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Insulin Receptor Signaling Through Non-Tyrosine Kinase Pathways: Evidence From Anti-Receptor Antibodies and Insulin Receptor Mutants

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Abstract Although there is general agreement that insulin receptor tyrosine kinase activity mediates many of the actions of insulin, two types of studies suggest that non-tyrosine kinase dependent pathways may also exist. First, both monoclonal and polyclonal antibodies to the receptor have been shown to mediate many of insulin's actions with little or no stimulation of receptor kinase. Second, insulin receptor mutants, with reduced or no tyrosine kinase activity, have been shown to mediate several actions of insulin. Non-tyrosine kinase pathways that could signal insulin effects through the insulin receptor include non-covalent activation of G proteins, phospholipase Cs, or docking proteins such as IRS-1. Further studies on the chemical structures of phospholipids and their hydrolysis products involved in insulin action will be required to sort out the underlying mechanisms of insulin action via non-tyrosine kinase dependent pathways.

Key words: anti-insulin receptor antibody, mutant receptors, non-tyrosine kinase pathways

Insulin is a major regulatory hormone for most cells. In the vast majority of patients with non-insulin dependent diabetes mellitus (NIDDM), insulin action is impaired [Reaven, 1984]. A decade of extensive research on the hormone insulin and its receptor has yielded much valuable information concerning insulin receptor signaling. However, the molecular mechanisms of insulin's actions are not completely understood.

The interaction of insulin with target cells is mediated by a specific tetrameric glycoprotein receptor located in the plasma membrane [Goldfine, 1987]. This receptor consists of two identical extracellular α-subunits (M_r = 130,000 each) that contain insulin binding sites and two identical transmembrane β-subunits (M_r = 95,000 each) that have tyrosine kinase activity in their intracellular domains. When insulin binds to the α-subunit of the receptor, tyrosine phosphorylation of the β-subunit is increased, β-subunit tyrosine kinase is activated, and various biological functions of insulin occur [Goldfine, 1987; Yarden and Ullrich, 1988].

Several independent lines of evidence suggest that insulin receptor tyrosine kinase activity may be important in many biological functions of insulin [Goldfine, 1987; Yarden and Ullrich, 1988]. First, insulin receptors mutated at the ATP binding site lose their activity to signal a wide variety of biological functions. Second, insulin receptors mutated at one or more of the key tyrosine autophosphorylation sites lose some biological functions. Third, when antibodies which react with the insulin receptor β-subunit are introduced into the cytoplasm of target cells, certain actions of insulin are blunted. However, the extensive search to determine putative targets for phosphorylation by the insulin receptor tyrosine kinase in vivo has yielded only limited information on their identities [Kasuga et al., 1990].

Despite the general agreement for the requirement for the tyrosine kinase activity and autophosphorylation of the receptor in insulin action, various studies have raised some interesting questions and have challenged the hypothesis [Simpson and Hedo, 1984; Zick et al., 1984; Ponzio et al., 1988; White et al., 1988; Debant et al., 1988; Soos et al., 1989; Hawley et al., 1989;

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Sung et al., 1989; Wilden et al., 1990; Gottschalk, 1991]. These studies have employed both polyclonal and monoclonal antibodies to the insulin receptor; and cells expressing insulin receptor constructs mutated in the tyrosine kinase domain. The present paper will review these studies on insulin receptor signaling through non-tyrosine kinase pathways.

STUDIES WITH ANTIBODIES TO INSULIN RECEPTOR

One interesting area of study has been antibodies to the insulin receptor. While certain of these antibodies, either polyclonal or monoclonal, have been shown to stimulate insulin receptor tyrosine kinase activity [Roth et al., 1983; Gherzi et al., 1987; Brindle et al., 1990], other antibodies appear to have little or no effect on receptor tyrosine kinase activity and receptor autophosphorylation [Zick et al., 1984; Simpson and Hedo, 1984; Ponzio et al., 1988; Hawley et al., 1989; Brunetti et al., 1989; Sung et al., 1989]. However, these latter antibodies have been shown to stimulate a wide spectrum of cellular functions in both primary cells and cultured cells.

