Common and Distinct Elements in Insulin and PDGF Signaling[a]

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INTRODUCTION

Receptor tyrosine kinases are distinguished by the presence of a single transmembrane spanning region and a well-conserved protein kinase catalytic domain that is absolutely required for transmembrane signaling.[1] Specificity arises because each receptor contains a distinct extracellular ligand-binding domain that recognizes its cognate ligand with high affinity. The growth factor or hormonal signal is relayed into the cell when the tyrosine kinase is activated during ligand binding.[1] However, the similarity of the catalytic domains among various tyrosine-kinase receptors raises questions about the mechanism maintaining the identity of the ligand-specific signal.

Insulin and platelet-derived growth factor (PDGF) receptors contain intrinsic tyrosine-kinase activity that is activated upon ligand binding.[1–14] *In vitro,* their catalytic domains are similar, as they recognize tyrosine residues near aspartic acid or glutamic acid residues; however, the biological effects of insulin and PDGF are quite distinct, even in the same cell background.[1,5,6] PDGF, but not insulin, triggers phosphatidylinositol (PI)-4,5-P_2 hydrolysis, generating two intracellular signals, diac-

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ylglycerol and inositol-1,4,5-P_3.[7] Moreover, early work resolved the mitogenic response of BALB/c 3T3 fibroblasts into two phases: competence and progression. Mitogenic competence is induced by a brief exposure to PDGF, and subsequent progression through the cell cycle requires continuous exposure to insulin/IGF-1 or EGF.[8–10] These observations predict that the insulin and PDGF receptor kinases engage distinct downstream elements that together lead to full biologic responses.

The means by which receptor-tyrosine kinases select their targets and thereby stimulate specific intracellular signaling pathways has been clarified by the identification of a conserved domain of approximately 100 amino acids, the Src homology 2 (SH2) domain.[11] The SH2 domain was originally found in Src-related tyrosine kinases[12] and later recognized in a surprisingly broad group of signal transduction proteins, including the phosphatidylinositol 3′-kinase, ras-GAP, and the phospholipase Cγ.[13] SH2 domains mediate protein-protein interactions by binding with high affinity to tyrosine-phosphorylated protein motifs. Although the phosphorylation of a tyrosine residue is the major controlling event or *on switch,* the selection of SH2 proteins seems to depend on the three amino acids carboxyterminal to the phosphotyrosine residue.[14] Indeed, structural analysis of SH2 domain/phosphopeptide complexes reveals the presence of both a phosphotyrosine recognition site and a binding pocket for the adjacent amino acids.[15,16]

In the case of EGF and PDGF receptors, autophosphorylation of specific tyrosine residues in the intracellular domain creates binding sites for certain SH2 proteins.[17,18] Thus, the phosphatidylinositol 3′-kinase, ras-GAP, and PLCγ each bind to different autophosphorylated sites in the PDGF receptor.[14,19–23] A variation of this model is employed by the insulin receptor. During insulin binding, the insulin receptor undergoes autophosphorylation in the intracellular juxtamembrane region[24] and in the regulatory and C-terminal regions.[25] Unlike the PDGF receptor, direct binding of SH2 proteins to the insulin receptor has been difficult to demonstrate. Autophosphorylation of the insulin receptor causes full activation of the tyrosine kinase[25,26] and, together with the juxtamembrane region, mediates the phosphorylation of IRS-1.[27,28] IRS-1 is the principal substrate of the insulin and IGF-1 receptors in most cells and tissues and may represent the first postreceptor event in insulin and IGF-1 signal transmission.[29,30]

IRS-1 contains multiple tyrosine phosphorylation sites, many of which reside in predicted SH2 domain-binding motifs.[14,28] During insulin stimulation, IRS-1 activates the PI 3′-kinase when it associates with the SH2 domains in the 85 kDa regulatory subunit, p85α.[31,32] Thus, IRS-1 serves as a "docking protein," to which SH2 proteins bind, and mediates insulin signaling by binding and regulating signaling molecules following insulin receptor-mediated tyrosine phosphorylation.[30]

This paper describes two levels of selectivity between insulin and PDGF receptor (PDGFr) signaling. The first level occurs when the insulin receptor, but not the PDGFr, phosphorylates IRS-1. The binding of SH2 proteins to IRS-1 during insulin signaling, and not directly to the insulin receptor as in the case of the PDGFr, may account for some of the differences in insulin and PDGF signaling. The second level of selectivity occurs because IRS-1 contains common and unique phosphorylation sites when compared to the autophosphorylation sites in the PDGF receptor. Differences in the biological effects of insulin and PDGF are likely to arise through the engagement of both common and distinct SH2 proteins by the cytosolic IRS-1 and the membrane-bound PDGF receptor.

MATERIAL AND METHODS

Cell Lines and Growth Factors

Mouse NIH/3T3 fibroblasts expressing the human insulin receptor (HIR 3.5) were the generous gift of Dr. Jonathan Whittaker.[33] HIR 3.5 cells were grown in DMEM-high glucose, supplemented with 10% fetal bovine serum. For insulin stimulation, recombinant human insulin (ELANCO) was added to a final concentration of 10^{-7} *M*. Recombinant human PDGF (B/B) was from Boeringer Mannheim or Upstate Biologicals, Inc., and was used at 100 ng/mL to stimulate cells.

Antibodies

αIRS-1 antibodies were protein A-purified polyclonal antibodies from rabbits immunized with recombinant baculovirus-produced IRS-1 protein.[34] Affinity-purified polyclonal antiphosphotyrosine antibodies (αPY) were as described.[35] Immunoprecipitating antibodies specific for the PtdIns 3′-kinase p85 were affinity-purified polyclonal antibodies against amino acids 321-724 of human p85, expressed as a GST fusion protein (αGSTp85)[36] or rabbit antipeptide antisera (Ab51).[36] αGRB-2 antibodies were generated against a GST fusion protein containing amino acids 50-152 of human GRB-2.[37] αPDGFr and αras-GAP antibodies and immunoblotting antibodies against p85 were purchased from Upstate Biotechnology, Inc. αPLCγ antibodies were the generous gifts of Drs. E. Y. Skolnik and J. Schlessinger (Department of Pharmacology, New York University) and Dr. G. Carpenter (Vanderbilt University, Nashville, TN).

Immunoprecipitation

Cells were grown to 80% confluence on 15 cm dishes (Costar) and made quiescent overnight in serum-free media containing 0.5% BSA (Fluka). Cells were stimulated with growth factor for 1 minute before extraction in 100 mM Tris (pH 8.0) containing 100 mM NaF, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/mL aprotinin, and 2 mM NaP_2O_4. Insoluble material was pelleted at 100,000 × *g* for 1 hour, and supernatants were incubated with 50 μL of αPLCγ, 25 μL of αGRB-2, 10 μL of αras-GAP, αPY (3 μg/mL), or αIRS-1 (10 μg/mL) overnight at 4 °C. Immune complexes were collected with Pansorbin Cells (Calbiochem), Protein A-Trisacryl (Pierce), or Protein A-Sepharose 6 MB (Pharmacia) and were washed three times in lysis buffer containing 0.1% SDS. The samples were denatured in Laemmli sample buffer containing 10 mM DTT and resolved by SDS-PAGE (7.5%).

