A Phosphotyrosyl Mimetic Peptide Reverses Impairment of Insulin-Stimulated Translocation of GLUT4 Caused by Overexpression of PTP1B in Rat Adipose Cells

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ABSTRACT: Biological actions of insulin are initiated by activation of the insulin receptor tyrosine kinase. Protein tyrosine phosphatases (PTPases) PTP1B and PTPα are known to dephosphorylate the insulin receptor and may contribute to insulin resistance in diseases such as diabetes. We previously reported that overexpression of PTP1B in rat adipose cells significantly impairs insulin-stimulated translocation of GLUT4 [Chen, H., et al. (1997) *J. Biol. Chem. 272*, 8026]. In the present study, we treated adipose cells with a PTPase inhibitor containing the phosphotyrosyl mimetic difluorophosphonomethyl phenylalanine (F_2Pmp) to determine whether we could improve the insulin resistance caused by overexpression of PTP1B or PTPα. Rat adipose cells transfected by electroporation with either PTP1B or PTPα were treated without or with the inhibitor, and effects on insulin-stimulated translocation of a cotransfected epitope-tagged GLUT4 were studied. The IC_{50} of the F_2Pmp-containing inhibitor is 180 nM for PTP1B and 10 mM for PTPα in vitro. As expected, in the absence of the inhibitor, overexpression of either PTP1B or PTPα caused a significant decrease in the amount of GLUT4 at the cell surface both in the absence and in the presence of insulin when compared with control cells transfected with epitope-tagged GLUT4 alone. Interestingly, the insulin resistance caused by overexpression of PTP1B (but not PTPα) was reversed by treating the transfected cells with the F_2Pmp-containing inhibitor. Furthermore, the inhibitor blocked the insulin-stimulated association of PTP1B with the insulin receptor. We conclude that the F_2Pmp-containing compound is a potent and specific inhibitor of overexpressed PTP1B that may be useful for designing rational therapies for treating insulin resistant diseases such as diabetes.

The biological actions of insulin are initiated when insulin binds to its receptor, resulting in stimulation of intrinsic receptor tyrosine kinase activity, autophosphorylation of the receptor, and subsequent phosphorylation of intracellular substrates (for reviews, see refs *1* and *2*). Since a proximal step in insulin signaling involves tyrosine phosphorylation of the insulin receptor and other substrates, it is likely that dephosphorylation of these molecules by protein tyrosine phosphatases (PTPases) is important for the modulation of insulin action (*3*). A number of PTPases, including PTP1B, LAR, PTPα, and PTPε, are capable of dephosphorylating the insulin receptor in intact cells (*4*−*9*). In addition, a role for PTP1B in the insulin resistance associated with diabetes and obesity has been suggested by some clinical studies in which correlations between levels of PTP1B expression in muscle and adipose tissue and insulin resistant states were found (*10*−*12*). Furthermore, we have recently demonstrated that overexpression of PTP1B in primary cultures of rat adipose cells results in significant impairment of insulin-stimulated translocation of GLUT4 (*13*). These studies suggest that PTP1B may play an important role in specifically modulating distinctive metabolic actions of insulin in contrast to mitogenic actions that are mediated by many receptor tyrosine kinases in addition to the insulin receptor. Therefore, it is of interest to identify and characterize specific inhibitors of PTP1B that may have therapeutic applications for ameliorating insulin resistance in diseases such as diabetes.

Because large families of receptor tyrosine kinases and PTPases are involved in the regulation of growth and other biological functions, an important feature of a useful PTPase inhibitor is specificity or selectivity for a particular PTPase. The crystal structures of the catalytic domain of wild-type PTP1B (*14*, *15*) and a catalytically inactive mutant of PTP1B (*16*) complexed with various compounds have been helpful for designing and understanding highly specific and potent inhibitors of PTP1B. Recently, a peptide with a nonhydrolyzable phosphotyrosyl mimetic difluorophosphonomethyl phenylalanine (F_2Pmp) residue based on one of the autophosphorylation sites in the epidermal growth factor (EGF) receptor was shown to be a potent inhibitor of PTP1B-mediated dephosphorylation of the insulin receptor in vitro ($IC_{50} \sim 200$ nM) (*17*). In the present study, we show that

