides, based on the ability of proteases to mimic insulin action (Seals and Czech 1980) to glycopeptides and free oligosaccharides amongst other more esoteric candidates. Clearly any mediator must either act in parallel to the IRS-1 and -2 system or downstream of it. A persistent mediator candidate, first implicated over 10 years ago but still being actively researched by some workers is a phospho-oligosaccharide (POS) fragment of a glycosylphosphatidylinositol (GPI) lipid proposed to be present in a number of mammalian cell types and is the subject of this minireview. The general structure of the canonical anchor type GPI molecule is shown in Figure 2. Below, I will consider the biochemical data that have been presented for and against a role for a POS factor in insulin signaling over the last decade and a half.

Early insulin mediator candidates

An early suggestion for an insulin mediator was made by Larner (1972), and the first investigation in detail of this factor was subsequently reported in 1979 (Jarret and Seals, 1979; Larner *et al.*, 1979). An acid extract of rat muscle tissue was subjected to paper chromatography to remove nucleotides and size fractionated by gel filtration. One fraction was found to be both ninhydrin positive, to have UV absorbance and bioactivity; this material inhibited cAMP-dependent protein kinase and activated both a phosphoprotein phosphatase and mitochondrial pyruvate dehydrogenase (PDH), two consequences of insulin action. The active component could be further purified by thin layer chromatography (TLC). Bioactivity was increased by prior treatment of the muscle tissue with insulin (Larner *et al.*, 1979). Using an *in vitro* system, containing plasma membranes and mitochondria, insulin was shown to decrease incorporation of ^{32}P into the α-subunit of PDH, resulting in increased PDH activity (Seals and Jarett, 1980). As the effect was completely dependent on the inclusion of plasma membranes in the *in vitro* system it was proposed that the mediator originated from these membranes.

Subsequently two antagonistic activities were reported to be present in insulin-treated material isolated from liver (Saltiel *et al.*, 1981, 1982). The 30,000 g supernatant from insulin-treated liver homogenate was lyophilized and ethanol extracted. When assayed the ethanol-soluble fraction inhibited, while the insoluble residue stimulated, PDH. Both activities required ATP, and the levels were dose-dependent for the initial insulin concentration used to stimulate the liver homogenate. Interestingly, the muscle-derived mediator did not require ATP (Seals and Jarett, 1980), suggesting possible differences between the