Immunoblotting

Immunoprecipitated proteins were resolved by 7.5% SDS-PAGE on standard apparatuses overnight at 5 mA or in Bio-Rad miniprotean apparatuses at 100 V. Gels were transferred to nitrocellulose membranes (Schleicker & Schuell) for 1.5 hours at 100 V in Towbin buffer containing 0.02% SDS and 20% methanol.[38] Membranes were blocked overnight at 4°C in 25 mM Tris-HCl (pH 7.4), containing 150 mM NaCl and 0.01% Tween-20 (wash buffer), containing 3% bovine serum albumin (Fluka). Membranes were then incubated for two hours at room temperature in wash buffer containing 3% BSA and antibody. Proteins were detected with αPY antibodies (used at a final concentration of 3 μg/mL) or αp85 antibodies (1 : 100 dilution of serum). The membranes were subsequently washed three times in wash buffer, re-blocked for 1 hour at room temperature in wash buffer with 3% BSA, and incubated with [^{125}I]protein A (ICN) (0.2 μCi/mL) for 1 hour in wash buffer containing 3% BSA. Blots were washed 4-5 times in wash buffer, dried, and exposed to autoradiography with Kodak X-AR film or imaged on a Molecular Dynamics Phosphorimager.

In vitro *PtdIns 3′-kinase Assay*

PtdIns 3′-kinase activity associated with immunoprecipitates was determined as previously described.[39] HIR 3.5 cells were made quiescent overnight in media containing 0.5% insulin-free BSA (Fluka). Cells were incubated in the presence or absence of growth factor for 10 minutes and washed once with phosphate-buffered saline, containing 100 μM sodium orthovanadate; and twice with 20 mM Tris pH 7.5, containing 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 100 μM sodium orthovanadate (A buffer). Cells were then solubilized in buffer A, containing 1% NP-40, 10% glycerol, and 1 mM PMSF. Insoluble material was removed by centrifugation at 10,000 × *g*, and supernatants were immunoprecipitated overnight. Precipitated material was collected on protein A sepharose beads (Pharmacia) and washed consecutively with PBS, containing 1% NP-40 and 100 μM Na_3VO_4 (three times); 100 mM Tris-HCl (pH 7.5),containing 500 mM LiCl and 100 μM sodium orthovanadate (three times); and 10 mM Tris-HCl (pH 7.5), containing 100 mM NaCl, 100 μM sodium orthovanadate, and 1 mM EDTA (twice). Some experiments were performed using 2 mM Na_3VO_4 in all wash and lysis buffers, with similar results. Pellets were resuspended in 50 μL of the final wash buffer. To each pellet was added 10 μL 100 mM $MgCl_2$ and 10 μL of a sonicated dispersion of PtdIns (2 μg/μL) (Avanti) in 10 mM Tris-HCl, pH 7.5, with 1 mM EGTA. Reactions were started by the addition of 5 μL of 880 mM ATP containing 30 μCi [^{32}P]ATP (NEN-DuPont) and 20 mM $MgCl_2$. After 10 minutes at room temperature, the reaction was stopped by the addition of 20 μL 8 N HCl and 160 μL $CHCl_3$: MeOH (1 : 1). The samples were centrifuged, and the lower organic phase was spotted on a silica gel TLC plate (Merck) that had been treated with 1% potassium oxalate. TLC plates were developed in $CHCl_3$: MeOH : H_2O : NH_4OH (60 : 47 : 11.3 : 2), dried, and visualized by autoradiography.

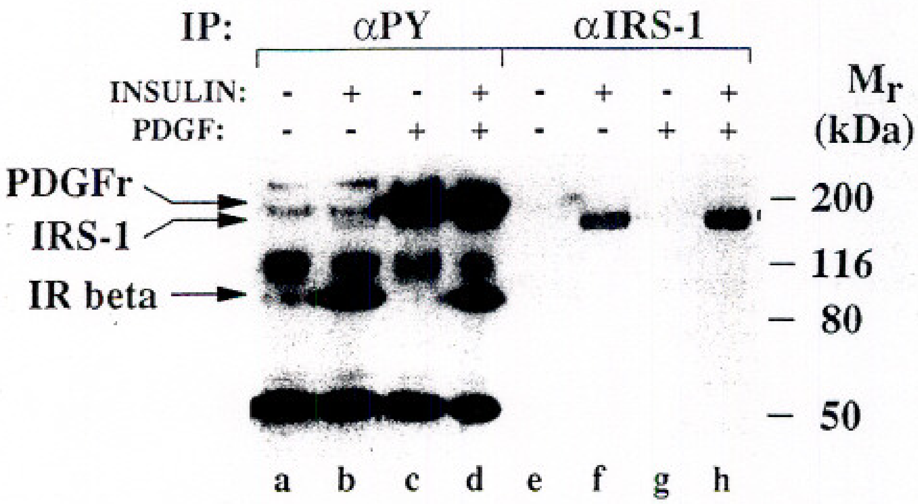

FIGURE 1. Treatment of HIR 3.5 cells with insulin, but not PDGF, stimulates the tyrosyl phosphorylation of IRS-1. Quiescent HIR 3.5 cells (lanes a and e) or HIR 3.5 cells stimulated with insulin (lanes b and f), PDGF (lanes c and g), or both (lanes d and h) were lysed and immunoprecipitated with αPY (lanes a-d) or αIRS-1 (lanes e-h) antibodies. Immunoprecipitates were collected, washed, resolved on SDS-PAGE, and transferred to nitrocellulose membranes. Tyrosyl phosphorylated proteins were detected by immunoblotting with αPY. Migration of molecular weight standards and the positions of the phosphorylated IR, IRS-1, and PDGFr are denoted.

RESULTS

Tyrosine Phosphorylation of IRS-1 in HIR 3.5 Cells during Insulin and PDGF Stimulation

We assessed insulin- and PDGF-stimulated tyrosine phosphorylation of cellular proteins in mouse 3T3 fibroblasts that express high levels of endogenous PDGFr and similar levels of stably transfected human insulin receptor (HIR 3.5 cells). Antiphosphotyrosine (αPY) immunoblots of proteins precipitated with αPY confirmed that insulin and PDGF stimulated tyrosine phosphorylation of the cognate receptor and demonstrated that similar amounts of each tyrosine phosphorylated receptor were present in HIR 3.5 cells (FIG. 1, lanes a-c). The effects of insulin and PDGF were independent, inasmuch as simultaneous stimulation with insulin and PDGF produced a composite result (FIG. 1, lanes a and d). Furthermore, a tyrosine-phosphorylated band at 175 kDa, just below the PDGFr, occurred during insulin stimulation alone or in combination with PDGF; the 175 kDa protein was not detected after PDGF stimulation alone. This protein is most likely IRS-1.[28] We used αIRS-1 antibodies to directly observe IRS-1 tyrosine phosphorylation during insulin and PDGF stimulation. An αPY immunoblot revealed that insulin, but not PDGF, stimulated IRS-1 tyrosine phosphorylation (FIG. 1, lanes e-h). These results demonstrate that IRS-1 is a substrate for the insulin receptor but not for the PDGFr in HIR 3.5 cells.