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this F_2Pmp-containing inhibitor can completely reverse the impairment in insulin-stimulated translocation of GLUT4 caused by overexpression of PTP1B in the physiologically relevant rat adipose cell. Furthermore, we demonstrate that this inhibitory peptide has specificity for PTP1B because under similar conditions, we were unable to reverse the impairment in insulin action caused by overexpression of PTPα. However, treatment of untransfected cells with the F_2Pmp-containing inhibitor did not appear to enhance insulin-stimulated translocation of GLUT4 (most likely because endogenous PTP1B is only expressed at low levels in rat adipose cells under our conditions). Nevertheless, because elevations in endogenous levels of PTP1B in muscle and adipose tissue may contribute to insulin resistance in obesity and diabetes (*10*, *12*), it remains possible that inhibitory compounds similar to the F_2Pmp-containing peptide characterized here may be useful for the treatment of these insulin resistant states.

MATERIALS AND METHODS

Expression Vectors. *pCIS2.* An expression vector containing a generic intron upstream of a cytomegalovirus promoter/enhancer that generates high levels of expression in rat adipose cells was used as the parent vector for subsequent constructions (*18*, *19*).

PTP1B-WT. cDNA encoding wild-type human PTP1B was subcloned into pCIS2 as described previously (*13*).

PTP1B-C/S. cDNA encoding a catalytically inactive mutant of human PTP1B with a cysteine to serine substitution at position 215 was subcloned into pCIS2 as described previously (*13*).

PTPα-WT. A 2.4 kb fragment containing the cDNA encoding wild-type human PTP-α (*20*) was subcloned into the *Xho*I−*Hpa*I sites in the multiple cloning region of pCIS2 (generous gift from J. Sap).

GLUT4-HA. This was constructed with the pCIS2 vector containing the cDNA encoding human GLUT4 with the influenza hemagglutinin epitope (HA1) inserted in the first exofacial loop of GLUT4 (*21*).

Synthesis of the F_2Pmp-Containing Inhibitor. Solid-phase synthesis of Ac-Asp-Ala-Asp-Glu-F_2Pmp-Leu-amide has been previously reported using the F_2Pmp reagent (*22*) bearing ethyl protection on the phosphonate hydroxyls (*17*). For the present study, the peptide inhibitor was prepared via a modified solid-phase procedure employing the F_2Pmp reagent lacking phosphonate protection (*23*).

Determination of Inhibitor Activity against PTP1B and PTPα in Vitro. The cDNA encoding the catalytic domain of human PTP1B (amino acids 1−321) was obtained using polymerase chain reaction (PCR) from a human fetal brain cDNA library (Stratagene). The PCR primers used were 5′-AGCTGGATCCATATGGAGATGGAAAAGGAGTT (encoding both a *Bam*HI and a *Nde*I site) and 3′-ACGCGAATTCTTAATTGTGTGGCTCCAGGATTCG (encoding an *Eco*RI site). The PCR product was digested with *Bam*HI and *Eco*RI and subcloned into a pUC118 vector. The PTP1B coding sequence was confirmed by DNA sequencing. The coding region for PTP1B was then cut from pUC118-PTP1B with *Nde*I and *Eco*RI and ligated into the corresponding sites of plasmid pT7-7. The PTP1B coding sequence was placed in-frame downstream of the phage T7 RNA polymerase promoter at the *Nde*I site of pT7-7 to provide the translational initiation at Met 1 of PTP1B. The resulting plasmid pT7-7/PTP1B was used to transform *Escherichia coli* BL21(DE3) cells. The expression of the recombinant PTP1B has been described previously (*16*). The pGEX plasmid containing the coding sequence for both of the PTPase domains of human PTPα was a generous gift from F. Jirik of the University of British Columbia (Vancouver, BC). The recombinant glutathione *S*-transferase (GST) fusion protein was purified, and the PTPα was cleaved off the fusion protein as described previously (*24*).