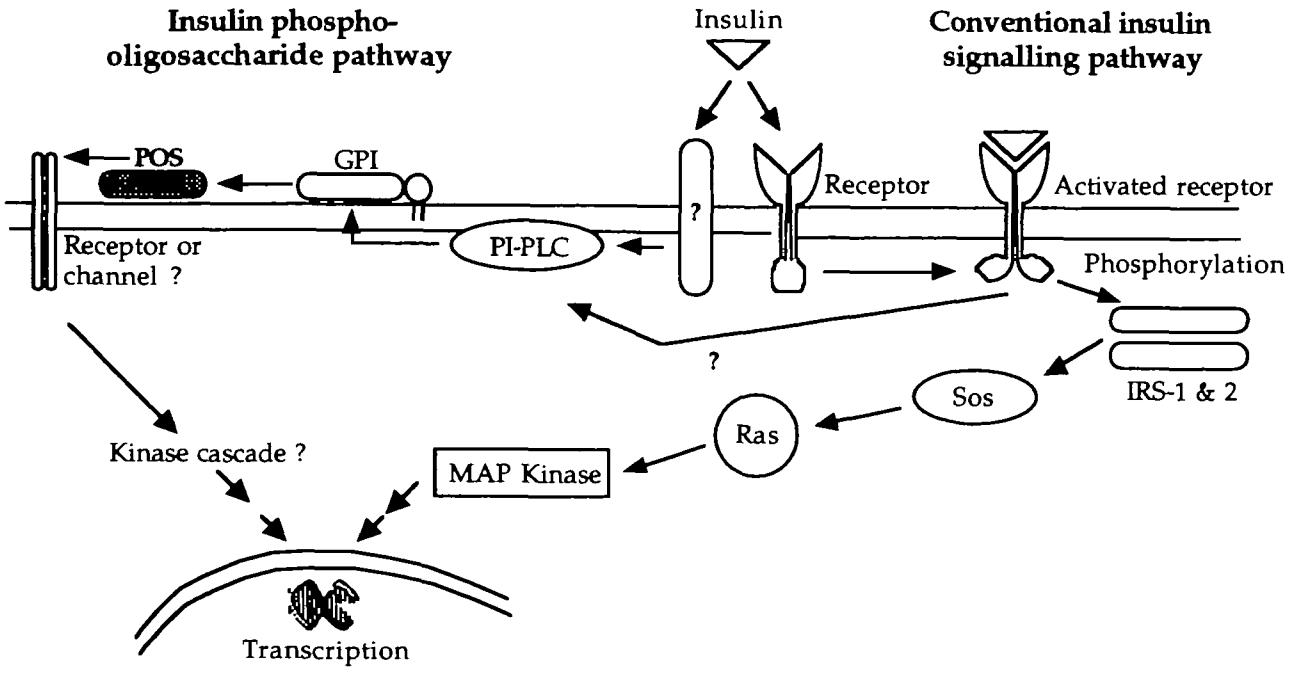

Fig. 1. Schematic model of the insulin signaling pathway. The conventional pathway (right hand pathway), via IRS-1 and -2 and the Sos/ras/Map kinase cascade has been well established. IRS-1 can also interact with a number of other molecules to produce a complex, branched pathway for downstream signal propagation, but these are not shown. The putative phospho-oligosaccharide pathway is shown at left. The definitive existence of this pathway and its relationship to the conventional receptor-based route is not known, but a simplified hypothetical version is shown that incorporates most of the features of the model. Insulin is proposed to generate a phospho-oligosaccharide (POS) fragment (shaded) via action of a PIPLC activity. The POS is then able to signal intracellular events, either by being taken up by the cell or bound through a receptor. The source of the signal for cleavage of the GPI lipid to generate POS may be independent of the classical pathway or alternatively may be derived from it at some point. Major difficulties with this model need to be resolved, and a crucial issue is the structure of the POS fragment itself, which remains unsolved.

mediators obtained from the two sources. The ethanol-soluble fraction also contained inhibitory activity for adenylate cyclase. By gel filtration both the inhibitory and stimulatory activities had molecular weights of 1–2 kDa, consistent with a small peptide or a glycan fragment.

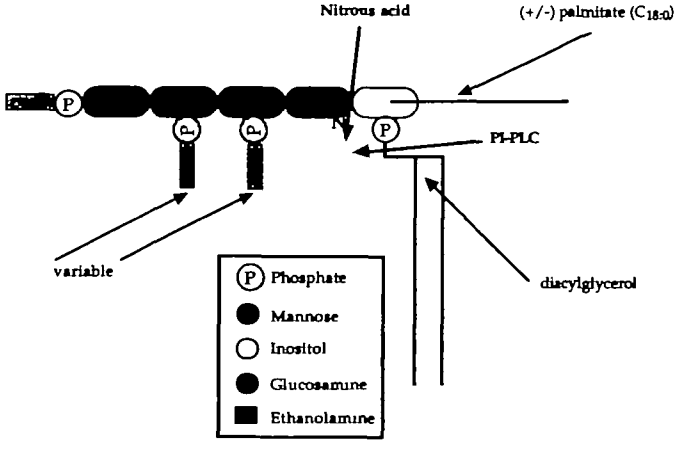

Fig. 2. Canonical structure of the mature glycosylphosphatidylinositol structure. The glycolipid headgroup is built up by sequential addition of monosaccharides and finally ethanolamine phosphate residues. All intermediate species can be detected in *in vitro* reactions, but their abundance is extremely low *in vivo*, suggesting that they are rapidly processed to the next species. The mature ethanolamine phosphate-containing lipids are readily observed in living cells by metabolic labeling techniques and can be isolated on a small scale. Protein-linked GPI-anchors often carry additional substituents, but the core is conserved.

In another study, insulin-stimulated adipocyte plasma membranes were used as a source of mediator (Zhang *et al.*, 1983). Assaying cAMP levels, these authors concluded that insulin generated 50-fold greater activity as mediator than input hormone, suggesting the presence of an amplification step, an important feature of many signal propagation pathways and implying the presence of a catalyst, that is, an enzyme. Furthermore, using a muscle mediator preparation on intact adipocytes, it was found that mediator activity could only account for a subset of insulin actions; while both insulin and mediator stimulated PDH and glycogen synthase, insulin alone could stimulate glucose oxidation and transport (Jarett *et al.*, 1985). This lack of effect on glucose oxidation reported here is at variance with a number of later studies (see below).

The mediator may be a GPI-lipid headgroup

From the above observations, a clear case could be made for the presence of some molecule in membrane fractions that mimicked insulin and potentially amplified the hormone signal. The mediator appears to consist of more than one discrete chemical identity and is active in both *in vivo* and *in vitro* assays. Obviously, the *in vivo* activity suggests the presence of either an extracellular receptor or an uptake mechanism for the mediator. A number of studies directed at identification of the chemical nature of the mediator pointed to a GPI fragment as being important.

