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Substrates and Signalling Complexes: The Tortured Path to Insulin Action

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Abstract In the last few years several potential substrates of the insulin receptor tyrosine kinase have been identified, purified, and their cDNAs isolated. These putative substrates include: 1) pp15, a fatty acid-binding protein; 2) pp120, a plasma membrane ecto-ATPase; 3) pp42, a MAP serine/threonine kinase; 4) pp85, a subunit of the Type 1 phosphatidylinositol kinase; and 5) pp185, a phosphatidylinositol kinase binding protein. Although the tyrosine phosphorylation of several of these substrates correlates with the signalling capabilities of various mutant receptors, the role of these substrates in mediating any one of insulin's many biological responses is still unknown. In addition, recent data indicate that the tyrosine phosphorylation of pp42 may in fact be due to autophosphorylation, thereby removing it from the list of putative substrates of the insulin receptor kinase. Finally, the present review discusses the question of whether signalling occurs as a result of the tyrosine phosphorylation of substrates or via the formation of signalling complexes.

Key words: tyrosine kinases, phosphatidylinositol kinases, insulin receptor

A major advance in our understanding of insulin action came from the discovery that the insulin receptor, like the receptors for several other growth factors, has an intrinsic tyrosine kinase activity [for several recent reviews, see references 1–4]. Subsequent studies of mutant receptors lacking kinase activity and inhibitors of the receptor kinase have implicated this enzymatic activity in many of insulin's biological actions. However, the role of the receptor kinase in at least one biological response (stimulation of pyruvate dehydrogenase) has been thrown into question by the finding that insulin can stimulate this biological response in cells expressing mutant insulin receptors which had been previously been shown to be devoid of kinase activity [5]. Additional studies will have to be done to confirm this finding.

An understanding of how insulin elicits a particular biological response now requires us to determine which proteins become tyrosine phosphorylated by the receptor kinase. One of the first (and most prominent) substrates of the insulin receptor tyrosine kinase is the β subunit of the receptor itself. Phosphorylation of at least two tyrosines in the kinase domain of the receptor (residues 1162 and 1163) results in a dramatic increase (more than a 100-fold) in the ability of the receptor to phosphorylate various exogenous substrates [6]. At least three other tyrosines (residue 1158 in the kinase domain and 1328 and 1334 in the carboxy tail) in the receptor β subunit have also been shown to become phosphorylated both in vitro and in vivo [1,6]. However, the reported effects of phosphorylation of these tyrosines on the signalling capabilities of the receptor remain controversial. For example, the kinase activity of a mutant receptor in which tyrosine 1158 was changed to phenylalanine was found in one study to have a severely compromised kinase activity, whereas in another study the same mutant receptor was found to have almost normal kinase activity [7,8]. It is also possible that other tyrosines in the receptor may become phosphorylated. For example, various reports have suggested that the two tyrosines in the juxtamembrane region (residues 965 and 972) may become phosphorylated, although other studies have questioned this [1,6]. A resolution of this point is very important since a mutant insulin receptor with tyrosine 972 changed to phenylalanine has been reported to have a defect in its

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ability to signal and phosphorylate various endogenous substrates [1]. This latter finding could be explained if tyrosine 972 was normally autophosphorylated and this autophosphorylation affected the ability of the receptor to phosphorylate various endogenous substrates.

SUBSTRATES OF THE INSULIN RECEPTOR KINASE

In addition to the receptor itself, insulin treatment of cells results in the tyrosine phosphorylation of numerous endogenous proteins of molecular weights ranging from 15,000 to 250,000 [9]. These phosphorylations can best be observed in cells overexpressing the receptor and when phosphatase inhibitors are also added to the cells. The goal of many groups over the last few years has been to identify these tyrosine phosphorylated proteins. Recently, a number of these proteins have been identified (Table I). One of the first such proteins identified was a 15-kDa protein that was phosphorylated in 3T3-L1 adipocytes treated with insulin and the phosphatase inhibitor, phenylarsine oxide [10]. When this protein was purified and a partial sequence was obtained, it turned out to be a member of the family of fatty acid–binding proteins. The purified protein was then shown to be phosphorylated in vitro with the purified insulin receptor. It is possible that tyrosine phosphorylation of a lipid binding protein may be important in insulin regulation of lipid metabolism. However, this abundant cellular protein is phosphorylated to a low stoichiometry in the intact cell and the role of this tyrosine phosphorylation is not known.