The PTP1B and PTPα phosphatase activity was assayed at 30 °C in a reaction mixture (0.2 mL) containing appropriate concentrations of *p*-nitrophenyl phosphate (*p*NPP) as the substrate. The buffer used was 50 mM 3,3-dimethylglutarate (pH 7.0) and 1 mM EDTA. The ionic strength of the solution was kept at 0.15 M using NaCl. The reaction was initiated by addition of enzyme and quenched after 2−3 min by addition of 1 mL of 1 N NaOH. The nonenzymatic hydrolysis of the substrate was corrected by measuring the control without the addition of enzyme. The amount of product *p*-nitrophenol was determined from the absorbance at 405 nm using a molar extinction coefficient of 18 000 M^{-1} cm^{-1}. Steady state kinetic parameters were evaluated by fitting directly the v versus [S] data to the Michaelis−Menten equation using KINETASYST (IntelliKinetics, State College, PA).

Inhibition constants for the F_2Pmp-containing inhibitor were determined for PTP1B and PTPα in the following manner. The initial rate at eight different *p*NPP concentrations (0.2−5K_m) was measured at three different fixed inhibitor concentrations (*25*). The inhibition constant and inhibition pattern were evaluated using direct curve-fitting program KINETASYST (IntelliKinetics).

Isolated Rat Adipose Cell Preparation. Isolated rat adipose cells were prepared from the epididymal fat pads of male rats (170−200 g, CD strain, Charles River Breeding Laboratories, Wilmington, MA) by collagenase digestion as described previously (*19*, *26*).

Electroporation. Isolated adipose cells were transfected by electroporation in the absence or presence of the inhibitor (final concentration of 2 μM) as described previously (*13*, *27*). Groups of cells were cotransfected with GLUT4-HA (2 μg/cuvette) and either pCIS2, PTP1B-WT, or PTPα-WT, (4 μg/cuvette). Cells from multiple cuvettes were pooled to obtain the necessary volume of cells for each experiment and cultured in 60 mm dishes.

Assay for Cell Surface Epitope-Tagged GLUT4. Twenty hours after electroporation, adipose cells were processed as described previously (*13*, *21*, *27*) and treated with insulin at concentrations ranging from 0 to 60 nM at 37 °C for 30 min. The amount of cell surface epitope-tagged GLUT4 was determined by using the anti-HA1 mouse monoclonal antibody HA-11 (Berkeley Antibody Co., Richmond, CA) as described previously (*13*, *28*). Total cellular levels of GLUT4-HA in each group of transfected cells were compared by immunoblotting as described previously (*13*).

Immunodetection of Recombinant PTP1B and Its Association with the Insulin Receptor. To determine if the inhibitor affected expression of recombinant PTP1B, we used a polyclonal antibody against PTP1B (UBI, Lake Placid, NY) to immunoblot membrane fractions isolated from adipose

cells transfected with pCIS2 or PTP1B-WT in the presence or absence of inhibitor as previously described (*13*). To examine the insulin-stimulated association of PTP1B with the insulin receptor, we transfected adipose cells with pCIS2 or PTP1B-C/S in the presence or absence of inhibitor (2 μM) and then, after overnight culture, treated the cells without or with insulin (100 nM, 2 min). The cells were then lysed, total membrane fractions prepared, and samples (150 μg of protein) immunoprecipitated with a monoclonal anti-PTP1B antibody (Oncogene Research Products, Cambridge, MA) followed by SDS-PAGE and immunblotting with a polyclonal antibody against the β-subunit of the insulin receptor (C-19; Santa Cruz Biotechnology, Santa Cruz, CA).

Statistical Analysis. Paired *t* tests were used to compare individual points where appropriate. Multiple analysis of variance (MANOVA) was used to compare insulin dose-response experiments. This method allows us to evaluate differences between entire dose-response curves of each experimental group and avoids the need to correct for multiple comparisons which is necessary if each individual insulin dose is compared for each experimental group. *p* values of <0.05 were considered to represent statistical significance.