Using stimulation of cAMP phosphodiesterase (PDE) as an assay an activity was characterized in organic solvent extracts of insulin-treated liver (Saltiel and Cuatrecasas, 1986), presum-

ably similar or identical to the ethanol-soluble mediator substance previously described (Saltiel *et al.*, 1981, 1982). Following extensive purification two low molecular weight factors were separated by anion exchange. Analysis by paper electrophoresis at low pH suggested that the factors differed in the number of phosphate or sulfate groups. Factors with identical chromatographic properties were also obtained by pretreatment of the liver starting material with *Staphylococcus aureas* PI-specific phospholipase C, and, most significantly, lipase pretreatment prevented further release of active factors upon subsequent insulin treatment. Additionally, a single species obtained by TLC from non-insulin-treated liver homogenate was also active, but it was not demonstrated that the TLC and PI-PLC derived factors were the same. However, both of the PI-PLC derived factors were inactivated by methylation, deamination with nitrous acid, periodate and strong acid or base treatments, all of which, though not highly specific, would be consistent with a GPI-type glycan or similar structure being important and suggestive of an activation of an endogenous PI-PLC by insulin stimulation. Furthermore, the factor was able to stimulate glucose oxidation and lipogenesis in intact adipocytes, similar to insulin (Saltiel and Sorbara-Cazan, 1987). Lipogenesis stimulation could be inhibited by competition with inositol-1-phosphate. Generation of an active factor released by PI-PLC was also demonstrated in a microsome *in vitro* system (Darnell *et al.*, 1988; Saltiel *et al.*, 1988). Therefore a connection between the active fractions, the putative GPI-headgroup they contain and demonstration that this molecule is the ultimate source of the various activities needed to be made. It is unfortunate that, despite a number of studies, this crucial question has still not been unequivocally answered over a decade later.

In a separate series of studies a mediator fraction was bulk isolated and characterized from rat liver homogenate. Using a ^{3}H-glucosamine-labeled PI-PLC sensitive lipid from H35 cells as a tracer (Mato *et al.*, 1987a), a comigrating TLC band was *S.aureus* PI-PLC treated and the resultant water-soluble fraction assayed for activity against rat adipocyte phospholipid methyltransferase (Kelly *et al.*, 1986, 1987a). The material ablated the isoproterenol-stimulated enhancement of the methyltransferase in a similar manner to insulin, but the magnitude of the responses obtained suggest that the substance was not highly potent. Activity was unaffected by NaF, EDTA, or EGTA, suggesting that a phosphorylation event or Ca^{2+}-dependent mechanisms were not involved. Subsequently, using ^{32}P-labeled adipocytes it was found that the POS fraction in fact prevented an isoproterenol-dependent increase in phosphorylation on serine in the methyltransferase, identical to the action of insulin (Kelly *et al.*, 1987a). However, the purification procedure for the POS was not very rigorous, and involved sequential TLC using first an acidic solvent, followed by rechromatography in a basic solvent. As the active fraction remained at the origin of the first TLC plate it is hard to evaluate what degree of purification had been achieved by this procedure. Nevertheless, this protocol became established as the standard method for production of the mediator. A composition of *chiro*-inositol, GlcN and galactose was reported for this material (Kelly *et al.*, 1987b). A second analysis, again on rat liver material, but isolated via anion exchange chromatography had a different composition. This mediator preparation activated PDH, but had a composition of mannose, GalN and *chiro*-inositol. However, mass spectrometric data indicate that the mediator was heavily contaminated with glucose, and later studies demonstrated considerable structural complexity in mediator fractions, limiting the utility of this information.

Using ^{3}H-glucosamine-labeling, Mato *et al.* (1987a) established the presence of an insulin-sensitive TLC peak in H35 cells, migrating between phosphatidic acid and phosphatidylcholine. After 24 h of labeling followed by stimulation *in vivo* with insulin, the peak was rapidly degraded by about 60% within 2 min. After 5 min of insulin stimulation the peak level had returned to near basal, suggestive of a very rapid resynthesis and also consistent with a small pool size. Most of the tritium was still present in the insulin-sensitive species as glucosamine and was sensitive to nitrous acid deamination. The peak did not incorporate radiolabeled inositol, serine, ethanolamine or mannose efficiently, but could be labeled with palmitic acid. By ^{32}P-labeling, the species was found to generate a lipid chromatographing close to PI following deamination, and most of the radiolabeled phosphate was recovered in the aqueous phase after this procedure, consistent with a GPI-type POS headgroup (Mato *et al.*, 1987b). However, as the evidence that the ^{32}P-labeled material and the ^{3}H-GlcN-labeled lipid are the same is based on co-chromatography in one TLC system, these data do not in themselves make a compelling case for an insulin-sensitive GPI. Also, as described below, the putative GPI-peak described here contains several species, not all of which exhibit GPI-type properties. The lack of mannose incorporation also suggests that the structure of this lipid must be rather unusual as mammalian GPI-anchor precursors can be readily labeled with ^{3}H-mannose (Stevens, 1992).