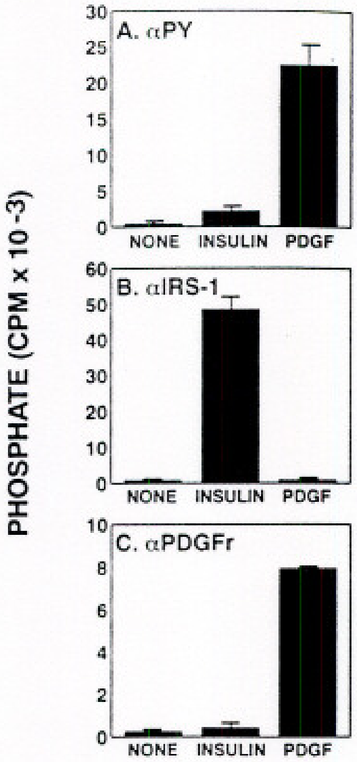

FIGURE 2. Association of PtdIns 3′-kinase with αPY, αIRS-1, and αPDGFr immunoprecipitates following growth factor stimulation of HIR 3.5 cells. Quiescent HIR 3.5 cells of HIR 3.5 cells stimulated with insulin or PDGF were lysed and immunoprecipitated with αPY(A), αIRS-1(B), and αPDGFr(C) antibodies. Immunoprecipitates were collected, washed, and assayed for associated PtdIns 3′-kinase. Results are expressed in cpm phosphate incorporated into PtdIns substrate during a 10-minute assay, +/−SEM. All assays represent triplicate measurements.

Association of PI 3′-Kinase with IRS-1

PI 3′-kinase binds to the tyrosine-phosphorylated YMXM motifs in various proteins, including the PDGF receptor and IRS-1.[14,28] We measured the amount of PI 3′-kinase activity detectable in αPY, αPDGFr, or αIRS-1 immunoprecipitates from HIR 3.5 cells treated with insulin or PDGF. Following insulin stimulation, PI 3′-kinase was detected in αPY and αIRS-1 immunoprecipitates (FIG. 2, A and B); however, much more PI 3′-kinase activity was found in αIRS-1 immunoprecipitates than in αPY immunoprecipitates, probably due to the greater efficiency with which αIRS-1 precipitates IRS-1 (compare levels of IRS-1 in αPY and αIRS-1 immunoprecipitates in FIG. 1). By contrast, no PI 3′-kinase activity was detected in αPDGFr immunoprecipitates following insulin stimulation (FIG. 2C).

Following PDGF stimulation, PI 3′-kinase activity associated strongly with αPY and αPDGFr immunoprecipitates (FIG. 2, A and C). This activity reflects the association of PI 3′-kinase with the PDGFr and is higher in αPY immunoprecipitates due to the greater efficiency of the αPY antibody than the αPDGFr antibody for immunoprecipitating the activated PDGFr (data not shown). During PDGF stimulation, there was no increase in PI 3′-kinase activity associated with IRS-1 (FIG. 2B), confirming that IRS-1 does not play a role in PDGF signaling to this enzyme. Because the tyrosine phosphorylation of proteins is known to be crucial for their association with PI 3′-kinase,[13,40–44] the absence of PI 3′-kinase in this case is entirely consistent with the lack of PDGF-stimulated tyrosine phosphorylation of IRS-1.

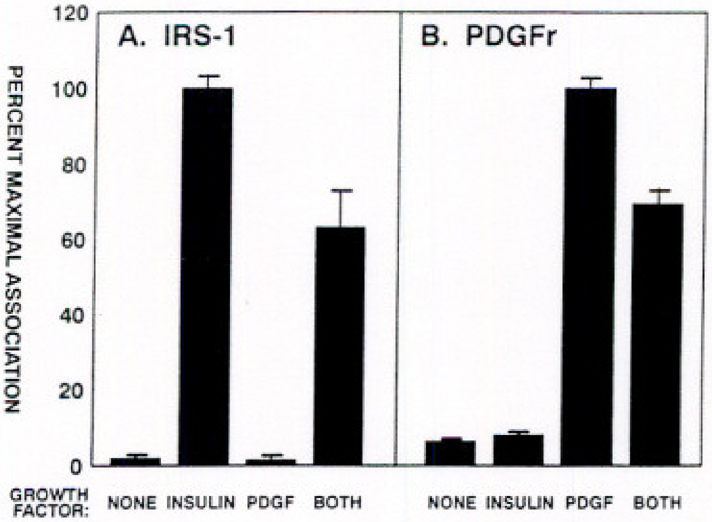

FIGURE 3. Simultaneous stimulation of HIR 3.5 cells with insulin and PDGF decreases the amount of PtdIns 3′-kinase activity associated with IRS-1(A) or the PDGFr(B), compared to insulin or PDGF alone. Quiescent HIR 3.5 cells or HIR 3.5 cells stimulated with insulin, PDGF, or both were lysed and immunoprecipitated with αIRS-1 or αPDGFr antibodies. PtdIns 3′-kinase activity associated with washed immunoprecipitates was determined and normalized to the maximum activity (100%) associated with each antibody (*e.g.*, insulin alone represents the maximum for αIRS-1 immunoprecipitates). Determinations were carried out in triplicate and are expressed ±SEM.

During stimulation of HIR 3.5 cells with both insulin and PDGF, the PDGFr and IRS-1 are available to associate with the PI 3′-kinase. The amount of PI 3′-kinase activity associated with the PDGFr was approximately 33% lower during insulin- and PDGF-stimulation than following stimulation with PDGF alone (FIG. 3B). Similarly, the amount of PI 3′-kinase activity associated with IRS-1 was also about 33% lower following double stimulation than following insulin stimulation alone (FIG. 3A). These results suggest that IRS-1 and the PDGFr bind the same intracellular pool of PI 3′-kinase and that following double stimulation they compete for this pool.