RESULTS

*In Vitro Determination of Inhibitor Activity against PTP1B and PTP*α. The effect of the F_2Pmp-containing phosphotyrosyl mimetic peptide on the PTP1B- and PTPα-catalyzed *p*NPP hydrolysis reaction was examined at 30 °C and pH 7.0. We found not only that the peptide inhibited the PTPase reaction catalyzed by PTP1B or PTPα but also that the mode of inhibition was competitive with respect to the substrate (data not shown). Interestingly, we also determined that the K_i value for the inhibitor was only 180 nM for PTP1B but 10 mM for PTPα, indicating remarkable selectivity of the inhibitor for PTP1B.

Effect of the PTPase Inhibitor on Adipose Cells Overexpressing PTP1B. After demonstrating in vitro inhibitory effects, we next determined whether the F_2Pmp-containing compound was also effective at blocking biological actions of PTP1B in vivo. We have previously reported that overexpression of PTP1B in adipose cells impairs the ability of insulin to stimulate recruitment of GLUT4 to the cell surface (*13*). Therefore, we cotransfected adipose cells with GLUT4-HA and either the empty expression vector pCIS2 (control) or PTP1B-WT in the absence or presence of the PTPase inhibitor (2 μM). Because the peptide inhibitor may have difficulty entering intact cells, we added the inhibitor to the media before electroporation. The process of electroporation should facilitate entry of the inhibitor into the cells. When membrane fractions derived from cells in each of the experimental groups were immunoblotted with an antibody against PTP1B, a small amount of endogenous PTP1B was detected in the control group (transfected with the empty vector, pCIS2), while high levels of recombinant PTP1B were detected in the groups transfected with PTP1B-WT (Figure 1). Since only ~5% of the adipose cells that have undergone electroporation are actually transfected (*21*), we estimate that there was at least 100-fold overexpression of PTP1B-WT relative to endogenous PTP1B in the transfected cells. Furthermore, the presence of the inhibitor did not appear to significantly affect the level of overexpression of PTP1B.

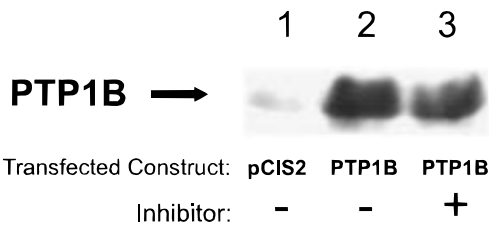

FIGURE 1: Overexpression of recombinant human PTP1B in rat adipose cells electroporated in the absence or presence of the PTPase inhibitor. Membrane fractions from each group containing 100 μg of total protein were subjected to SDS−PAGE and immunoblotted with an antibody against PTP1B. A representative blot is shown from an experiment that was repeated independently three times.