Myocytes also contain an insulin-sensitive GlcN-labeled lipid (Saltiel *et al.*, 1987). The lipid exhibited the same rapid loss and reappearance kinetics as the H35 cell lipid, and additionally the radiolabel became water soluble during the degradation of the lipid. Both the water-soluble fragment and the PI-PLC-generated product of the lipid were similar by chromatography to the previously described cAMP PDE modulator from liver (Saltiel and Cuatrecasas, 1986), and the PI-PLC product could modulate cAMP PDE. Also, the lipid fraction could be labeled with inositol in addition to GlcN and insulin stimulation produced ^{3}H-dimyristoylglycerol in ^{3}H-myristate-labeled myocytes. All incorporated radiolabels exhibited the rapid decrease and resynthesis kinetics (Saltiel *et al.*, 1987) as observed for the insulin-sensitive ^{3}H-GlcN-labeled species of H35 cells (Mato *et al.*, 1987a). In a separate study a GPI-specific PLC was isolated from rat liver (Fox *et al.*, 1987), but the significance of this to insulin signaling is difficult to evaluate, especially in light of the large number of lipases that have been subsequently discovered to be important in signaling events in the mammalian cell. In a later study of this kind an insulin-sensitive GPI was labeled with ^{3}H-inositol, GlcN, Gal, glycerol, and myristate (Suzuki *et al.*, 1991). Following PI-PLC treatment the monosaccharides were water soluble, while the glycerol and myristate were recovered as a diacylglycerol. The glycan fraction could modulate glucose oxidation and lipogenesis, but had no effect on glucose transport. Glucose oxidation was earlier suggested to not be affected by the mediator (Jarett *et al.*, 1985). Analysis of the glycan fraction indicated the presence of two different insulin-sensitive glycan fragments, suggesting more than one insulin mediator substance. Alternatively, several lipids may be insulin sensitive, but only a subset of which may generate active mediator fragments.

A PI-PLC POS fraction from rat liver was active at modulating the phosphorylation state of a number of proteins when

added to intact adipocytes in a manner indistinguishable from insulin and mimicked the ability of insulin to block isoprotenerol action (Alemany *et al.*, 1987). Activity was abolished by deamination. At suboptimal concentrations insulin and the POS fraction were additive in effect, but if either was maximal the other had no effect implying signaling via the same system. The POS fraction did not induce lipolysis or glucose transport, but was active on broken cells, all of which imply that any signal does not progress via the insulin plasma membrane receptor. Similarly, purified ^{3}H-GlcN-labeled rat liver lipid fractions following PI-PLC treatment blocked isoprotenerol lipolysis stimulation (Kelly *et al.*, 1987b) while the intact, non PI-PLC treated lipid had no activity. This mediator lacked any ability to modulate glucose oxidation as measured by CO_2 production. Again this preparation was not very potent, with the POS from ~5% of a liver being as active as 100 μU of insulin. A further study also showed that a POS preparation could influence both lactate production and fructose bisphosphate levels (Bruni *et al.*, 1990) and Macaulay and Larkins (1990) found that the putative mediator was not turned over in the streptozotocin-diabetic rat model. This latter observation could be interpreted as suggestive evidence for a role for defects in GPI-biosynthesis in certain types of diabetes.

In T cells appearance of a putative GPI-lipid paralleled expression of the insulin receptor (Gaulton *et al.*, 1988). The lipid was sensitive to PI-PLC, rapidly degraded when insulin was added to the T cell culture and the aqueous-soluble fraction following PI-PLC inhibited cAMP-dependent protein kinase. Using the standard purification (Kelly *et al.*, 1986), the lipid was shown to label with ^{3}H-inositol, to migrate slower that *lyso*PI in a basic TLC system (*lyso*PI would be anticipated to be PI-PLC sensitive and the resultant inositol-phosphate could potentially possess some biological activity), to preferentially incorporate ^{3}H-palmitic and myristic acids compared with other fatty acids, and was cleaved by nitrous acid. However, only 50% of the peak was cleaved, and less than 20% was PI-PLC sensitive, indicating that the putative GPI peak contained much else besides. Also, partial cleavage raises the possibility that the PI-PLC sensitive and nitrous acid sensitive fractions are separate molecules within the identified peak.