In order to determine the nature of proteins associated with the 85 kDa subunit (p85) of PI 3′-kinase following stimulation with insulin, PDGF, or both, we prepared immunoprecipitates with antibodies against p85, washed them as for a PI 3′-kinase assay, and analyzed the associated proteins by SDS-PAGE and immunoblotting with αPY (FIG. 4). After PDGF stimulation, the PDGFr was the major phosphotyrosine-containing protein associated with PI 3′-kinase (FIG. 4, lanes e and f). After insulin stimulation, IRS-1 was the major phosphoprotein associated with p85; however, a small amount of insulin receptor β subunit and two unidentified 120 and 140 kDa proteins were also detected (FIG. 4, lanes c and d). During simultaneous insulin and PDGF stimulation, both the PDGFr and IRS-1 were associated with p85. However, the amounts of IRS-1 and PDGFr associated with p85 was reduced compared to stimulation with insulin or PDGF alone (FIG. 4, lanes g and h). These results confirm that the PDGFr and IRS-1 compete for a common pool of p85 during growth-factor stimulation.

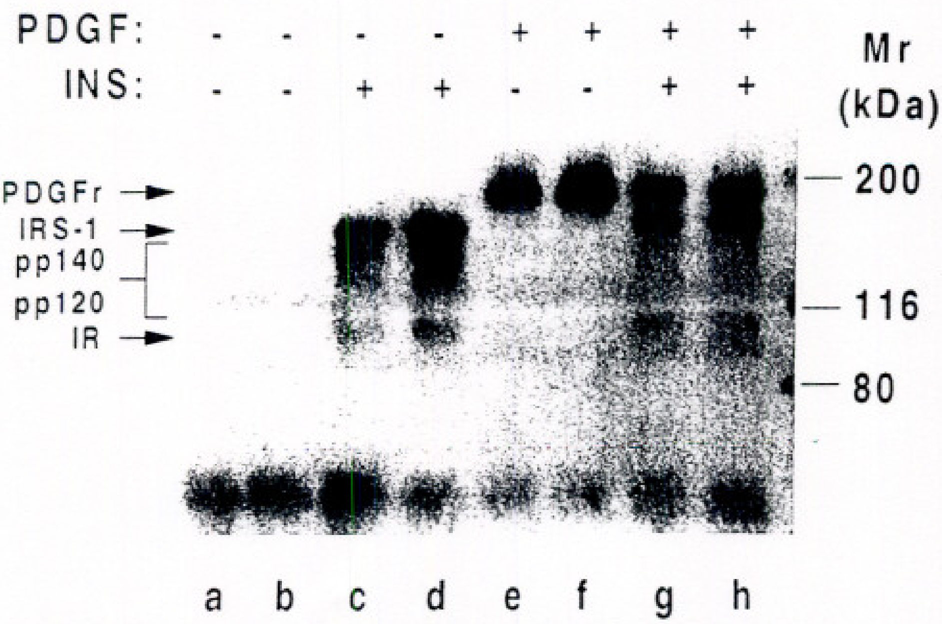

FIGURE 4. Tyrosyl phosphoproteins associated with PtdIns 3′-kinase following growth factor stimulation of HIR 3.5 cells. Quiescent HIR 3.5 cells (lanes a and b) or HIR 3.5 cells stimulated with insulin (lanes c and d), PDGF (lanes e and f), or both (lanes g and h) were lysed and immunoprecipitated with αp85 antibodies: Ab51 (lanes a, c, e, and g) or αGSTp85 (lanes b, d, f, and h). Immunoprecipitates were washed as for a PtdIns 3′-kinase assay, resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with αPY. Migration of molecular weight markers and the positions of tyrosine phosphorylated IRS-1 and the PDGFr are shown.

Activation of PI 3′-kinase by Insulin and PDGF

The stimulation of cells with growth factors, including insulin and PDGF, increases the cellular levels of $PI(3,4)P_2$ and $PI(3,4,5)P_3$, which is attributed to the stimulation of PI 3′-kinase.[39,40,45] Consistent with this result, insulin stimulation of PI 3′-kinase is observed in p85 immunoprecipitates. This activation appears to occur by the binding of tyrosine phosphorylated IRS-1 to the SH2 domains of its regulatory subunit p85.[31,32] Oddly, analogous assays have been unable to detect such activation following stimulation with PDGF.[36] In order to compare the activation of PI 3′-kinase with IRS-1 and the PDGFr, we assayed the activity of PI 3′-kinase in immunoprecipitates from HIR 3.5 cells using two antibodies (Ab51 and αGSTp85) directed against the p85 subunit of PI 3′-kinase. PI 3′-kinase activity in Ab51 immunoprecipitates from HIR 3.5 cells was stimulated approximately 4-fold following insulin stimulation and 2.4-fold by PDGF (100 nM) (FIG. 5A). However, using αGSTp85, only a 2.4-fold activation of PI 3′-kinase was detected with insulin, and no stimulation was observed with PDGF (FIG. 5B). Parallel immunoprecipitates immunoblotted with αp85 showed similar amounts of p85 present following stimulation with insulin, PDGF, or both (data not shown). Thus the ability to detect stimulated PI 3′-kinase activity depends both on the antibody used and the intrinsic strength of the activating signal.

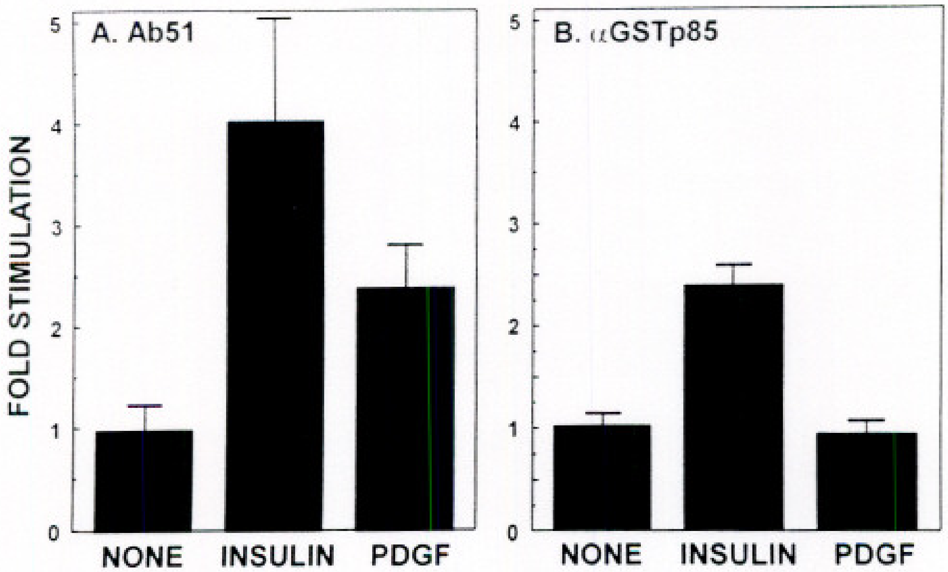

FIGURE 5. Insulin and PDGF stimulation increases the activity of PtdIns 3′-kinase in αp85 immunoprecipitates. αp85 immunoprecipitates were prepared from lysates of unstimulated (control) HIR 3.5 cells, or HIR 3.5 cells stimulated with insulin or PDGF using two different αp85 antibodies, Ab51(A), and αGSTp85(B). Immunoprecipitates were washed, and associated PtdIns 3′-kinase activity was assayed. Activity is plotted as fold stimulation over basal ±SEM. Data represents the aggregate of four experiments.

