Adapter Function of Protein-tyrosine Phosphatase 1D in Insulin Receptor/Insulin Receptor Substrate-1 Interaction*  

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Insulin signal transduction involves the multisite docking protein insulin receptor substrate-1 (IRS-1) and a number of Src homology-2 (SH2) domain factors, including p85/p110 phosphatidylinositol 3-kinase, p110 GTPase-activating protein, and the phosphotyrosine-specific phosphatase PTP1D. In transfected baby hamster kidney cells, Rat1 fibroblasts, and normal IM9 lymphoblasts, PTP1D directly binds activated insulin receptor. This interaction is mediated by catalytic domain-proximal SH2 determinants of the phosphatase and phosphotyrosine 1146 of the activated insulin receptor. While the receptor and the phosphatase do not serve as substrates for each other, their interaction promotes IRS-1 binding to the receptor, indicating that PTP1D functions as an adapter for insulin receptor and IRS-1. The formation of a multiprotein signaling complex involving the insulin receptor, PTP1D, and IRS-1 enhances cellular glucose uptake, a critical process in the physiological action of insulin.

Protein-tyrosine phosphatase 1D (PTP1D), also known as SH-PTP2 (1), SH-PTP3 (2), PTP2C (3), and Syp (4), is a cytosolic enzyme with two Src homology 2 (SH2) domains that is expressed in a wide variety of cell types (1, 3). Upon stimulation of cells with different growth factors, PTP1D becomes tyrosine-phosphorylated and associates with activated receptor-type or cytoplasmic tyrosine kinases via its SH2 domains (4–6). Interaction with the platelet-derived growth factor receptor (PDGFR) kinase results in activation of PTP1D phosphatase activity (5).

In addition to directly interacting with activated protein-tyrosine kinases, PTP1D also binds to insulin receptor substrate-1 (IRS-1) (7), the major substrate of the insulin receptor (IR) tyrosine kinase, which is thought to serve as a “docking protein” required for the recruitment of different SH2 domain-containing proteins to the IR signaling pathway. Previous reports indicate that insulin stimulation of the IR causes IRS-1 association with the 85-kDa noncatalytic subunit of phosphatidylinositol 3-kinase and the adapter proteins Grb2 and Nck (reviewed in Ref. 8). The physiological significance of the interaction of IRS-1 with these SH2 domain proteins, however, remains unclear. Recent studies involving catalytically inactive PTP1D have demonstrated that rather than playing a negative regulatory role, PTP1D is required for the transduction of signals triggered by activation of the PDGFR (9, 10) and the IR (11–13). Upon PDGFR activation, PTP1D becomes phosphorylated on tyrosines and appears to serve as an adapter, linking PDGFR to the Grb2-Sos complex (9). In contrast to the PDGFR pathway, IR activation does not result in PTP1D phosphorylation on tyrosine residues (7), indicating that the positive effect of corresponding PTP1D in IRS signaling is mediated by another mechanism.

In the present study, we demonstrate direct interaction between activated IR and PTP1D, primarily involving the SH2 domain proximal to the region specifying phosphatase activity. Phosphorylation of Tyr-1146 in the IR promotes binding of IRS-1 and formation of a complex that enhances insulin-dependent glucose uptake. Our data suggest a positive role for PTP1D in insulin action through its critical involvement in the formation of a multiprotein signaling complex.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Protein A-Sepharose, wheat germ agglutinin (WGA)-Sepharose, and glutathione-Sepharose were purchased from Pharmacia Biotech Inc. All other reagents were obtained from Sigma. Antibodies used were affinity-purified rabbit polyclonal anti-PTP1D antibodies (5), mouse monoclonal anti-phosphotyrosine antibody pY20 (14), mouse monoclonal anti-IR 83-14 antibody (15), and rabbit polyclonal anti-IRS-1 antibody raised against a C-terminal IRS-1 peptide, STYASIDFKQPEQDRQ (provided by R. Lammers). As secondary antibodies, goat anti-mouse or anti-rabbit conjugates (Bio-Rad) were used. For immunoblot detection, the ECL system was utilized. Stripping and reprobing of blots was performed according to the manufacturers’ recommendations.

Cell Culture, Transient Expression, and Lysis Procedure—BHK-IR cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. PTP1D cDNA expression vector (5) or control plasmid were transiently expressed in BHK-IR cells (16). After 16 h of starvation, cells were stimulated with insulin (10–7 M) for 20 min, lysed in buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 2 mM sodium orthovanadate, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin; IR was immunoprecipitated with monoconal 83-14 antibodies; and PTP1D was detected in IR immunoprecipitates by immunoblotting.