Further characterization of the insulin mediator by amine-directed derivatization and isolation from intact cells suggested that the lipid was mainly located in the outer membrane leaflet (Alvarez *et al.*, 1988), and that the water soluble headgroup produced by PI-PLC action consisted of at least three species differing in their degree of phosphorylation (Merida *et al.*, 1988). In an analysis of several cell types most GPI was present externally (Varela *et al.*, 1990). Again this material was insulin sensitive and could be removed with nitrous acid or *B.cereus* PI-PLC. Additionally, β-galactosidase pretreatment of cells reduced the responsiveness to insulin (galactose had been proposed as a component of the mediator by Mato *et al.*, 1987b), but an obvious problem with this experiment is that the galactosidase would act on all surface glycoconjugates, with potentially many effects, including possible alteration of the insulin receptor itself. These workers went on to show that in aged animals (24-month-old rats) there was both a decrease in the putative mediator peak and a corresponding decreased responsiveness to insulin and POS of glycogen synthesis (Sanchez Arias *et al.*, 1993). However, again no mechanistic connection was made between glycogen synthesis and the putative mediator peak.

In an attempt to define structural features of the mediator, monosaccharide phosphates were used as antagonists for insulin-induced protein phosphorylation (Stralfors and Alemany 1990). Inositol-6-phosphate was found to block insulin, while both inositol-6-phosphate and glucose-6-phosphate could prevent POS action. The compounds were apparently not taken up by the cells and did not alter the phosphorylation state of the receptor. In a more elegant approach the effect of antibodies to the GPI-anchor of the variant surface glycoprotein (VSG) of *Trypanosoma brucei* on POS activity was assessed (Romero *et al.*, 1990). Using the cross-reactive determinant as a method to eliminate antiprotein antibodies and to select for specificities that recognize the GPI-anchor only, the final antibody preparation could antagonize insulin activation of PDH or POS inhibition of protein kinase A. The antibodies were active *in vivo*, but had no influence on glucose uptake or dimyristyl glycerol production. Using the antibodies as an affinity matrix, both PI-PLC-cleaved VSG and pronase-digested VSG (anchor fragment) were bound, but the intact glycoprotein was not, suggesting that PI-PLC-processing was essential for recognition. Furthermore, material retained by the column could stimulate PDH. Therefore, for the first time a structurally defined, albeit immunologically, GPI-molecule appeared to contain insulinomimetic activity.

If, then, insulin is able to promote turnover of a plasma membrane GPI-lipid, presumably through activation of a specific lipase, there is a real possibility that this pathway might also involve GPI-anchored proteins. Several studies support this model. For example, an alkaline phosphatase activity (detected using *p*-nitrophenylphosphate) is released from myocytes upon insulin treatment in addition to a PDH activator (Romero *et al.*, 1988). Both the phosphatase and PDH stimulator shedding were inhibited by *p*-aminobenzamindine, and the PDH stimulatory activity was lost by nitrous acid treatment. As *p*-aminobenzamindine is a protease inhibitor it is possible that proteolysis is involved. Similarly, lipoprotein lipase, a GPI-anchored protein, is shed from NIH3T3 L1 adipocytes in the presence of insulin (Chan *et al.*, 1988) and surface expression of a number of proteins in these cells is sensitive to insulin (Lisanti *et al.*, 1989). Obviously, if the surface expression level of GPI-anchored proteins are somehow controlled by insulin, this would have important consequences for both the possible mechanism of production of an insulin mediator and for the physiological actions of the hormone. In a later study release of placental alkaline phosphatase (PLAP) from Hep2 cells was monitored using a monoclonal antibody (Roberts *et al.*, 1990). PI-PLC, EGF, and insulin can release PLAP, as can GTPγS in a broken cell system. However, as growth factors may lead to membrane ruffling and reorganization and the released PLAP was not shown to contain the cross reactive determinant (a hallmark of PI-PLC cleavage of a GPI-anchor), it is possible that shedding was caused by some other effect, and a mechanistic connection could not be directly proven with these data. In a more recent study insulin stimulated the release of lipoprotein lipase (LPL) and a GPI-anchored cAMP binding protein (GCer). Both insulin and glimepiride converted ~30% of the proteins to a soluble form which retained the inositol phosphate (Muller *et al.*, 1994). The cleaved GPI-moieties were apparently retained by the cells through an inositol phosphate receptor. All of these studies imply that a PI-PLC activity is either activated or mobilized by insulin stimulation. Clearly, if the POS precursor glycolipid is similarly localized in the external leaflet of the plasma membrane, both POS production

and GPI-anchor cleavage could be essentially the same process.