The ability of the PDGF receptor and IRS-1 to activate PI 3′-kinase was directly compared in immunoprecipitates. PI 3′-kinase activity in αIRS-1 and αPDGFr immunoprecipitates was measured and normalized against the amount of p85 found in the immunoprecipitates by immunoblotting to account for the relative efficiency of the immunoprecipitations (FIG. 6). The specific activity of the IRS-1- and PDGFr-associated PI 3′-kinase was estimated from the ratio of PI 3′-kinase activity to p85 protein. Using this method, PI 3′-kinase associated with IRS-1 was approximately threefold more active than that associated with the PDGFr (FIG. 6).

Formation of Multimolecular Complexes by IRS-1 and the PDGFr

PDGF-stimulated autophosphorylation of the PDGFr causes the formation of a multimolecular complex containing PI 3′-kinase, ras-GAP, GRB-2, PLCγ, c-fyn, and likely other SH2 proteins.[17,19,21,22,46] In order to assess whether IRS-1 acts similarly following insulin receptor-dependent tyrosine phosphorylation, we assessed the binding of ras-GAP, GRB-2, and PLCγ to IRS-1.

Insulin and PDGF stimulated the association-tyrosyl phosphoproteins in immunoprecipitates prepared with antibodies against ras-GAP, GRB-2, and PLCγ (FIG. 7). Following stimulation with PDGF, a 185-200 kDa band, corresponding to the PDGFr, was observed in αras-GAP, αGRB-2, and αPLCγ immunoprecipitates (FIG. 7). A

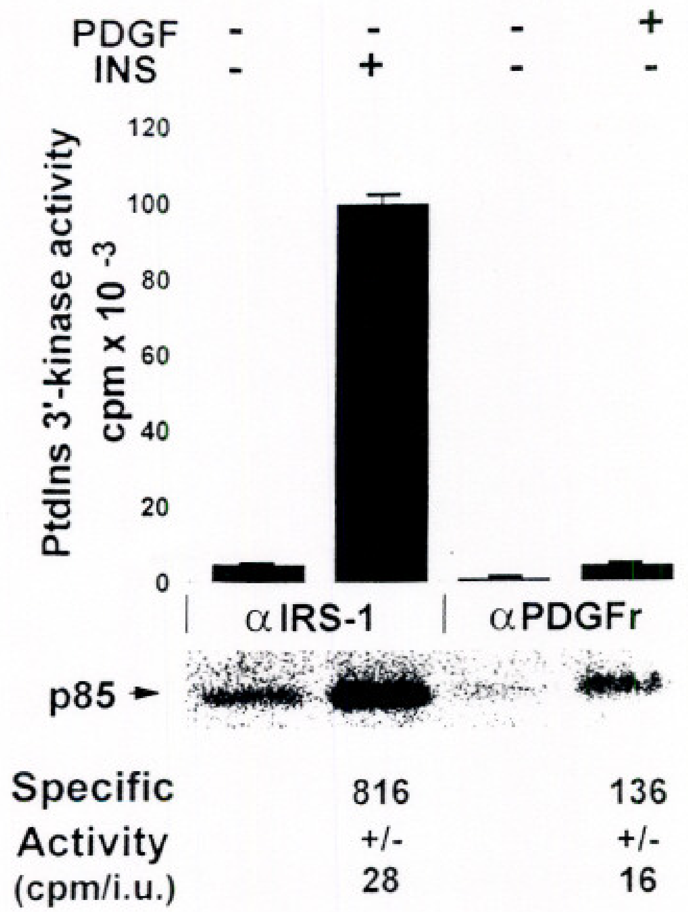

FIGURE 6. PtdIns 3′-kinase associated with IRS-1 is more highly activated than that associated with the PDGFr. αIRS-1 and αPDGFr immunoprecipitates were prepared from unstimulated, insulin-stimulated, or PDGF-stimulated HIR 3.5 cells. Immunoprecipitates were washed and assayed for associated PtdIns 3′-kinase activity or resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with αp85 antibodies. Specific activity of PtdIns 3′-kinase associated with IRS-1 and the PDGFr was determined by calculating the ratio of associated PtdIns 3′-kinase activity (cpm) to associated p85 (phosphorimager units (I.U.)). One example of such an experiment is represented. The specific activity represents the aggregate result of two such experiments.

140 kDa band, corresponding to the tyrosine-phosphorylated PLCγ, was observed in αPLCγ immunoprecipitates from PDGF-stimulated cells (Fig. 7). Following stimulation with insulin, a tyrosyl phosphoprotein migrating at 180 kDa was associated with αGRB-2 immunoprecipitates, confirming that GRB-2 also associates with IRS-1.[37,47] However, no novel tyrosyl phosphoproteins were observed in either αras-GAP or αPLCγ immunoprecipitates prepared from insulin-stimulated cells, suggesting that neither ras-GAP nor PLCγ associate with IRS-1 following insulin stimulation and that PLCγ is not tyrosine phosphorylated following insulin stimulation (Fig. 7). Thus,