Rat1 cells stably expressing human IR isotype A (Rat1-IR) were maintained in Dulbecco’s modified Eagle’s/F12 medium supplemented with 10% fetal calf serum. After 16 h of starvation, cells were stimulated with insulin (10–7 M) for 20 min and lysed in the same lysis buffer. Human lymphoblastic IM-9 cells (ATCC: CRL 159) were grown in RPMI medium supplemented with 10% fetal calf serum. Prior to insulin stimulation, cells were starved for 16 h in serum-free medium. After insulin stimulation (20 min, 10–7 M cells were lysed in the same lysis buffer, PTP1D was immunoprecipitated from crude lysates of unstimulated IM-9 cells with polyclonal affinity-purified antibodies, and IR was immunoprecipitated from unstimulated or stimulated cells with 83-14 antibodies. PTP1D was detected in Western blotting with polyclonal anti-PTP1D antibodies.

Insulin Receptor Purification—For IR isolation, BHK-IR cells were starved for 16 h and then lysed in lysis buffer and mixed with 200 μl of...
RESULTS AND DISCUSSION

PTP1D/Insulin Receptor Association in Intact Cells—The SH2 phosphotyrosine-specific phosphatase PTP1D has been shown to associate with the epidermal growth factor-R, HER2, and PDGFR in transfected cells based on its homology to the Drosophila corkscrew gene product, and it was originally suggested to play a positive role in the transmission of cellular growth signals (5). Moreover, in vitro experiments indicate that it may also bind to the tyrosine-phosphorylated receptor for insulin, although this has not been substantiated in vivo (18–21).

To further examine the role of PTP1D in insulin action, we used the stably transfected baby hamster kidney cells (BHK-IR) (16) and the Rat1 fibroblasts (Rat1-IR) (17), expressing the human IR, and nontransfected human lymphoblastic IM-9 cells. Upon stimulation of intact cells with insulin, PTP1D was detected in IR immunoprecipitates of all three cell lines using anti-PTP1D antibodies and immunoblot analysis. In BHK-IR and IM-9 cells (Fig. 1 and in Rat1-IR fibroblasts (data not shown), PTP1D associated with the IR in an insulin-dependent manner. Based on this immunoprecipitation analysis, we estimated that in IM-9 cells approximately 2–5% of total cellular PTP1D associated with the receptor, which is similar to earlier findings for PTP1D association with the PDGFR (22). In contrast to PDGFR and in accordance with a previous report (7), PTP1D tyrosine phosphorylation was not observed upon insulin treatment of BHK-IR, Rat1-IR, or IM-9 cells (not shown). Taken together, these results demonstrate that in intact cells PTP1D does not serve as a substrate for the IR tyrosine kinase but associates with the IR in an activation-dependent manner.

SH2 Domain Involvement in IR/PTP1D Interaction—To investigate whether tyrosine-phosphorylated IR associates directly with PTP1D SH2 domains, different GST-fusion proteins with the N-terminal (N) and C-terminal (C) SH2 domains, N-GST, C-GST, and (N+C)-GST, were used in conjunction with partially purified and in vitro autophosphorylated IR protein. As shown in Fig. 2A, tyrosine-phosphorylated IR associated efficiently with the (N+C)-GST protein. Much lower, but still significant binding was found for the C-GST fusion construct. In contrast, only traces of IR were detected when the N-GST fusion protein was used.

Further proof of the involvement of the PTP1D C-terminal SH2 domain in its interaction with the IR was obtained in association experiments with PTP1D mutants containing lysine substitutions of arginine residues that are known to be critical for SH2 binding functions. As shown in Fig. 2B, PTP1D/R138K, which contains an intact C-terminal SH2 domain, was still able to associate with activated IR, while PTP1D/R138K was not. From these results we concluded that PTP1D associates directly with activated IR through SH2 domain-mediated interactions and that the C-SH2 domain of PTP1D is necessary but not sufficient for full PTP1D binding capacity to the IR. While the N-terminal SH2 domain was found previously to be responsible for PTP1D binding to activated epidermal growth factor-R, PDGFR, and tyrosine-phosphorylated IRS-1 (23), no proteins have so far been reported to associate with the PTP1D/IR interaction.
PTP1D Adapter Function

To further demonstrate Tyr-1146 involvement in the interaction of activated IR and PTP1D, wild type receptor and mutant proteins with a C-terminal 43-amino acid deletion (NCT) or a tyrosine to phenylalanine mutation at position 1146 (RY1146F) were partially purified from transfected BHK-IR cells and after autophosphorylation used in an in vitro association experiment with the PTP1D (N+C)-GST fusion protein.