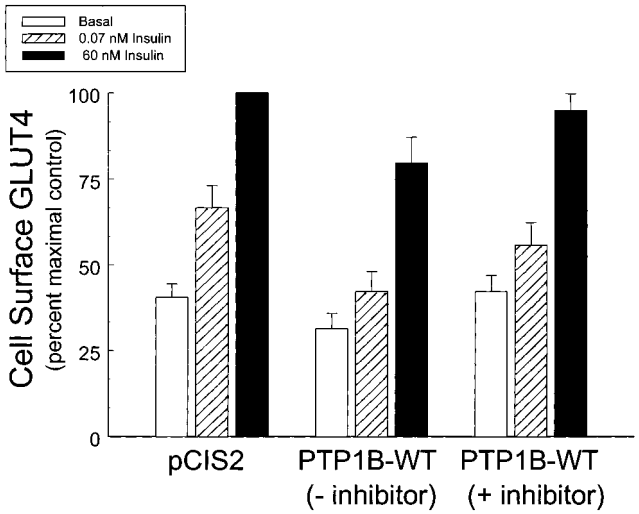

FIGURE 2: Effect of the PTPase inhibitor on translocation of GLUT4 in response to insulin stimulation in rat adipose cells overexpressing PTP1B. Cells were cotransfected with GLUT4-HA and either pCIS2 (control) or PTP1B-WT (in the absence or presence of 2 μM PTPase inhibitor). Data are expressed as a percentage of the amount of cell surface GLUT4-HA in the presence of a maximally effective insulin concentration for the control group. Cells overexpressing PTP1B-WT in the absence of inhibitor had a significant decrease in the amount of cell surface GLUT4-HA both in the absence and in the presence of insulin when compared to control cells ($p < 0.0001$). The response of cells overexpressing PTP1B-WT in the presence of the inhibitor was not significantly different from that of the control cells ($p > 0.23$). For cells overexpressing PTP1B-WT, the presence of the inhibitor was associated with a significant increase in the amount of cell surface GLUT4 ($p < 0.003$ by MANOVA; $p < 0.03$ for each insulin dose using the paired *t* test). Results shown are the means ± SEM of seven independent experiments.

After overexpression of the recombinant PTP1B was verified, the amount of GLUT4 recruited to the cell surface in response to varying doses of insulin was assessed for each experimental group. Control cells (without overexpression of PTP1B) had a 2.5-fold increase in the amount of cell surface GLUT4 following treatment with a maximally stimulating dose of insulin (60 nM) (Figure 2). Consistent with our previously reported results (*13*), overexpression of PTP1B in the absence of the PTPase inhibitor resulted in a significant decrease in the amount of cell surface GLUT4 both in the absence and in the presence of insulin when compared with the control cells. Interestingly, the decrease in the amount of cell surface GLUT4 caused by overexpres-

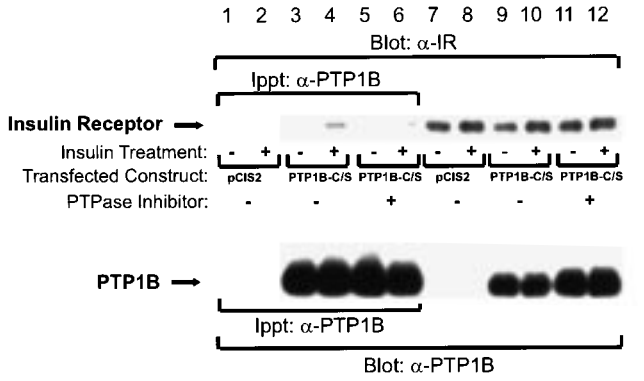

FIGURE 3: PTPase inhibitor impairs insulin-stimulated association of PTP1B with the insulin receptor. Adipose cells were transfected with pCIS2 (empty vector) or PTP1B-C/S in the presence or absence of the inhibitor. The cells were then treated without or with insulin (100 nM, 2 min), and membrane fractions (150 μg of protein) were immunoprecipitated with 2 μg of an anti-PTP1B antibody. The samples were then subjected to SDS−PAGE followed by immunoblotting with antibodies against either the insulin receptor or PTP1B (lanes 1−6). As a control, aliquots of the same samples containing 50 μg of total protein (without immunoprecipitation) were immunblotted with antibodies against either the insulin receptor or PTP1B (lanes 7−12). A representative blot is shown from an experiment that was repeated independently three times.