In mouse fibroblast 3T3 cells transfected with and expressing normal human insulin receptors, we demonstrated that several species-specific monoclonal antibodies to the human insulin receptor including MA-5, in contrast to insulin, failed to stimulate tyrosine phosphorylation of the receptor and an endogenous substrate, pp185 (IRS-1) (Fig. 1A). When tyrosine kinase activity of the receptor was measured using exogenous substrates such as histone 2B and poly(Glu,Tyr), these monoclonal antibodies did not increase this function [Hawley et al., 1989; Sung et al., 1989]. However, these antibodies were able to elicit a variety of biological functions including glucose uptake, amino acid uptake, and thymidine incorporation [Brunetti et al., 1989]. Strikingly, MA-5, a monoclonal antibody which had little or no effect on receptor tyrosine kinase activity, activated ribosomal protein S6 kinase with a dose response that was very similar to insulin (Fig. 1B).

Soos et al. [1989] also reported that several of their monoclonal antibodies could mimic insulin's actions in mammalian cells without stimulation of receptor tyrosine kinase. It should be pointed out, however, that others have suggested that these agonist receptor antibodies may induce low levels of receptor tyrosine kinase activity and receptor autophosphorylation [Gherzi et al., 1987; Steele-Perkins and Roth, 1990]. However, in contrast to insulin, studies with antibodies failed to demonstrate a direct correlation between receptor tyrosine kinase activity and biological functions. The most potent monoclonal antibodies did not stimulate receptor kinase activity to the same extent as insulin, even though in some studies these antibodies have been found to be almost as potent as insulin in stimulating certain biological response [Steele-Perkins and Roth, 1990]. Furthermore, at low concentrations of antibodies which did not activate receptor tyrosine kinase in a sensitive plate assay there was a significant increase of some biological functions [Steele-Perkins and Roth, 1990].

STUDIES WITH INSULIN RECEPTOR MUTANTS

In order to study the potential relationships between insulin receptor tyrosine kinase activation, receptor autophosphorylation, and biological functions of insulin, we and others have employed insulin receptors that have been mutated in the tyrosine kinase domain. In normal insulin receptors, insulin activation of receptor tyrosine kinase very rapidly leads to the autophosphorylation of a cluster of three major tyrosines at residues 1158, 1162, and 1163 followed by other minor tyrosine phosphorylation at 1328 and 1334 [White and Kahn, 1989]. Insulin receptor mutants used for the studies include ones with single, double, or triple major tyrosine residues changed to phenylalanines and with lysine 1030 in the ATP binding site changed to other amino acids.

In Chinese hamster ovary (CHO) cells expressing receptor mutants in which tyrosine at 1146 (equivalent to tyrosine 1158 in the exon 11 variant) was changed to phenylalanine, receptor autophosphorylation was reduced 60–70%, which was insufficient for full activation of the receptor kinase during in vitro assays toward exogenous substrates [Wilden et al., 1990]. In contrast, others have reported normal receptor kinase activity with these mutants [Zhang et al., 1991]. These receptors neither internalized insulin normally nor stimulated DNA synthesis in the presence of insulin. However, these receptors mediated normal insulin stimulated glycogen synthesis [Wilden et al., 1990].