In addition to a possible involvement in surface protein stability several other functions have been proposed for this pathway. For example, in NGF sensitive PC12 cells a putative GPI species labeled with myristate was found to be rapidly degraded, but in NGF-resistant PC12 no such hydrolysis was detected (Chan *et al.*, 1989). This presumably implicates the NGF receptor directly in this process. Unfortunately cross reactivity of the NGF and insulin mediators was not assessed. Also, insulin and POS have been proposed to elicit effects of specific neurons in *Aplysia*, and insulin causes release of POS activity from isolated *Aplysia* ganglia (Shapiro *et al.*, 1991). Evidence for POS in rat brain tissue has been published (Velazquez *et al.*, 1993), but no structural information are available as yet. Evidence was also presented in a later paper that IL-2 also modulated a GPI-type lipid, and that the PI-PLC-derived headgroup could synergize with IL-2 in growth promotion of CTIL-2 cells (Merida *et al.*, 1990). These data are somewhat confusing with hindsight as the CTIL-2 line is a lymphocyte line, and it is now known that T and B cells synthesize a complex spectrum of readily metabolically labeled GPI-lipids (Stevens, 1993), but in this study these other structurally characterized GPI-species were apparently not detected. It is also curious that the mediator shows consistent PI-PLC sensitivity as many of the GPI-lipid species studied in the higher animals are inositol-acylated, rendering them insensitive to PLC action.

Persistent and emerging problems with the mediator

Following reports of *in vitro* synthesis of the insulin-sensitive GPI species (Darnell *et al.*, 1988) came a contradictory paper that called into question many of these findings (Thakkar *et al.*, 1990). These workers found that an inositol-labeled lipid with the same TLC migration as the candidate mediator was cleaved by PI-PLC but not by GPI-PLC or GPI-PLD, calling into doubt the assignment as a GPI. A similar species was found to be synthesized by liver microsomes prepared from type II diabetics, previously suggested to lack the ability to make the lipid (Darnell *et al.*, 1988). Also, if GPI-PLC or PLD were included during the synthesis period, the peak was still generated, suggesting that conventional GPI biosynthesis was not involved. These data must be treated with some caution however as the GPI lipases are quite specific and ablation of GPI-biosynthesis was not demonstrated. The candidate peak was not nitrous acid sensitive nor was it affected by insulin treatment. On reexamination the peak co-chromatographed with *lyso*-PI, and by composition analysis contained only inositol and glucose, but not GlcN, galactose or GalN at variance with the data from earlier reports (see above). While these observations may not be completely relevant to the *in vivo* studies, they clearly do raise significant doubt as to the validity of some of the earlier work. A later study, by Whatmore *et al.* (1993) also raised concern over some of the earlier reports. Here the authors had labeled rat hepatocytes with a battery of ^{3}H-precursors and looked for insulin sensitive GPI species. Again a lipid with the same TLC migration as the H35 cell line derived mediator candidate (Mato *et al.*, 1987b) was isolated and labeled with GlcN, GalN, Gal, and palmitate, but not with inositol or myristate. Despite the cells being insulin responsive, this peak was not affected by insulin treatment or by PI-PLC or nitrous acid.

While Thakkar *et al.* (1990) may have presented the first direct evidence that there might be a problem with the GPI-insulin connection, a number of other aspects of the model were also of concern. The complete lack of structural data, despite a large number of publications, and no information on the purity of the POS fraction were clearly problematic. These issues remain outstanding at the time of writing. The absence of enrichment tabulation and rigorous structural analysis has made it difficult to ascribe the biological activity of the POS fraction to GPI with real confidence. Indeed, in several cases data suggest that the POS fraction is in fact quite impure (e.g., Larner *et al.*, 1988; Parpal *et al.*, 1995). The purification procedure first reported by Kelly *et al.* (1987b) is also not particularly rigorous as the initial organic extract is first chromatographed on a low pH system with the bioactivity remaining at the origin. As this does not represent chromatography *sensu stricto* the POS was purified really by a single chromatographic step, and therefore conceivably contains a plethora of molecules. Several of the reports discussed elsewhere in this article certainly point to there being several GPI-molecules present in the POS fraction. What else may be present remains an open question.

Also, a number of potential conceptual problems with the model have emerged. One major concern is a topological inconsistency; data suggest an externally orientated GPI species, but any PI-PLC activity would presumably originate in the cytosol. Additionally, the activity of the POS fractions in broken cells suggested that the POS acts at some internal site, and therefore, if derived from a surface oriented precursor, a transport process, that is, a channel or a pump, must be operating. Finally, there are inconsistencies with the more recent understanding of mammalian GPIs. In mammals most of the precursors are PI-PLC resistant due to inositol acylation (Stevens, 1992), and a great many species are synthesized in T cells and hepatocytes. The relationship of the insulin mediator GPI peak to these other lipids is not known, but it is possible that some of the activities ascribed to the mediator may be induced in these molecules via some PI-PLC digestion, as suggested by some of the studies using GPI-fragments derived from cell surface proteins (see below).

Newer evidence

Recent advances in methodology, GPI-biology and signal transduction have meant that evaluation of the role of POS in insulin and other signaling events has become more sophisticated, and several studies from the last 5 years have reexamined the case for a bioactive glycan with structural homology to GPIs. The effects of deletions in the GPI-biosynthetic pathway, manipulation of the conventional insulin signaling path through the receptor, and the availability of a defined GPI-headgroup from the VSG and other molecules have all been utilized.