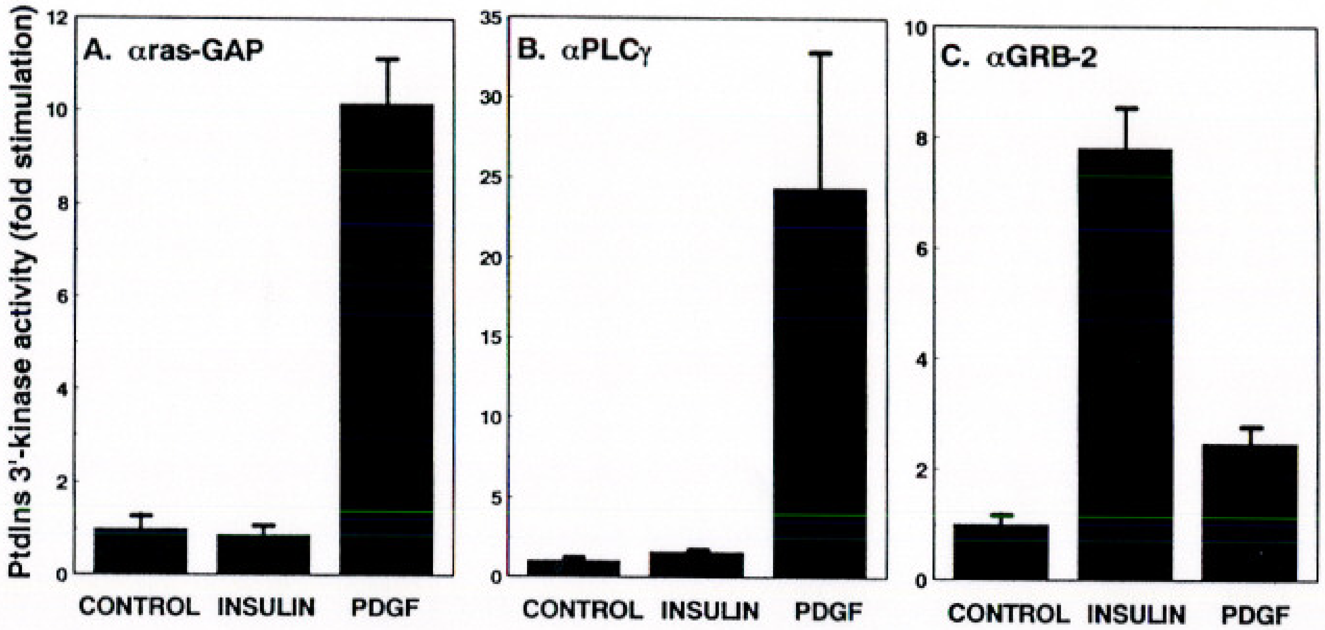

FIGURE 7. Tyrosyl phosphoproteins associated with PLCγ, GRB-2, and ras-GAP in growth-factor stimulated HIR 3.5 cells. Quiescent HIR 3.5 cells (A) or HIR 3.5 cells stimulated with insulin (B) or PDGF (C) were lysed and incubated with αras-GAP (A), αPLCγ (B), or αGRB-2 (C) antibodies. Immunoprecipitates were washed, resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with αPY antibodies. Migration of molecular weight markers and the positions of tyrosine-phosphorylated PDGFr, IRS-1, insulin receptor β subunit, and PLCγ are indicated.

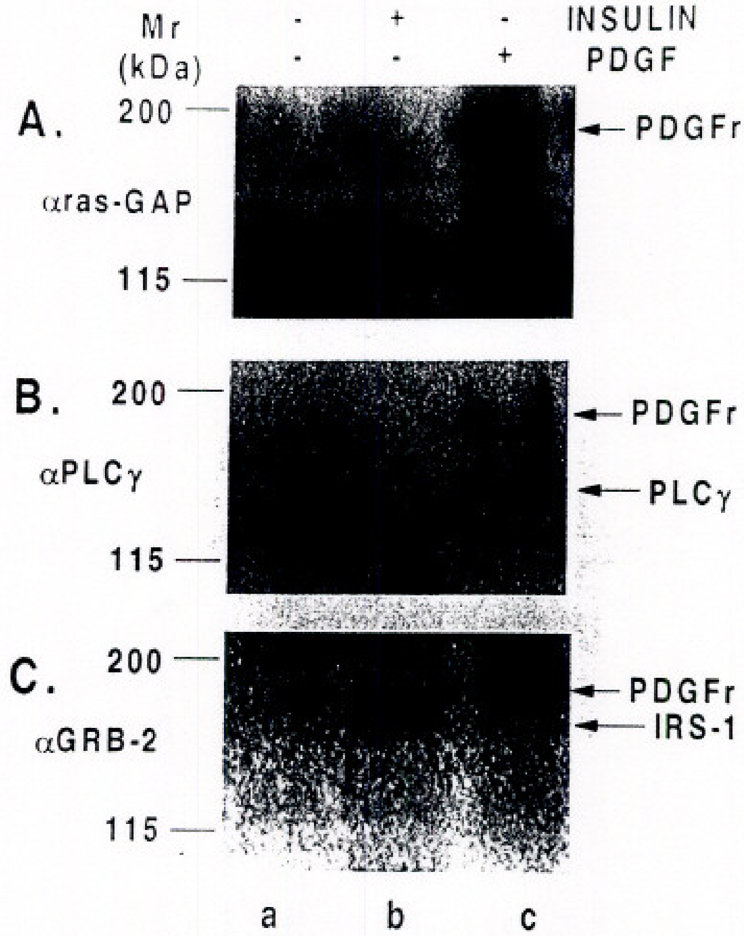

FIGURE 8. PtdIns 3′-kinase activity associated with αras-GAP(A), αPLCγ(B), and αGRB-2(C), immunoprecipitates from growth factor-stimulated HIR 3.5 cells. Quiescent HIR 3.5 cells or HIR 3.5 cells stimulated with insulin or PDGF were lysed and incubated with αras-GAP, αPLCγ, or αGRB-2 antibodies. Immunoprecipitates were washed and assayed for associated PtdIns 3′-kinase activity. Activity is plotted as fold stimulation over basal ± SEM and represents the average of duplicate or triplicate determinations.

IRS-1 and the PDGFr associate with overlapping yet distinct pools of SH2 domain-containing signaling elements.

In order to investigate whether a single tyrosine phosphorylated PDGFr or IRS-1 molecule formed multimolecular complexes containing PI 3′-kinase, GRB-2, ras-GAP, and PLCγ, we measured PI 3′-kinase activity in αras-GAP, αGRB-2, and αPLCγ immunoprecipitates from HIR 3.5 cells following stimulation with insulin or PDGF (Fig. 8). PI 3′-kinase associated with αras-GAP and αPLCγ immunoprecipitates increased 10- and 23-fold, respectively, following PDGF stimulation, whereas the associated PI 3′-kinase remained unchanged following stimulation with insulin

(FIG. 8). However, in αGRB-2 immunoprecipitates there were 8- and 3-fold increases in the amount of associated PI 3′-kinase activity following insulin and PDGF stimulation, respectively. Thus, the PDGFr forms a multimolecular complex containing PI 3′-kinase, ras-GAP, GRB-2, and PLCγ, whereas IRS-1 associates with GRB-2 and PI 3′-kinase. This undoubtedly contributes to the unique nature of the insulin and PDGF signals observed in 3T3 fibroblasts.