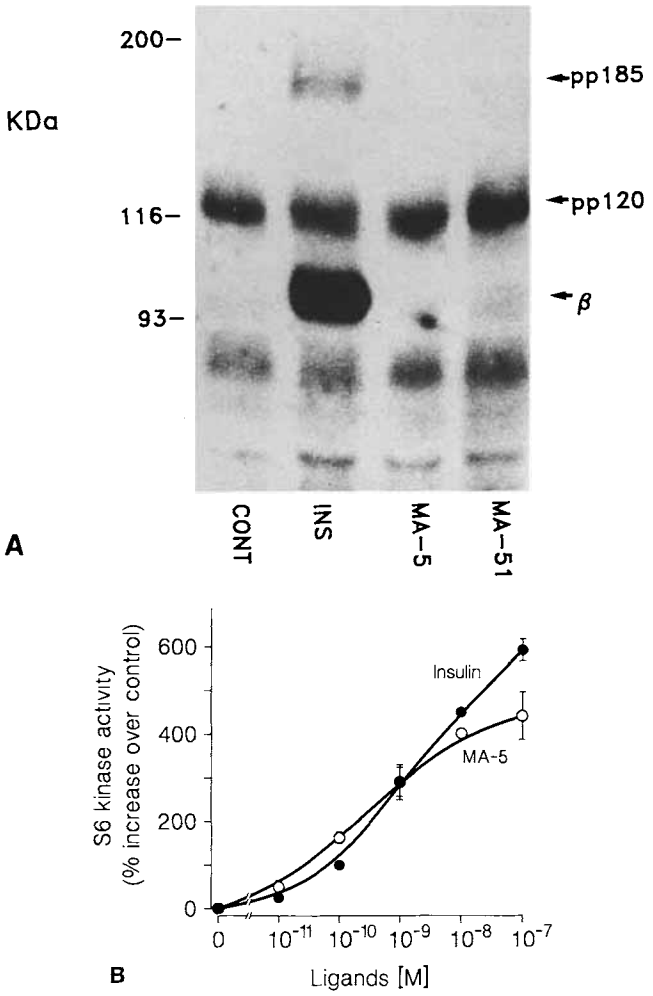

Fig. 1. A: Tyrosine autophosphorylation of insulin receptors and tyrosine phosphorylation of pp185 (IRS-1) in intact 3T3 cells expressing human insulin receptors (3T3/HIR) by 100 nM insulin (INS), and monoclonal antibodies MA-5 and MA-51 using Western blotting. Cells were treated with various agents and the solubilized cellular lysates were then analyzed by 7.5% SDS-PAGE and Western blotting with an antiphosphotyrosine antibody. Proteins containing phosphotyrosine residues were visualized by autoradiography. β, insulin receptor β-subunit. **B:** Concentration dependence of S6 kinase activation by MA-5 (○) and insulin (●) in 3T3/HIR cells. Cells were incubated with various concentrations of agents. Soluble cellular lysates were then prepared and assayed for S6 kinase activity. The data are presented as percent increase over control and are mean ± S.E.M. Note that MA-5, which does not increase insulin receptor and pp185 tyrosine phosphorylation, has a potency similar to insulin.

In CHO cells expressing receptors in which tyrosine residues 1162 and 1163 were replaced by phenylalanine, there was a total inhibition of the insulin-mediated tyrosine kinase activity toward exogenous substrates [Ellis et al., 1986; Debant et al., 1988]. These receptors could still autophosphorylate, but to a diminished level when compared with normal receptors. These receptors did not mediate metabolic effects of insulin, such as glucose uptake and glycogen synthesis. In contrast, these receptors fully mediated thymidine incorporation into DNA when compared with normal receptors [Debant et al., 1988].

When all three major tyrosines in the insulin receptor were mutated to phenylalanines and overexpressed in HTC rat hepatoma cells, there was no enhancement of receptor tyrosine kinase activity both in vivo and in vitro as assessed by receptor autophosphorylation, phosphorylation of pp185, and phosphorylation of exogenous substrates (Fig. 2A) [Sung et al., 1989; Rafaeloff et al., 1991]. In these cells, insulin via the mutant receptors induced amino acid uptake and thymi-