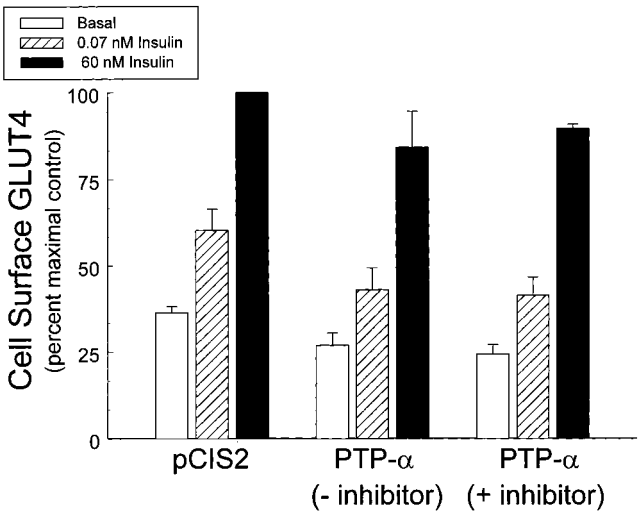

FIGURE 4: Effect of the PTPase inhibitor on translocation of GLUT4 in response to insulin stimulation in rat adipose cells overexpressing PTPα. Cells were cotransfected with GLUT4-HA and either pCIS2 (control) or PTPα-WT (in the absence or presence of 2 μM PTPase inhibitor). Cells overexpressing PTPα in the absence of inhibitor had a significant decrease in the levels of cell surface GLUT4-HA ($p < 0.0003$). The response of cells overexpressing PTPα in either the absence or presence of inhibitor was similar ($p > 0.92$). Results shown are the means ± SEM of five independent experiments.

sion of PTP1B was reversed in the presence of the F_2Pmp-containing inhibitor. That is, for cells overexpressing PTP1B, the level of cell surface GLUT4 at every insulin dose tested was significantly greater in the presence of inhibitor. In addition, there was no significant difference between the dose-response curves for the control group and the group of cells overexpressing PTP1B and treated with the inhibitor. To rule out the possibility that our results were influenced by effects of the inhibitor on expression of the GLUT4-HA reporter, we performed immunoblotting experiments with an anti-HA antibody to assess total levels of GLUT4-HA expression in the presence and absence of the inhibitor. We found that all groups of cells expressed comparable total levels of GLUT4-HA (data not shown). Taken together, our results demonstrate that the F_2Pmp-containing inhibitor can reverse the impairment of insulin-stimulated translocation of GLUT4 caused by overexpression of PTP1B in adipose cells.

Effect of the PTPase Inhibitor on Interactions between PTP1B and the Insulin Receptor. Others have previously shown that PTP1B can dephosphorylate the activated insulin receptor (*5*, *6*). Therefore, one potential mechanism for explaining the ability of our PTPase inhibitor to reverse the impairment in insulin-stimulated translocation of GLUT4 caused by overexpression of PTP1B involves the ability of the inhibitor to block the interaction of PTP1B with the activated insulin receptor. To examine this possibility, we transfected adipose cells with PTP1B-C/S in the absence or presence of inhibitor and then treated cells without or with insulin. If the insulin receptor is a direct substrate for PTP1B, the catalytically inactive mutant PTP1B-C/S would be predicted to bind to (but not dephosphorylate) the activated insulin receptor. Indeed, significant coimmunoprecipitation of PTP1B-C/S and the insulin receptor was observed upon insulin stimulation of cells overexpressing PTP1B-C/S in the absence of inhibitor (Figure 3). However, in the presence of inhibitor, the extent of insulin-stimulated association between PTP1B and the insulin receptor was substantially decreased (Figure 3). Therefore, the PTPase inhibitor interferes with the insulin-stimulated association of PTP1B with the insulin receptor. Since the F_2Pmp-containing peptide inhibits PTP1B activity by occupying the enzyme catalytic site, the reduced extent of association between PTP1B and the insulin receptor in the presence of inhibitor suggests that this interaction involves the PTP1B catalytic site and phosphotyrosine residues on the insulin receptor. This finding may help explain the biological actions of the inhibitor that we observed.

*Effect of the PTPase Inhibitor on Adipose Cells Overexpressing PTP*α. To determine if the inhibitory effects of the F_2Pmp-containing peptide in adipose cells were specific for PTP1B or would also be observed with overexpression of other PTPases, we studied adipose cells overexpressing PTPα. Recently, we reported that overexpression of PTPα in adipose cells also significantly impairs insulin-stimulated translocation of GLUT4 (*29*). As expected, adipose cells overexpressing PTPα in the absence of the PTPase inhibitor showed a significant decrease in the amount of cell surface GLUT4 in both the absence and presence of insulin when compared with control cells (Figure 4). In contrast to our results with overexpression of PTP1B, treatment of cells with the PTPase inhibitor did not significantly reverse the impairment of insulin-stimulated translocation of GLUT4 caused by overexpression of PTPα (Figure 4). Thus, the effects (or lack of effects) of the inhibitor on biological functions of overexpressed PTPases in adipose cells reflect the selectivity of the F_2Pmp-containing peptide for PTP1B that we demonstrated in vitro.