As shown in Fig. 4 (lanes 4 and 5), both IR and IRΔCT bound to the PTP1D SH2 domain fusion protein, while the Y1146F mutant did not (Fig. 4, lane 6). Thus, using two independent approaches, we identified phosphotyrosine 1146 as a binding site for the signal-transducing phosphatase PTP1D. This is of particular interest since this residue has been implicated in the initiation of the IR autophosphorylation cascade and positive insulin signal transmission (Ref. 25; reviewed in Refs. 8 and 26).

We are unable to explain the divergent findings by others, which reported phosphotyrosine 1322 as the IR binding site for Syt/PTP1D (18–21). Comparison of the SH2 domain-GST fusion protein/R binding experiments indicates, however, that only marginal competition was achieved with pY1322 peptide at a concentration of 750 μM (21), while our data demonstrate complete inhibition of association at 100 μM of pY1146 phosphopeptide. This suggests Tyr(P)-1322 as a potentially secondary binding site or as a site of weak interaction with the N-terminal SH2 domain in vitro.

PTP1D Is an Adapter for IR/IRS-1 Interaction—The significance of a direct interaction of a phosphotyrosine-specific phosphatase with the activated IR was first investigated in PTP1D/IRS-1 expression vector cotransfection experiments of BHK-IR cells, which yielded no significant effects on either IR or IRS-1 phosphorylation characteristics (not shown). Next, we examined the question of whether PTP1D may function as an adapter protein for a secondary signal transducer, a possibility that had been shown to be viable in the context of the PDGFR signal (9, 10). Therefore, and because of the demonstrated association with tyrosine-phosphorylated IRS-1 (7), we analyzed the influence of IRS-1 on PTP1D/IR interactions. As shown in Fig. 5B, IRS-1 overexpression in BHK-IR cells did not significantly change the amount of PTP1D associated with IR upon insulin stimulation (lanes 4 and 8). In contrast, co-over-

Fig. 2 Association of activated IR with PTP1D SH2 domains in vitro. A, binding of semipurified autophosphorylated IR to different PTP1D SH2 domain-GST-fusion proteins. B, effect of SH2 domain point mutation on PTP1D/IR interaction. PTP1D (lanes 1 and 2), PTP1D/R32K (lanes 3 and 4), and PTP1D/R138K (lanes 5 and 6) were transiently expressed in BHK-IR cells. Cells were left unstimulated (lanes 1, 3, and 5) or stimulated with insulin (lanes 2, 4, and 6) for 20 min. After cell lysis, IR was immunoprecipitated with monoclonal antibody 83-14, and after gel electrophoresis, the presence of PTP1D wild type and mutant proteins in IR immunoprecipitates was detected by immunoblot analysis with anti-PTP1D antibody. C, identification of IR binding site for PTP1D. Phosphotyrosine peptides representing IR cytoplasmic domain tyrosine residues and upstream (5) and downstream (5) flanking amino acids were employed in in vitro association competition experiments between semipurified IR and the (N+C)-GST fusion protein. Control, no phosphotyrosine peptide added.

C-proximal SH2 domain.

IR/PTP1D Association Is Mediated by IR Phosphotyrosine 1146—To map the phosphotyrosine residue in the IR molecule involved in the observed interaction between the IR and the (N+C)-GST fusion protein, in vitro competition experiments were performed with phosphodipeptides corresponding to possible tyrosine phosphorylation sites in the cytoplasmic domain of the IR at amino acid positions 953, 960, 972, 999, 1075, 1110, 1146, 1150, 1151, 1198, 1215, 1316, and 1322 (24). Phosphotyrosine peptide 1146 completely prevented association of the IR and the (N+C)-GST fusion proteins at concentrations of 100 μM (Fig. 2C) and even as low as 30 μM (data not shown). Under the same conditions, none of the other phosphopeptides examined had an effect on this interaction (Fig. 2C).

Of the two major phosphotyrosine-containing proteins from crude lysates of insulin-stimulated BHK-IR cells that bound to the (N+C)-GST fusion protein, the 95-kDa band was identified as the IR β-chain, and the second of about 180 kDa was likely to represent endogenous IRS-1 (Fig. 3). Interestingly, phosphopeptide 1146 completely blocked IR β-chain association with the (N+C)-GST construct, while the change of 180-kDa protein binding was within the experimental range of variation (Fig. 3, lanes 4 and 5). This result indicated that binding of the tyrosine-phosphorylated 180-kDa protein to the SH2 domain fusion protein was independent of the IR interaction and that it occurred through a binding motif different from that of phosphopeptide 1146.