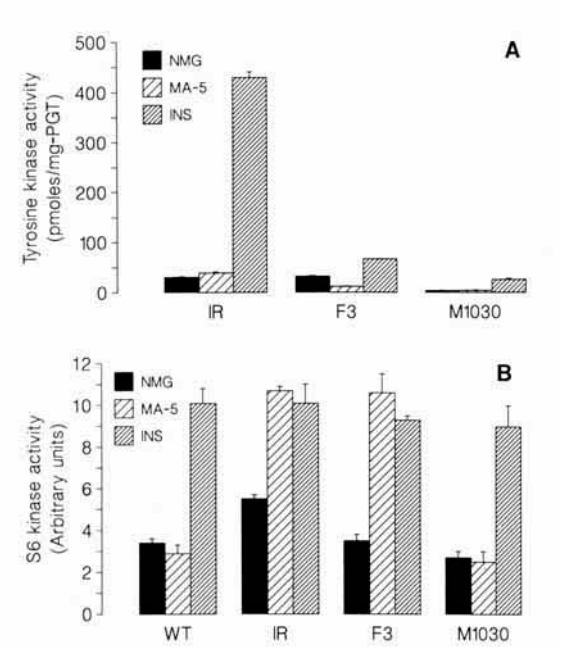

Fig. 2. A: Insulin receptor tyrosine kinase activity in various HTC cell lines. Wheat germ extracts of cells containing the same amount of insulin receptors (5 ng) were preincubated with either 100 nM NMG (normal mouse IgG), 100 nM MA-5, or insulin and then assayed for their tyrosine kinase activities with the substrate poly (Glu,Tyr). Cell lines used include wild type HTC cells (WT) transfected with and expressing normal insulin receptors (IR), receptors mutated at autophosphorylation sites 1158, 1162, 1163 (F3), and in the ATP binding site (M1030). **B:** S6 kinase activation by 100 nM insulin and MA-5 in various HTC cell lines. Cells were incubated with agents. Soluble cellular lysates were then prepared and assayed for S6 kinase activity. Note that in IR and F3 cell lines, MA-5 stimulated S6 kinase activity without activation of receptor tyrosine kinase.

dine incorporation [Rafaeloff et al., 1991]. These studies indicate therefore a dissociation between insulin receptor autophosphorylation, pp185 phosphorylation, kinase activity, and transmembrane signaling.

In the receptor mutant in which lysine 1030 in the ATP binding site was mutated to other amino acids, there was a complete loss of receptor tyrosine kinase activity in response to insulin both in vivo and in vitro [Goldfine, 1987; Yarden and Ullrich, 1988]. In cells expressing these receptors, insulin failed to stimulate glucose uptake, glycogen synthesis, thymidine incorporation, S6 kinase activation, receptor internalization, and receptor downregulation. Recently, Gottschalk [1991] reported that via these kinase defective receptors insulin could stimulate pyruvate dehydrogenase activity. These data demonstrated that the activation of insulin receptor tyrosine kinase does not play an obligatory role in the insulin receptor signaling pathway that stimulates this enzyme.

An interesting observation comes from studies with receptor mutants in which tyrosine 960 (equivalent to tyrosine 972 in the exon 11 variant) was changed to phenylalanine [White et al., 1988]. In these receptors, insulin-induced activation of receptor tyrosine kinase was normal both in vivo and in vitro, although phosphorylation of pp185 in vivo was barely detected. In the cells expressing these receptors, insulin failed to stimulate amino acid uptake, glycogen synthesis, and thymidine incorporation. White et al. [1988] suggested that receptor autophosphorylation was not sufficient for the insulin response, and a region of the insulin receptor around Tyr 960 may facilitate phosphorylation of pp185 required for transmission of the insulin signal.

Taken together, these data clearly demonstrated that there is a dissociation in insulin receptor signaling between 1) receptor autophosphorylation and receptor tyrosine kinase activity, 2) the former two functions and pp185 phosphorylation, and 3) individual biological functions of insulin.