Another protein of M_r 120 kDa was first observed to be tyrosine phosphorylated in liver extracts. This protein was subsequently shown to be also tyrosine phosphorylated in intact liver cells in response to insulin. This protein has now been identified as an ecto-ATPase [11]. Again, the role of this phosphorylation is not known but could possibly affect the enzymatic activity of this protein.

A more interesting potential substrate of the insulin receptor that has been identified is a 42-kDa protein. This protein was identified as a serine/threonine kinase called the MAP kinase since it could phosphorylate MAP2 in vitro [12]. In the intact cell this enzyme may phosphorylate another serine/threonine kinase which phosphorylates the ribosomal protein S6. Tyrosine phosphorylation of the MAP2 kinase was shown to activate its enzymatic activity [12]. Thus, this kinase was thought to be a "switch" kinase, an enzyme that would switch the tyrosine kinase activity of the insulin receptor to the more common serine/threonine phosphorylations that occur in response to insulin and other growth factors. Since several of insulin's biological actions are mediated by changes in the serine/threonine phosphorylation state of various target proteins [6], this was an exciting potential substrate for the insulin receptor tyrosine kinase. The purification and sequencing of this protein has allowed the isolation of its cDNA [13]. These studies have revealed that there is a family of related MAP kinases (also called ERK, for extracellular signal-regulated kinase). It is therefore very dissappointing that the most recent studies of this enzyme have revealed that its tyrosine phosphorylation appears to be due to an autophosphorylation reaction and not via the tyrosine phosphorylation of this enzyme by a tyrosine kinase [14]. Thus, pp42 should no longer be considered a potential substrate of the insulin receptor kinase although an understanding of its regulation and identification of its substrates may still be important in understanding how insulin works.

TABLE I. Putative Substrates of the Insulin Receptor Tyrosine Kinase

Substrate	Properties	M_r (kDa)
Fatty acid-binding protein	Binds lipids and calcium	15
Ecto ATPase	Has ATPase activity	120
MAP kinase	Serine/threonine kinase	42
Type I PtdIns kinase	Phosphorylates PtdIns at 3-position	85
IRS-1	Binds the type I PtdIns kinase	185

PHOSPHATIDYLINOSITOL KINASE AS A SUBSTRATE OF THE INSULIN RECEPTOR KINASE VS. AS A SIGNALLING COMPLEX

One substrate of the platelet-derived growth factor (PDGF) receptor tyrosine kinase is called the type 1 phosphatidylinositol (PtdIns) kinase [15]. This enzyme phosphorylates PtdIns at the D-3 position of the inositol ring [16]. The type I PtdIns kinase activity also appears to be stimulated in cells treated with insulin since the amount of PtdIns 3,4-P and PtdIns 3,4,5-P increased in cells treated with insulin [17]. These PtdIns derivatives, unlike the more abundant

PtdIns 4,5-P, do not appear to be cleaved by any known phospholipase C [18]. It is not known what role these compounds play in the cell. Treatment of cells with either insulin or PDGF also stimulates an increase in the amount of type 1 PtdIns kinase activity which can be precipitated from cell extracts with anti-phosphotyrosine antibodies [19]. In the case of the insulin treated cells, only a small fraction (approximately 5%) of the tyrosine phosphorylated PtdIns kinase activity can be precipitated with anti-receptor antibodies. These results suggest that either the PtdIns kinase itself or a tightly associated protein becomes tyrosine phosphorylated in insulin-treated cells. It is likely that this phosphorylation is directly mediated via the insulin receptor kinase since the insulin-stimulated tyrosine phosphorylation of PtdIns kinase can occur even at 4°C, a temperature where little movement of intracellular proteins occurs [19]. Moreover, the receptor can be covalently cross-linked to the PtdIns kinase in the intact cell [20]. However, the direct phosphorylation of PtdIns kinase in vitro by the insulin receptor has not yet been formally demonstrated.