Effect of the Inhibitor in Adipose Cells Not Overexpressing PTPases. Since specific PTPase inhibitors may be potential therapeutic agents for the treatment of diseases associated with insulin resistance such as diabetes, we inquired whether the F_2Pmp-containing peptide would enhance insulin-stimulated translocation of GLUT4 in adipose cells not

overexpressing any PTPase. The insulin dose-response curve of control adipose cells transfected with the GLUT4-HA reporter in the absence of the inhibitor showed a 2.7-fold increase in the amount of cell surface GLUT4 upon maximal insulin stimulation ($ED_{50} \sim 0.06$ nM) (data not shown). The insulin dose-response curve of cells treated with the PTPase inhibitor (2 μM) was not significantly different from that of the control cells at the insulin concentrations tested (0, 0.024, 0.072, 0.3, or 60 nM; $ED_{50} \sim 0.06$ nM; $p > 0.13$). The failure of the PTPase inhibitor to significantly enhance insulin-stimulated translocation of GLUT4 under conditions where PTP1B is not overexpressed implies that the low levels of endogenous PTP1B in rat adipose cells do not contribute significantly to metabolic effects of insulin under our experimental conditions.

DISCUSSION

Metabolic actions of insulin are initiated by activation of the insulin receptor tyrosine kinase. Therefore, specific inhibition of particular PTPases that are negative modulators of insulin action may have the potential to improve insulin sensitivity and lead to the development of therapeutic agents for the treatment of diseases such as diabetes that are associated with insulin resistance. PTP1B is an attractive target for inhibition because levels of expression for PTP1B in muscle and adipose tissue correlate with the degree of insulin resistance in subjects with obesity or diabetes (*10*, *12*). In addition, PTP1B is known to dephosphorylate the insulin receptor in intact cells (*4*−*6*). Furthermore, we have previously reported that overexpression of PTP1B in rat adipose cells impairs insulin-stimulated translocation of GLUT4 (*13*).

*In Vitro Determination of Inhibitor Activity against PTP1B and PTP*α. The PTPase inhibitor used in the present study is based on a peptide sequence Ac-Asp-Ala-Asp-Glu-pTyr-Leu-amide, which was originally shown to be a high-affinity substrate for the rat PTP1 phosphatase ($K_m = 13.2$ μM) (*30*). A modified peptide was prepared that contains the nonhydrolyzable phosphotyrosyl mimetic F_2Pmp instead of the pTyr residue (the phosphoryl ester oxygen is replaced by a difluoromethylene unit) (*31*). While maintaining a pK_a2 value similar to that of the parent phosphate (*32*), the aryl difluorophosphonate is not cleaved by phosphatases (*33*). This compound exhibits extremely potent inhibition of the PTP1B enzyme in vitro ($IC_{50} = 100$ nM) and prevents the dephosphorylation of purified phosphorylated insulin receptors by PTP1B (*17*). Subsequent studies have confirmed the high PTPase affinity of a variety of F_2Pmp-containing peptides (*34*), as well as small-molecule aryl difluoromethylphosphonates (*35*). In the present study, our results have confirmed the potency of the F_2Pmp-containing inhibitor against PTP1B. Moreover, we have shown that this inhibitor is also highly selective because the K_i of the inhibitor for PTPα (a transmembrane PTPase also known to dephosphorylate the insulin receptor) is approximately 50 times greater than the K_i for PTP1B.