STUDIES WITH BOTH RECEPTOR ANTIBODIES AND RECEPTOR MUTANTS

In CHO cells expressing insulin receptors mutated at tyrosines 1162 and 1163, insulin failed to stimulate glucose uptake and glycogen synthesis [Debant et al., 1988]. However, in these cells polyclonal antibodies to the insulin receptor could restore the effects of insulin on the above biological functions, suggesting a role of receptor cross-linking [Debant et al., 1989].

In HTC rat hepatoma cells expressing normal insulin receptors, there was an increase in the sensitivity of cells to insulin to stimulate S6 kinase [Sung, 1991]. In HTC cells expressing insulin receptors mutated at tyrosines 1158, 1162, and 1163, a species-specific monoclonal antibody to the insulin receptor (MA-5) with little or no effect on tyrosine kinase activity did not stimulate receptor tyrosine kinase activity (Fig. 2A). Moreover, MA-5 activated S6 kinase via both normal human receptors (IR) and triple tyrosine mutants (F3), but not via ATP binding site mutants (M1030) (Fig. 2B) [Sung et al., 1991]. Employment of both receptor mutants lacking tyrosine kinase activity and receptor antibodies with little or no effect on stimulation of receptor tyrosine kinase clearly demonstrated that stimulation of insulin receptor tyrosine ki-

nase per se is not essential to elicit certain biological effects of insulin.

MECHANISM(S) OF INSULIN ACTION

The detailed mechanisms by which the insulin receptor transmits its signal is still uncertain. In a model involving a protein phosphorylation cascade, receptor tyrosine kinase plays a pivotal role to induce receptor autophosphorylation, activation of receptor tyrosine kinase, and subsequent phosphorylation of one or more cellular substrates [Goldfine, 1987; Yarden and Ullrich, 1988; Kahn and White, 1988]. In vitro, one insulin action, activation of S6 kinase from Xenopus [Sturgill et al., 1988] but not from mammalian cells [Price et al., 1990], has been partially reconstituted in vitro in the presence of microtubule-associated protein 2 kinase (MAP-2 kinase). Insulin receptor tyrosine kinase, however, failed to directly phosphorylate and activate MAP-2 kinase. The most recent studies of MAP-2 kinase proposed that autophosphorylation is involved in its activation in vitro [Seger et al., 1991].

As previously reviewed from the studies with anti-receptor antibodies and receptor mutants, not all insulin's actions can be explained by the protein phosphorylation cascade model. Also there is no evidence that insulin alters phosphorylation of the glucose transporter or any proteins at the glucose transport pathway [White et al., 1988]. These data have led to a second model of action in which receptor autophosphorylation is viewed as changing the conformation of the receptor β-subunit, allowing it to interact noncovalently with some other effector system [Kahn and White, 1988; Maddux and Goldfine, 1991] (Fig. 3). Some anti-receptor antibodies could cause the same conformational change in the receptor β-subunit without receptor autophosphorylation. In receptors mutated at three major tyrosines 1158, 1162, and 1163, Maddux and Goldfine [1991] demonstrated an insulin-induced conformational change similar to that with normal receptors. These results may account for some of insulin's actions such as amino acid uptake and thymidine incorporation in these receptor mutants, but not for S6 kinase activation [Rafaeloff et al., 1991; Sung, 1991].

Linking activated insulin receptor with intracellular effector systems may be mediated via a G protein or a ras-related protein. First, Pertus-