DISCUSSION

Insulin and platelet-derived growth factor employ a common theme for signaling transmission that involves ligand-induced phosphotyrosine binding motifs that associate specifically with proteins containing SH2 domains. In the case of insulin signaling, the SH2 proteins are recruited to tyrosine-phosphorylated IRS-1, whereas during PDGF stimulation the SH2 proteins associate directly with autophosphorylation sites in the PDGF receptor (FIG. 9).[19,36,43,48] In a few cases, insulin and PDGF engage the same SH2 proteins, as shown here for PtdIns 3′-kinase and GRB-2. In other instances, unique elements are selected, as illustrated by the association of PLCγ and ras-GAP with the PDGF receptor but not IRS-1. The unique response of cells to insulin or PDGF, as proposed earlier, is likely to result partly from the cohort of SH2 proteins that are engaged during ligand stimulation. At the molecular level, this selectivity is now understood by the fact that IRS-1 and the PDGF receptor contain common and distinct tyrosine-phosphorylated motifs that are predicted to associate with various SH2 proteins.[14,19,28,37,43,49]

IRS-1 is not tyrosine phosphorylated by the PDGF receptor in HIR 3.5 cells. Thus, IRS-1 is selected for phosphorylation by a subset of tyrosine kinases, including the receptors for insulin, IGF-1, and IL-4.[28,29,50–52] The purified insulin receptor phosphorylates recombinant IRS-1 protein *in vitro*, but the purified PDGF receptor does not, even though it phosphorylates similar motifs in its kinase insert domain (M. G. Myers Jr., M. F. White *et al*, unpublished observations). Thus, a unique domain in the insulin receptor mediates the specific recognition of IRS-1, whereas this element must be absent in the PDGF receptor. Mutational analysis of the insulin and IL-4 receptors suggests that the receptor side of this recognition may be mediated by a tyrosine-containing NPXY motif found in these receptors.[27,53] Recent analysis of IRS-1 structure suggests that one or two regions in the N-terminus (*e.g.*, the pleckstrin homology domain) of IRS-1 may form the cognate for the interaction with these receptors (M. G. Myers Jr., M. F. White, *et al*, unpublished data).

The utilization of IRS-1 as a principal docking unit for the SH2 proteins in the insulin receptor pathway may explain some of the differences between the insulin and PDGF signals. Although the PDGF receptor is an integral membrane protein, IRS-1 is largely cytosolic: This may target associated proteins in a different manner than the PDGF receptor does or than the insulin receptor would if it directly bound SH2 proteins. Indeed, IRS-1 confers additional flexibility to the insulin signal, as both membrane-bound and soluble forms are found in insulin-stimulated cells;[47,54] a small but detectable fraction of the PtdIns 3′-kinase is bound to the insulin receptor in Chinese hamster ovary cells, and this complex is found in intracellular membrane fractions in rat adipocytes.[47,54] Moreover, the direct association with the receptor

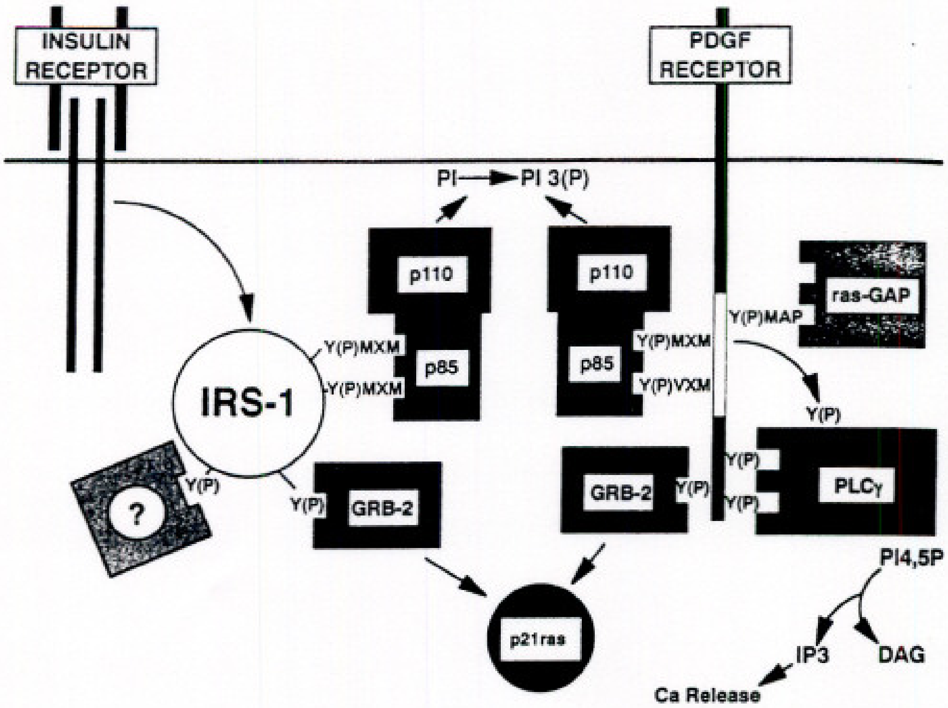

FIGURE 9. A model of the signaling pathways used by the receptors for insulin and PDGF. Stimulation of the insulin and PDGF receptors causes activation of the receptor-tyrosine kinase and receptor autophosphorylation. However, in the case of the insulin receptor, the activated insulin receptor-tyrosine kinase also phosphorylates IRS-1. The tyrosine-phosphorylated PDGFr and IRS-1 then act to nucleate binding of different but overlapping sets of SH2 domain-containing proteins. IRS-1 binds both GRB-2 and PtdIns 3′-kinase, whereas the PDGFr associates with GRB-2, PtdIns 3′-kinase, ras-GAP, and PLCγ. The association of PtdIns 3′-kinase with IRS-1 is more activating than its association with the PDGFr. Thus the insulin-signaling pathway differs from that of PDGF by the insertion of a nonreceptor "docking protein" (IRS-1), in the signaling pathways it uses, and in the way one common element (PtdIns 3′-kinase) is regulated.

tyrosine kinase adds the additional possibility of regulation of SH2 proteins by tyrosine phosphorylation, as demonstrated for PDGF receptor-bound PLCγ. As IRS-1 has no demonstrable catalytic activity,[28] it cannot exercise this form of regulation on bound proteins without the specific association of an auxiliary kinase.

The association of PtdIns 3′-kinase with tyrosyl phosphorylated proteins, such as IRS-1 and the PDGFr, is mediated by the binding of SH2 domains in the 85 kDa subunit of PtdIns 3′-kinase.[31,32,36,55] Ptd Ins 3′-kinase does not associate with IRS-1 following PDGF stimulation because IRS-1 is not tyrosine phosphorylated by the PDGF receptor in the intact cell; however, the pDGFr and IRS-1 compete for a common pool of enzyme when both molecules are tyrosine phosphorylated. The predominant proteins associated with PtdIns 3′-kinase following insulin stimulation are IRS-1 and a small amount of the insulin receptor;[31,47] IRS-1, PtdIns 3′-kinase, and the insulin receptor form a ternary complex in which the insulin receptor is bound to IRS-1, which mediates binding to PtdIns 3′-kinase.[47] Two other tyrosyl-phosphorylated protein bands observed in association with PtdIns 3′-kinase following insulin stimulation[29,31] represent degradation products of IRS-1 (M. G. Myers Jr. & M. F. White, unpublished observations). Following PDGF stimulation, the PDGF receptor represents the only αPY-detectable PtdIns 3′-kinase-associated tyrosyl phosphoprotein.

PtdIns 3′-kinase is activated by insulin[31,32] and PDGF treatment of cells. Initial reports indicated that p85 becomes tyrosine phosphorylated following stimulation of cells with PDGF and that this may regulate the activity of PtdIns 3′-kinase.[56–59] p85 undergoes a conformational change following the binding of tyrosine-phosphorylated YMXM motifs;[16,60,61] this conformational change is one possible mechanism by which the activating signal may be transmitted from p85 to the 110 kDa catalytic subunit.