To further demonstrate Tyr-1146 involvement in the interaction of activated IR and PTP1D, wild type receptor and mutant proteins with a C-terminal 43-amino acid deletion (IRΔCT) or a tyrosine to phenylalanine mutation at position 1146 (RY1146F) were partially purified from transfected BHK cells and after autophosphorylation used in an in vitro association experiment with the PTP1D (N+C)-GST fusion protein.

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Fig. 3 Anti-phosphotyrosine immunoblot analysis of IR and 180-kDa protein association with PTP1D (N+C)-GST fusion protein. Lanes 1 and 2, crude lysates of starved or insulin-stimulated BHK-IR cells, respectively. Lanes 3–5, association of proteins from crude lysate of insulin-stimulated BHK-IR cells associated with GST protein control (lane 3), PTP1D (N+C)-GST fusion protein (lane 4), and (N+C)-GST fusion protein in the presence of 1146 peptide (lane 5). Phosphotyrosine-containing proteins were detected by immunoblotting with monoclonal anti-phosphotyrosine antibodies (SE2).

Fig. 4 Y1146F mutation abrogates binding of PTP1D (N+C)-GST fusion protein to IR. BHK-21 cells were separately transfected with IR, IRΔCT, and IRY1146F expression plasmids. IR and Y1146F mutant proteins were partially purified on WGA-Sepharose and auto-

phosphorylated in vitro. Lanes 4–6 represent bind of activated IR constructs (IR, IRΔCT, and IRY1146F, respectively) to the PTP1D (N+C)-GST fusion protein. Lanes 1–3, negative control (binding of the same constructs to GST protein). Activated IR and IR mutants were detected by immunoblotting with monoclonal anti-phosphotyrosine antibody (SE2).
expression of PTP1D with IRS-1 in BHK-IR cells dramatically increased the amount of IRS-1 in IR immunoprecipitates from insulin-stimulated cells (Fig. 5A, lanes 6 and 8). This result sheds new light on previous reports that demonstrated interaction of IR and IRS-1 in Chinese hamster ovary cells upon insulin stimulation (27, 28). Although as we demonstrated earlier, tyrosine 960 of the IR is required for IRS-1 phosphorylation on tyrosine (29), the significance of its involvement had not been clear. Our results now strongly support a type of IRS-1 interaction in which PTP1D serves as an adapter molecule that physically links tyrosine 1146 within the core region of the IR tyrosine kinase with a so far unidentified molecule that participates in the IRS-1 complex necessary for efficient substrate phosphorylation.

PTP1D Modulation of Insulin-dependent Glucose Uptake—Finally, to assess whether the IRS-1 coupling function represents an important link in the cellular insulin signal transduction cascade, BHK-IR cells were transfected with PTP1D or IRS-1 cDNAs, and glucose uptake was measured. Representative results are shown in Fig. 6. In agreement with previously published data (8, 26), IRS-1 expression led to an increased insulin-dependent glucose uptake in BHK-IR cells. However, coexpression of PTP1D and IRS-1 resulted in an additional enhancement of glucose transport, strongly suggesting that overexpression of PTP1D allows recruitment of additional IRS-1 molecules into the IR signaling complex, causing significant stimulation of insulin-dependent glucose uptake. The extent of glucose transport induction was the same when a catalytically inactive mutant of PTP1D was employed in these experiments, indicating that phosphatase function was not required for the stimulating effect.

A positive role for PTP1D in receptor-type kinase-mediated signal transduction was initially suggested because of its structural homology to the Drosofla corkscrew gene product, which by genetic criteria had been identified as a downstream transducer of the torso receptor-type kinase signal (1, 30). Recently this proposed function was confirmed by the demonstration of inhibitory effects on insulin-induced mitogenic responses in cells overexpressing a mutant of PTP1D (11–13). While neither the reaction partners of PTP1D nor the pathway requiring an active phosphatase in insulin-induced mitogenic signaling are currently identified, it is clear that the positive role in insulin action suggested by the adapter function of PTP1D between the IR and IRS-1 represents a different mechanism, since it does not require an intact catalytic function. The overall significance of the PTP1D adapter function for the various cellular responses induced by insulin in different cell types is currently unclear, but our results strongly suggest that it is involved in a critical aspect of the insulin signal, the regulation of glucose uptake.

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REFERENCES

PTP1D Adapter Function