The MVB2 T cell line has a defect in GPI-biosynthesis and is devoid of GPI-anchored proteins at the cell surface. However, the cells still synthesize a GlcN-labeled peak that comigrates with the insulin-sensitive species from previous studies (Avila *et al.*, 1992). The peak labels to the same extent with a variety of precursors in the MVB2 and the parental line, but only 20% of it is cleaved by nitrous acid. Substantially more than this proportion displays the transient degradation on insulin stimulation, suggesting that in addition to the putative GPI-species in the peak, that there is a further insulin-sensitive species. This appreciation of an increased complexity in the

putative mediator lipid peak is also supported by data using PHA-stimulated T cells and an antibody reactive to the GPI-headgroup (Gaulton, 1991). In this study four GPI-lipids were identified, but only two showed sensitivity to insulin, with the usual rapid degradation and resynthesis. Polar glycan fragments were detected in the cell medium. As an antibody was used in this study, the insulin-induced turnover was finally shown to affect bulk material, rather than movement of a radiolabeled tracer.

Manipulations of receptor levels have been used by several workers, and the influence on mediator activity assessed. Gottschalk (1992) studied rat embryo fibroblasts that overexpressed functional or tyrosine kinase deficient human insulin receptor. Despite identifying two GlcN-labeled insulin sensitive lipids, one of which was endoceramidase sensitive, the presence of functional human insulin receptor at elevated levels had no influence of the stability of these species. Additionally, a transient decrease by about 10% in PI levels was observed, mirrored by an increase in the amount of the insulin-sensitive endoceramidase resistant species. As PI is a major cellular phospholipid, turnover of even 10% indicates a far larger mass of lipid being metabolized than earlier data would suggest. Most importantly, the data here suggest that the insulin receptor is not directly involved in any lipid-mediated effects of the hormone. This conclusion is directly contradicted by another study using CHO cell lines expressing different levels of the human insulin receptor (Macaulay *et al.*, 1992). The transient turnover of the putative mediator was found to be increased in cells expressing a greater level of insulin receptor, and that turnover could be stimulated by up to 100-fold less insulin in the highest expressers compared with the control cells. However, as the putative GPI peak was only degraded by ~7% in the control cells, and this only doubled in the overexpressing cells which had 600 times the control level of receptor, the effects are not very dramatic. In a recent study of GPI-biosynthesis mutants, the ability of the insulin receptor to signal correctly was assessed (Lazar *et al.*, 1994). These authors made use of two K562 cell mutants in early steps of GPI-biosynthesis, the IA mutant (fails to make GlcNAc-PI) and the IVD mutant (fails to deNacetylate GlcNAc-PI to GlcN-PI). When introduced into these cells by transfection, the human insulin receptor was able to respond to insulin as normal by receptor autophosphorylation, phosphorylation of IRS-1 and downstream interaction with Shc and Grb2. However, glycogen synthesis was completely insulin insensitive in the IA cells and barely sensitive in the IVD cells. In broken cells glucose-6-phosphate could stimulate glycogen synthase, ruling out a simple defect in the target enzyme. Overall, these data do provide a case for a insulin-signaling pathway in mammalian cells that requires ongoing GPI-biosynthesis.

Using a defined fragment from VSG or the human erythrocyte acetylcholinesterase (huEAchE), both of which have fully defined GPI-anchor structures (Ferguson *et al.*, 1988; Roberts *et al.*, 1988) has produced data consistent with this type of molecule having some signaling properties. A pronase fragment from the 118 VSG molecule, purified by anion exchange HPLC, was able to inhibit isoproterenol-stimulated lipolysis, although high doses were required (100 μM) compared with insulin (used at 10 nM) (Misek and Saltiel, 1992). The activity showed similar properties to the POS fractions and was destroyed by mild acid or deamination, suggesting that a cyclic inositol phosphate was important. Most interestingly okadaic acid, a type 1 and 2A phosphatase inhibitor, was able to block the antilipolytic action of the VSG fragment at lower doses than required to block the effect of insulin. In a later study these authors obtained evidence for specific inhibition of isoproterenol-stimulated phosphorylation of a 70 kDa protein in rat epididymal adipocytes by the VSG fragment (Misek and Saltiel, 1994). The effect was specific (other phosphoproteins were unaffected); mild acid, nitrous acid, and okadaic acid sensitive; and required high concentrations of stimulator (150 μM).

In a complementary approach the C-terminal GPI fragment from huEAchE was shown to antagonize glucagon stimulation of glycogen phosphorylase (Deeg *et al.*, 1993). This fragment had a potency greater than the VSG fragment, being equivalent to 10 nM insulin at ~10 μM, also significantly greater than the potencies of the earlier POS preparations. In addition cyclic inositol-1,2-phosphate could mimic the effect of the GPI-fragment.