In the case of PDGF, the receptor appears to become tightly associated with the PtdIns kinase [21]. The receptor could therefore be used to purify the PtdIns kinase [22]. This enzyme was shown to consist of two subunits, an 85- and 110-kDa component. The 85-kDa subunit has been sequenced, cloned, and expressed [22]. It does not appear to have PtdIns kinase activity, suggesting that the 110-kDa subunit has catalytic activity. The 85-kDa subunit has been shown to be tyrosine phosphorylated by the PDGF receptor and its sequence was found to contain two regions which were homologous to regions in the Src tyrosine kinase (called Src Homology or SH regions). It has been proposed that the SH regions of various proteins serve as high-affinity binding sites of other tyrosine phosphorylated proteins [23]. Thus, it would be predicted that the SH regions of the PtdIns kinase would bind with high affinity to a particular phosphorylated tyrosine in the PDGF receptor. This would yield a signalling complex. It has been proposed that the formation of this complex is what increases the activity of the PtdIns kinase in the cell since by complexing with the PDGF receptor the PtdIns kinase would be brought to the membrane, the site of its substrate. According to this model, tyrosine phosphorylation of PtdIns kinase could even be the turn-off signal by enhancing the dissociation of the enzyme from the receptor.

In the case of the PDGF receptor, another substrate is a specific phospholipase C (PLC-γ1) [15]. (This enzyme does not appear to be tyrosine phosphorylated by the insulin receptor.) This substrate, like the PtdIns kinase, also contains SH regions and complexes with the PDGF receptor although the particular phosphorylated tyrosine recognized in the receptor appears to differ for these two substrates [24]. Since the complex formation with the PDGF receptor would also bring this PLC to the membrane where its substrate is present, it could also be imagined that the formation of this complex would be the cause of the increase of PLC activity in the cell. However, recent studies of mutant PLCs in which the tyrosine phosphorylation sites have been changed to phenylalanines have shown that some of these mutant PLCs still make a complex with the receptor but do not mediate an increase in PLC activity in the intact cell [25]. These results therefore indicate that it is the tyrosine phosphorylation of PLC-γ that stimulates its increase in activity and not the formation of a complex with the PDGF receptor. Future studies will be required to verify if this is also true for the PtdIns kinase.

In the case of the insulin receptor, in contrast to the PDGF receptor, only a small percentage of PtdIns kinase appears to associate with the receptor in immunoprecipitation studies [19]. It is not known whether the PtdIns kinase which is associated with the receptor is tyrosine phosphorylated or not. It is possible that the small amount of receptor-associated PtdIns kinase is associated with the receptor only transiently while it is being phosphorylated. Once phosphorylated, the kinase may then be released.

It also is not clear whether the PtdIns kinase which becomes immunoprecipitable with anti-phosphotyrosine antibodies after insulin treatment is associated with the same 85-kDa protein that associates with the PDGF receptor. Recently, the insulin receptor substrate pp185 (also called IRS-1 for insulin receptor substrate-1) has been purified, sequenced, and its cDNA isolated [26]. It does not contain any SH regions. However, antibodies to this protein were shown to precipitate type 1 PtdIns kinase activity from insulin stimulated cells. Thus it is possible to envision several models for the interaction of the insulin receptor with the type 1 PtdIns kinase (Fig. 1). According to the first model, the