Others have suggested that p85 becomes tyrosine phosphorylated following stimulation of cells with PDGF and that this may regulate the activity of PtdIns 3′-kinase.[41,57,59,62] We and others, however, have previously been unable to detect the tyrosine phosphorylation of p85 following stimulation with a variety of growth factors, including insulin, EGF, and PDGF.[31,36] Furthermore, in this report, we are unable to detect the phosphorylation of p85 following PDGF or insulin stimulation, although the tyrosine phosphorylation of PLC is readily observed following stimulation with PDGF (FIG. 7). Thus, phosphorylation of p85 does not represent a functional difference between the regulation of PtdIns 3′-kinase by IRS-1 and the PDGFr.

Insulin, through IRS-1, stimulates the activity of PtdIns 3′-kinase more effectively than the PDGF receptor. At the molecular level, this may be due to the presence of a 6 YMXM motif in IRS-1 compared to one YMXM and a related YVXM motif in the PDGF receptor. Moreover, autophosphorylation studies with the PDGF receptor suggest that only one of these sites are phosphorylated in a given receptor molecule,[20] whereas multiple tyrosines are phosphorylated in IRS-1, including several YMXM motifs.[63] PtdIns 3′-kinase may be fully activated only when both of its SH2 domains are occupied: Activation of PtdIns 3′-kinase by monophosphopeptides *in vitro* is biphasic and only occurs at high peptide concentrations, but activation by phosphorylated IRS-1 (which contains multiple tyrosyl phosphorylated YMXM motifs) or phosphopeptides containing two tyrosine phosphorylated YMXM motifs is monophasic and orders of magnitude more sensitive (ref. 31 and J. M. Backer *et al.*, unpublished data).

The consequences of PtdIns 3′-kinase activation are unclear. In yeast, VPS34 (the apparent homologue of the PtdIns 3′-kinase catalytic subunit) is essential for the efficient sorting of vacuolar hydrolases.[64,65] PtdIns 3′-kinase may control similar functions in mammalian cells during tyrosine-kinase signaling. In this case, the PDGF and insulin effect could be different, as the PDGF receptor and IRS-1 could target PtdIns 3′-kinase to alternate cellular compartments.[54,66] Moreover, the higher activation of PtdIns 3′-kinase by insulin may result in different signals than PDGF.

The PDGFr binds multiple SH2 proteins, including PtdIns 3′-kinase, ras-GAP, GRB-2, and PLCγ; and PDGF stimulation increases the amount of PtdIns 3′-kinase activity associated with αras-GAP, αGRB-2, and αPLCγ immunoprecipitates, suggesting that the PDGF receptor recruits SH2 proteins into a large signaling complex during PDGF stimulation. IRS-1 does not bind ras-GAP or PLCγ, but recruits PtdIns 3′-kinase and GRB-2 into a signaling complex. The lack of interaction between ras-GAP and PLCγ with IRS-1 is not surprising inasmuch as none of the potential tyrosine phosphorylation sites on IRS-1 contain the surrounding amino acid sequences thought to be necessary for recognition by the SH2 domains of ras-GAP and PLCγ.[14,19,22,28] PLCγ is tyrosine phosphorylated following PDGF stimulation, and PDGF increases turnover of phosphatidylinositol secondary to the activation of PLCγ,[7] whereas insulin does not stimulate PLCγ; this is likely because IRS-1 does not bind PLCγ or activate it by tyrosine phosphorylation.

The SH2 domains from ras-GAP, PLCγ, GRB-2, and p85 have been used as affinity reagents to determine whether these proteins associate with certain cellular phosphoproteins.[32,37,67–69] Although no binding of IRS-1 to the ras-GAP SH2 domains has been demonstrable, the binding of IRS-1 to the SH2 domains of GRB-2, p85, and PLCγ has been reported *in vitro*.[32,37,67,68] In light of the data presented here, the binding of IRS-1 to PLCγ SH2 domains likely represents an *in vitro* artifact. Cautious evaluation of the binding of phosphoproteins to large amounts of SH2 domain fusion proteins *in vitro* is necessary, as SH2 domains display a moderate affinity for phosphotyrosine alone,[70] and, under *in vitro* conditions, interactions that may not occur *in vivo* may be observed.

PtdIns 3′-kinase and GRB-2 are common elements in the PDGF and insulin-signaling cascades, whereas ras-GAP and PLCγ are restricted to the PDGF signal. This likely accounts for some of the differences between the insulin and PDGF signals. Furthermore, among the common elements (PtdIns 3′-kinase and GRB-2), engagement by IRS-1 and the PDGF receptor appears to differ. It also seems likely that as more SH2 domain-containing proteins become known and are investigated fully, more molecules that associate with IRS-1 and the PDGF receptor will be discovered. The elucidation of which of these new elements is unique to the IRS-1 and PDGF receptor systems will more fully explain the differences between the cellular responses to PDGF and insulin.

SUMMARY

The receptors for insulin and PDGF are tyrosine kinases that mediate distinct effects in identical cellular backgrounds. Each receptor must therefore engage a unique subset of the available signaling elements—at least partly through the selection of proteins with src-homology 2 domains (SH2 proteins). Autophosphorylation sites in the PDGFr directly bind SH2 proteins, whereas activation of the insulin receptor leads to phosphorylation of IRS-1, which in turn binds SH2 proteins. In HIR 3.5 cells, which contain similar numbers of PDGF and insulin receptors, insulin, but not PDGF, stimulated tyrosyl phosphorylation of IRS-1. Similarly, insulin, but not PDGF, treatment of HIR 3.5 stimulated the association of IRS-1 with PtdIns 3′-kinase, although PDGF stimulated the association of PtdIns 3′-kinase with the tyrosine-phosphorylated PDGFr. Association with IRS-1 activated PtdIns 3′-kinase more effectively than association with the PDGFr. Whereas the PDGFr associated with PtdIns 3′-kinase, ras-GAP, GRB-2, and phospholipase Cγ, only GRB-2 and PtdIns 3′-kinase associated with IRS-1. Moreover, PDGF, but not insulin, caused tyrosine

phosphorylation of phospholipase Cγ in HIR 3.5 cells. Thus, the insulin signal differs from that of PDGF by the insertion of a cytosolic, nonreceptor SH2 domain docking protein (IRS-1). Furthermore, IRS-1 binds a different subset of SH2 domain-containing proteins than does the PDGFr and regulates at least one common element (PtdIns 3′-kinase) differently than the PDGFr. These results support the hypothesis that IRS-1 differentiates the signals generated by the insulin receptor and PDGFr tyrosine kinases by binding and regulating a specific subset of SH2 domain-containing signaling molecules.

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