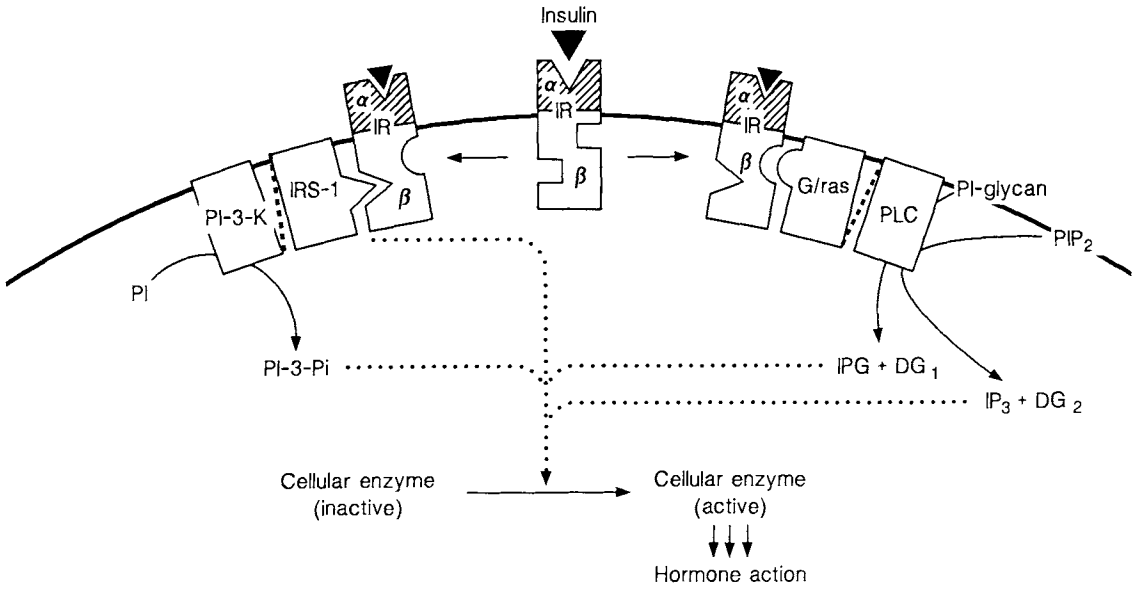

Fig. 3. Model of insulin receptor signaling via potential non-tyrosine kinase pathways. Insulin first interacts with its receptor α-subunit. This interaction then leads to a change in receptor β-subunit conformation without activation of tyrosine kinase. This change then allows interaction with either G/ras protein or IRS-1 which further interact non-covalently with PLC or PI-3-K, respectively. Activated PLC may then hydrolyze a unique PI-glycan or PIP_2 into appropriate molecules which then activate cellular enzymes leading to hormone action. Similarly, activated PI-3-K may also activate cellular enzymes to elicit hormone action. DG_1, diacylglycerol; DG_2, 1,2-diacylglycerol; G/ras, G protein or ras-related protein; IP_3, inositol 1,4,5,-trisphosphate; IPG, inositol phosphate glycan; IR, insulin receptor; IRS-1, insulin receptor substrate 1; PI, phosphatidyl inositol; PI-glycan, phosphatidylinositol glycan; PIP_2, phosphatidyl inositol 4,5-bisphosphate; PLC, phospholipase C (PI unique PLC or $PLC_{\beta 1}$).

sis toxin treatment, which inhibits the guanyl nucleotide binding protein Gi, inhibits some of insulin's actions [Houslay, 1986]. Studies with streptozotocin- or alloxan-induced diabetic rats demonstrated that the G protein involved in insulin action is not Gi, but a rather unique G protein [Gawler et al., 1987]. Second, in oocytes and hepatocytes, antibodies to ras-related proteins prevent insulin stimulation of oocyte maturation and glycogen synthesis, respectively [Korn et al., 1987]. Studies with BC_3H-1 cells [Luttrell et al., 1990] suggested that interaction of insulin-insulin receptor complex with G proteins does not require receptor tyrosine kinase activity.

Intracellular effector systems might include phosphatidylinositol-3-kinase (PI-3-kinase) or phosphatidylinositol specific phospholipase C (PI-PLC). PI-3-kinase appears to be activated in cells treated with insulin [Ruderman et al., 1990], is subject to tyrosine phosphorylation, a small fraction of phosphorylated enzymes shown to be associated with insulin receptor [Roth et al., 1991], and contains Src homology 2 domain for potential interaction with other cellular signaling proteins such as pp185 (also called IRS-1) [Sun et al., 1991]. IRS-1 was recently proposed as a multisite docking protein to bind signal-transducing molecule containing SH domains 2 and 3 [Sun et al., 1991] (Fig. 3). Physiological significance of PI-3-kinase, however, is not clear.