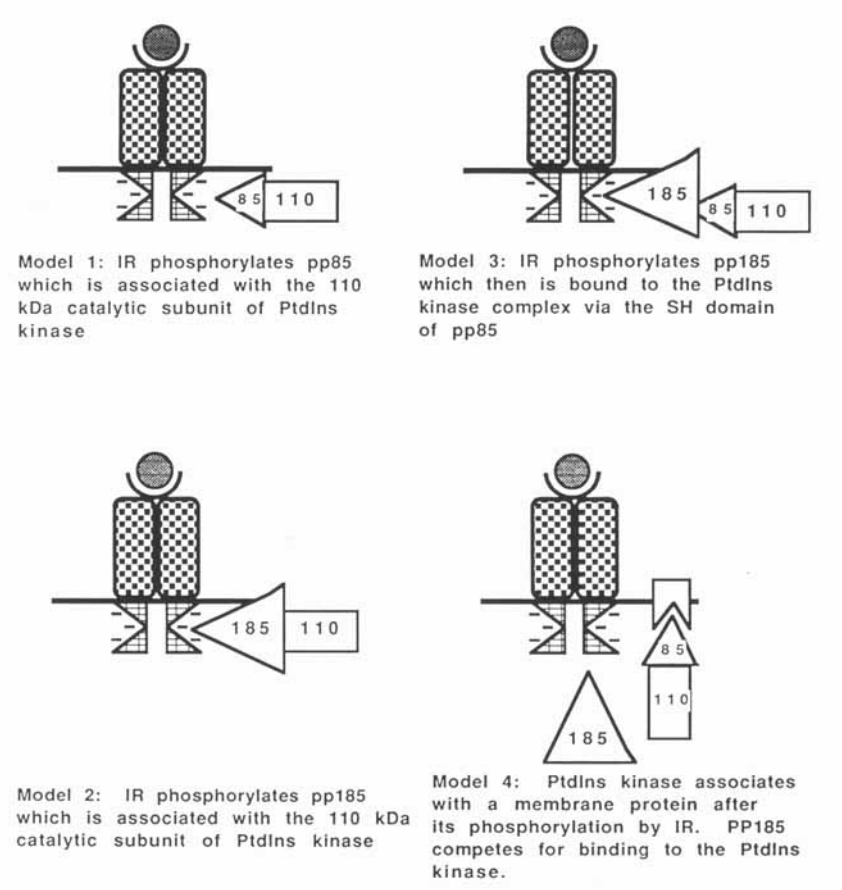

Fig. 1. Models for the interaction of the insulin receptor (IR), the PtdIns kinase and pp185.

insulin receptor, like the PDGF receptor, phosphorylates the 85-kDa subunit of the PtdIns kinase. In the second model, the 110-kDa catalytic unit of PtdIns kinase associates directly with pp185 which is phosphorylated by the insulin receptor. A third model would be that this 185-kDa protein is phosphorylated by the insulin receptor kinase and then is bound by the 85-kDa subunit of the PtdIns kinase. In this regard, the sequence of the 185-kDa contains six tyrosines that are in the same motif (YMXM) as the putative consensus site for binding to the 85-kDa subunit of the PtdIns kinase [15]. In contrast, the insulin receptor contains only one such sequence in its carboxy tail that is a partial consensus site for pp85 binding (YXXM). Moreover, mutant receptors which lack this tyrosine (residue 1334) still appear to phosphorylate the PtdIns kinase normally [20]. Thus, it is more likely that the PtdIns kinase would bind to a tyrosine phosphorylated pp185 than to the autophosphorylated insulin receptor.

However, it is not clear why the insulin receptor would need to phosphorylate another protein that would then bind to the PtdIns kinase. Since pp185 appears to be primarily a cytosolic protein, one could even speculate that the binding of pp185 to the 85-kDa subunit of the PtdIns kinase may be a way of inhibiting the activity of this kinase (Model 4, Fig. 1). According to this model, there are other less abundant membrane proteins which contain the consensus site for PtdIns kinase binding. After phosphorylation of these membrane proteins by the insulin receptor, the 85-kDa subunit of the PtdIns kinase could bind to such proteins and this would serve to bring this enzyme to the membrane where its substrate is present. Evidence for an insulin-induced movement of a portion (approximately 25%) of the PtdIns kinase activity to the membrane has been presented [20,27]. Thus, the tyrosine phosphorylation of a cytosolic protein like pp185 which binds to the 85-kDa subunit of the PtdIns kinase could serve to decrease the amount of PtdIns kinase activity in the cell by competing with these membrane proteins for binding to the PtdIns kinase. These different

models should now be testable with the availability of the cDNA clones for pp85 and pp185.