Immunoaffinity purified GPI-anchored proteins MSP-1 and MSP-2 from the malaria parasite *P.falciparum* were assayed for their ability to stimulate macrophages, to release TNF and IL-1 and to affect adipocyte glucose metabolism (Schofield and Hackett, 1993). Intact protein and the GPI-anchor fragment could induce TNF, while disruption of the lipid structure destroyed activity. MSP-2 additionally stimulated IL-1 production. When administered *in vivo* to mice the GPI fragments lead to cachexia, which was blocked by anti-TNF antibodies. A GPI fragment prepared from the 118 VSG was also shown to stimulate C3H macrophages to secrete IL-1α and TNF over a several-hour period (Tachado and Schofield, 1994). Addition of the VSG fragment lead to tyrosine-phosphorylation of multiple proteins followed by IL-1α release. Both of these effects were blocked by genistein and tyrphostin, inhibitors of protein tyrosine kinases. TNF release was somewhat delayed, and may be induced by the IL-1α. Significantly, the GPI was much more potent in this system, being used at 1 μM. Preclearing with antibody against GPI prevented IL-1α induction by parasite extracts. Taken together, these two studies are highly suggestive of bioactivity for these GPI-fragments in the macrophage system.

A very recent study reexamined the heterogeneity of the putative mediator peak in rat adipocytes (Parpal *et al.*, 1995). Using ^{32}P as radiolabel three distinct water-soluble products were obtained by PI-PLC cleavage of the mediator peak. On analysis on a Glycopak N HPLC system only one of the products, migrating as a pentasaccharide, was sensitive to deamination. This product itself contained at least three subspecies, evidence indeed that the putative mediator peak contains many distinct molecules. The nitrous acid sensitive species was found to be enriched in caveolae, glycolipid-rich regions of the cell membrane containing a higher than average level of GPI-anchored proteins. Analysis of ^{3}H-galactose-labeled hepatoma cells was in agreement with this; in this case less than 5% of the putative mediator peak was PI-PLC sensitive, and only a portion of this was cleavable with nitrous acid. This final fraction however had the ability to modulate phosphorylation of 116 and 84 kDa protein species, could block isoproterenol kinase stimulation and had the same turnover kinetics on insulin stimulation as reported for the mediator peak. Therefore, this more rigorous examination is suggestive that a minor fraction of the mediator peak is, in fact, the true mediator. This may, to some extent, explain the low potency of the mediator in a number of the studies described above, as the phosphate

content, habitually used as an estimate of mass, is quite probably misleading.

Concluding remarks

A final and critical evaluation of the potential role that POS may play in signal transduction is difficult to arrive at. Early studies were focused on a mediator fraction that was of low potency and was clearly a mixture of many molecules. Information such as composition is obviously of little utility when dealing with such fractions for prediction of the structure of a single active component. Additionally, problems concerning the origin of the mediator and conflicting reports concerning the presence of a mediator lipid clearly require resolution. However, much more convincing are the reports of bioactivity within GPI-fragments prepared form both mammalian and protozoan sources, and these factors being of higher purity show correspondingly increased potency. The use of a heterologous source also removes some of the possibility for artefactual activity from homologous sources due to the presence of cytokines or other bioactive compounds. These analyses do not, of course, approach the question of the nature of an endogenous insulin mediator. The need for a detailed structural analysis of highly purified material and the identification of interacting components is clearly required before this potentially very important issue can be resolved.

A report that caveolin is a substrate for insulin-stimulated phosphorylation (Mastick *et al.*, 1995) perhaps suggests that this molecule may be involved in GPI-insulin connection. Phosphorylation of caveolin may lead to alterations in plasma membrane organization and hence to cleavage of otherwise stable GPI structures, although the caveolin story has become controversial recently. Finally, it is interesting to note that phosphorylation of IRS-1 follows similar kinetics as turnover of the putative mediator peak on insulin treatment (Milarski *et al.*, 1995), and therefore POS mediator action, if it exists, is quite likely tightly coupled to the conventional signaling pathway.

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Abbreviations

Gal(N), galactose(amine); GlcN, glucosamine; GPI, glycosylphosphatidylinositol; huEAchE, human erythrocyte acetylcholinesterase; IFN, interferon; IGF, insulin-like growth factor; IL, interleukin; IRS, insulin receptor substrate; MAP, mitogen-activated protein; PDE, phosphodiesterase; PDH, pyruvate dehydrogenase; PI, phosphatidylinositol; PLC(D), phospholipase C(D); POS, phosphooligosaccharide; SH2(3), src homology domain 2(3); TLC, thin layer chromatography; VSG, variant surface glycoprotein.

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