*Effect of the PTPase Inhibitor on Adipose Cells Overexpressing PTP1B or PTP*α. In previous studies, we demonstrated that the significant impairment of insulin-stimulated translocation of GLUT4 caused by overexpression of either PTP1B or PTPα in rat adipose cells was dependent on the presence of an intact PTPase domain (*13*, *29*). That is, overexpression of point mutants of these PTPases that were catalytically inactive did not affect translocation of GLUT4. Therefore, a potent, specific PTPase inhibitor might be predicted to modulate metabolic actions of insulin. In the present study, we achieve ∼100-fold overexpression of recombinant PTP1B comparable to levels previously reported (estimated from scanning densitometry of immunoblots and assuming a transfection efficiency of 5%) (*13*). Consistent with the importance of an active PTPase domain, the expected impairment of insulin-stimulated translocation of GLUT4 caused by overexpression of PTP1B was reversed in the presence of the F_2Pmp-containing inhibitor. This is the first direct demonstration that an F_2Pmp-containing peptide can inhibit a biological function of PTP1B in intact cells. As previously mentioned, others have directly demonstrated that PTP1B is capable of dephosphorylating the activated insulin receptor in intact cells (*4*−*6*). The relatively low 5% transfection efficiency of our system precludes a direct demonstration of the effect of overexpressed PTP1B to dephosphorylate the insulin receptor (due to the high background created by phosphorylated insulin receptors in nontransfected cells) (*21*). Nevertheless, using a catalytically inactive mutant of PTP1B we were able to directly demonstrate that the inhibitor can interfere with the interaction between PTP1B and the activated insulin receptor, suggesting that PTP1B impairs insulin action, at least in part, by dephosphorylating the insulin receptor.

Because the peptide inhibitor will not easily cross the plasma membrane, we used electroporation to facilitate the entry of the inhibitor into adipose cells. It is possible that after overnight incubation, some of the inhibitor has been degraded. Unfortunately, we are unable to measure the final concentration of inhibitor in the small fraction of cells that are actually transfected with the GLUT4-HA reporter and the PTPase constructs. Nevertheless, the initial concentration of 2 μM represents an upper bound, and the actual concentration present after overnight incubation is clearly sufficient to reverse the effects of the overexpressed PTP1B and significantly impair its interaction with the phosphorylated insulin receptor.

In contrast to our results with overexpression of PTP1B, we found that the F_2Pmp-containing inhibitor did not alter the effects of overexpression of PTPα to impair translocation of GLUT4 in adipose cells. PTP1B and PTPα most likely interfere with insulin-stimulated translocation of GLUT4 by dephosphorylating the insulin receptor and/or other substrates shared in common because the inhibitory effects of PTP1B and PTPα are not additive (L.-N. Cong and M. J. Quon, unpublished observations). Thus, the selectivity of the PTPase inhibitor for PTP1B that we demonstrated in vitro is also reflected in our biological assay in intact cells.

Effect of the Inhibitor in Adipose Cells Not Overexpressing PTPases. Since one potential application for PTPase inhibitors is to enhance insulin-stimulated glucose transport, we tested effects of the F_2Pmp-containing peptide in adipose cells transfected with only the GLUT4-HA reporter. Under these conditions, we were not able to demonstrate any significant effect of the inhibitor in enhancing translocation of GLUT4. However, this may be due to the low levels of endogenous PTP1B present in rat adipose cells (Figure 1). That is, in our experimental system, endogenous PTP1B may

not be a significant negative modulator of metabolic actions of insulin. However, because levels of PTP1B appear to be elevated in muscle and adipose tissue from obese and diabetic subjects (*10*, *12*) and elevated levels of PTP1B have been implicated as a factor contributing to insulin resistance in pathological states such as obesity and diabetes, it remains possible that PTPase inhibitors similar to the one described here would be useful for ameliorating insulin resistance and improving metabolic functions of insulin under certain conditions.

Conclusion. In summary, we have shown that an F_2Pmp-containing peptide can act as a specific inhibitor of PTP1B in intact cells and can reverse the impairment of insulin-stimulated translocation of GLUT4 caused by overexpression of PTP1B. The effect of the inhibitor on insulin action relates, at least in part, to its ability to block the interaction of PTP1B with the phosphorylated insulin receptor. We conclude that specific PTP1B inhibitors similar to the one described here may be useful for treating diseases such as diabetes where elevated levels of expression of PTP1B have been implicated in the pathophysiology of insulin resistance.

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