It also is not clear at the present time whether the type 1 PtdIns kinase is important in mediating none, one, or several of insulin's biological responses. To attempt to test this, we have analyzed the ability of various mutant receptors to mediate the phosphorylation of the PtdIns kinase. A mutant receptor which has had tyrosine 1162 and 1163 changed to phenylalanines had been reported to be capable of stimulating thymidine incorporation as well as the wild-type receptor but to show only a partial capability of mediating short-term metabolic responses like stimulation of glucose uptake [28]. However, in our studies this mutant receptor exhibited a decreased ability to stimulate thymidine incorporation and other responses [20]. The ability of this mutant receptor to mediate the phosphorylation of the PtdIns kinase was similarly decreased. In a study by Kapeller et al., a mutant receptor which has had tyrosine 972 changed to phenylalanine exhibited a decreased ability to mediate the tyrosine phosphorylation of the type 1 PtdIns kinase, in agreement with the prior studies showing that this mutant receptor exhibits a decreased ability to stimulate various responses, although this receptor has been found to exhibit a normal level of autophosphorylation and exogenous substrate kinase activity in vitro [29]. It is possible that this tyrosine serves in the substrate recognition site of the receptor. Thus, studies of the ability of various mutant receptors to phosphorylate PtdIns kinase have so far shown a close correlation with the abilities of these mutant receptors to stimulate biological responses. These studies do not, however, prove a role for the PtdIns kinase in mediating any particular response in the cell.

One of the main questions concerning the type 1 PtdIns kinase involves what this enzyme does in the cell. In vitro, the enzyme can phosphorylate PtdIns, PtdIns 4-P, and PtdIns 4,5-P at the D-3 position of the inositol ring [15]. It is possible that the enzyme has other substrates in the intact cell that we do not yet know about. In the intact cell, the levels of PtdIns 3,4-P and PtdIns 3,4,5-P appear to increase in response to insulin and PDGF [15]. These compounds could elicit various effects by binding to different proteins. Potential candidate proteins would include proteins that have been shown to bind phospholipids such as the serine/threonine kinase protein kinase C and the GTPase-activating proteins GAP and NF-1 [30–32]. These are also good candidates because there is some evidence that growth factors may affect their activities in the intact cell [33,34]. However, attempts at demonstrating direct binding of labeled PtdIns 3-P and PtdIns 3,4,5-P to these proteins in vitro were unsuccessful (K.K. and R.A.R., unpublished studies). Another candidate would be a protein called profilin, which causes depolymerization of actin [35]. One study has shown a correlation between actin depolymerization and generation of PtdIns 3-P derivatives in neutrophils treated with chemotactic factors [36]. These studies with neutrophils indicate that a role for the type I PtdIns kinase need not be limited to a mitogenic response.

FUTURE PROSPECTS

From the above discussion, it is apparent that we still have a long way to go in understanding the detailed biochemical mechanisms of how insulin elicits its subsequent biological responses. Although several substrates of the insulin receptor kinase have now been identified (Table I), we do not know what the function of any of these substrates is in the cell. Even in the case of the PtdIns kinase, where we know what products its enzymatic activity generates, these novel PtdIns derivatives are present in very low concentrations in the cell and their actions are unknown. We also do not know whether the tyrosine phosphorylation of any of the known substrates is required to elicit any particular biological response. Even though the tyrosine phosphorylation of some of these substrates has been shown to correlate with the ability of various mutant receptors to elicit biological responses, this does not prove that the phosphorylation of this particular substrate causes the subsequent response since there are many other substrates whose phosphorylation could also correlate with receptor kinase activity. With the availability of the cDNA clones for several of the substrates, it should be possible to directly test the role of a particular substrate in a specific response in the near future.

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