Insulin action on cells also activates one or more specific PI-PLC that acts on phosphatidylinositol (PI)-glycan to generate an inositol phosphate glycan (IPG) capable of mimicking several of insulin's actions on cells and myristoyl diacylglycerol [Low and Saltiel, 1988]. Fox et al. [1987] purified PI-glycan specific PLC from liver plasma membranes and proposed it as a potential target of insulin action. Romero et al. [1988] and Farese et al. [1988] also confirmed that insulin releases these two mediators although there is disagreement about the structure and source of the diacylglycerol, which may be derived from phosphatidylcholine rather than PI-glycan [Farese et al., 1988]. Difficulties with the PI-glycan hypothesis, however, relate to a lack of knowledge about its chemical structure and that of its IPG products, its location in the plasma membrane, the mechanism by which insulin promotes its cleavage, and the mechanisms of action of IPG on its target enzymes (Exton, 1991). Furthermore, Cooper et al. [1987] demonstrated, although it is controversial [Spach et al., 1986], that insulin stimulated protein kinase C in BC_3H-1 myocytes, which may be mediated by increase in diacylglycerol.

FUTURE PROSPECTS

Despite significant advances in the past few years on the chemistry and biology of insulin and its receptor, the molecular events that couple the interaction of insulin and its receptor to the regulation of cellular functions remain uncertain. Progress in this area has been complicated by the pleiotropic nature of insulin actions.

Several lines of evidence indicate that a unique G protein whose nature is unknown may be involved in insulin receptor signaling. Recently, a new G protein, Gq, has been purified and this Gq protein was demonstrated to activate $PLC_{\beta 1}$ isozyme in vitro resulting in hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into two byproducts, inositol 1,4,5-trisphosphate and 1,2-diacylglycerol [Taylor et al., 1991].

PLC_γ, the major PLC isozyme which has been shown to be involved in EGF receptor and PDGF receptor signaling, does not appear to be involved in insulin receptor signaling (Meisenhelder et al., 1989). As discussed previously, insulin treatment of BC_3H-1 myocytes increased diacylglycerol content in cells [Farese et al., 1988]. The discovery of new Gq protein and its ability to activate $PLC_{\beta 1}$ isozyme suggests that in addition to PI-glycan, PIP_2, a common substrate in other receptor signaling, may serve as an alternative substrate in insulin action to generate diacyglyacol (Fig. 3). In fact, insulin could increase phosphorylation of $PLC_{\beta 1}$ isozyme in insulin-responsive cells (personal communications). The reason that PIP_2 hydrolysis has not been easily detected in insulin action could be due to the low amount of $PLC_{\beta 1}$ in cells. A more sensitive detection method may be required, therefore, to study the effects of insulin on activation of $PLC_{\beta 1}$ and PIP_2 hydrolysis resulting in an increase of diacylglycerol and inositol trisphosphate. Diacylglycerol produced in insulin action could then activate protein kinase C in a certain type of cells [Farese et al., 1988]. Further studies are necessary to understand the extent to which protein kinase C operates, the isozyme involved, and its mechanism of activation [Exton, 1991].

Taken together, it appears that insulin receptor signaling is complex and requires multiple pathways including both receptor tyrosine kinase dependent and independent pathways. To

further understand these signaling mechanisms, one cannot eliminate one mechanism for another. Rather, one needs to study all aspects of signaling, including protein phosphorylation at both tyrosine and serine/threonine residues, protein phosphatases, protein kinase inhibitors, conformational changes in receptor β-subunit, involvement of G proteins, second messenger molecules, etc.

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