Selective Down-regulation of the Insulin Receptor Signal by Protein-tyrosine Phosphatases α and ε*

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Binding of insulin to its receptor (IR) causes rapid autophosphorylation with concomitant activation of its tyrosine kinase which transmits the signal by phosphorylating cellular substrates. The IR activity is controlled by protein-tyrosine phosphatases, but those directly involved in regulating the insulin receptor and its signaling pathways have not yet been identified. Using baby hamster kidney cells overexpressing the IR and a novel insulin-based selection principle, we established stable cell lines with functionally coupled expression of the IR and protein-tyrosine phosphatases. The two closely related protein-tyrosine phosphatases α and ε were identified as negative regulators of IR tyrosine kinase.

Insulin is an important regulator of different metabolic processes and plays a key role in the control of blood glucose. Defects related to its synthesis or signaling lead to diabetes mellitus. Binding of insulin to its receptor causes rapid autophosphorylation of several tyrosine residues in the intracellular part of the β-subunit. Three closely positioned tyrosine residues (the tyrosine 1150 domain) must all be phosphorylated to obtain full activity of the insulin receptor tyrosine kinase (IRTK) which transmits the signal further downstream by tyrosine phosphorylation of other cellular substrates, including insulin receptor substrate-1 (IRS-1) (1–4). The structural basis for the function of the tyrosine triplet has been provided by recent x-ray crystallographic studies of IRTK that showed tyrosine 1150 to be autoinhibitory in its unphosphorylated state (5).

Several studies clearly indicate that the activity of the autophosphorylated IRTK can be reversed by dephosphorylation in vitro (reviewed in Goldstein (6)) (7, 8), with the triphosphorylated form being the most sensitive target for protein-tyrosine phosphatases (PTPs) as compared to the di- and monophosphorylated forms (8). It is, therefore, tempting to speculate that this tyrosine triplet function as a control switch of IRTK activity. Indeed, the IRTK appears to be tightly regulated by PTP-mediated dephosphorylation in vivo (9–11). The intimate coupling of PTPs to the insulin signaling pathway is further evidenced by the finding that insulin differentially regulates PTP activity in rat hepatoma cells (12) and in livers from alloxan diabetic rats (13). However, little is known about the identity of the PTPs involved in IRTK regulation.

To identify PTPs that negatively regulate the IRTK activity we developed a novel selection principle that allows establishment of stable cell lines with functionally coupled overexpression of IR and inhibitory PTPs. For this purpose we used a previously established baby hamster kidney cell line (BHK-IR) (14) which exhibits high levels of IR activity. Similar and responds to insulin stimulation with complete growth inhibition of adherent cells. PTPs that impede or block the insulin signal cannot consequently be identified by their capacity to restore cell growth. This effect was found to be induced through direct activation of the insulin receptor with half-maximal effect at physiological relevant concentrations of the hormone. Therefore, the BHK-IR cell line can be used to identify PTPs with activity toward the receptor itself. Twelve different PTPs were analyzed by this procedure, and the closely related receptor-like phosphatases, PTPα (15) and PTPδ (16), were found to be efficient negative regulators of the IRTK.

MATERIALS AND METHODS

Antibodies—Antibodies used were mouse monoclonal anti-phospho-tyrosine antibody SE.2 (17), mouse monoclonal anti-PTP1B (Oncogene Science), mouse monoclonal anti-CD45, and polyclonal rabbit anti-peptide antisera against the other PTPs and the IRTK C terminus (CT104). Secondary antibody preparations (goat anti-mouse and goat anti-rabbit antibodies) were obtained from Bio-Rad.

Cell Culture—BHK cell lines were maintained at 37 °C under 5% CO₂ in Dulbecco’s modified Eagle’s medium containing 4.5 g liter⁻¹ glucose, 10% fetal calf serum, 2 mM L-glutamine, 1 mM methotrexate, and penicillin/streptomycin. Stable BHK-IR/PTP cell lines were maintained in complete medium in the presence of 100 nM insulin. The parental cell line BHK-IR was grown under the same conditions without insulin. BHK-IR was previously (14) established by transfection of the cell line BHK tkts13 with CDNA encoding the insulin receptor (ATCC CRL 1632) (18, 19). Insulin Dose-response and Time Course Experiments—For analysis of the insulin dose response, BHK-IR cells were seeded at a density of 1 × 10⁴ cells/well in 6-well plates and incubated for 20 h at the conditions described above. Human insulin was added corresponding to the indicated final concentrations, and the plates were incubated for further 24 h, after which the numbers of adherent and nonadherent cells were determined using a Coulter counter (triplicate). Similar conditions were used for the time course experiments, except that the final insulin concentration was 100 nM and that the numbers of adherent and nonadherent cells were determined at the indicated time intervals.

Growth Curves—Five 6-well plates were seeded with 2 × 10⁶ cells/well. After 24 h the number of adherent cells per well was determined in one plate (day 0). Insulin was added to the remaining four plates at a final concentration of 100 nM to three wells per plate (−insulin) with the other three wells as controls (−insulin). After a further 24 h of incubation the number of adherent cells was determined in one plate (day 1) while the remaining plates were washed three times to remove nonadherent cells, and fresh medium with and without insulin was added. This procedure was repeated every 24 h for the next 3 days.

Establishment of Stable Cell Lines—For the rescue experiments,

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‡ The abbreviations used are: IRTK, insulin receptor tyrosine kinase; IR, insulin receptor; IRS-1, insulin receptor substrate-1; BHK, baby hamster kidney cells; PTP, protein-tyrosine phosphatase; TC, T cell; LAR, leukocyte common antigen-related protein.
BHk-IR cells were seeded at a density of 5 × 10⁴ per 6-cm tissue culture dish and incubated for 20 h. Six μg of expression plasmids containing cDNAs encoding PTPs were transfected into the BHk-IR cells as described by Chen and Okayama (20) with empty vectors as a negative control. Eighteen hours later the cells were washed once, and fresh medium was added. For the next 12 days the cells were treated daily with insulin (100 nM final concentration), and nonadherent cells were removed. The rescued clones were then visualized by staining in 0.5% crystal violet (w/v) in 30% (v/v) methanol.

Western Blot Analysis—To analyze the influence of PTPs on the pattern of tyrosine-phosphorylated proteins, all cells were grown under standardized conditions. Exponentially growing cells were seeded in 14-cm tissue culture dishes (Nunc™ delta, Nunc Denmark) in complete Dulbecco’s modified Eagle’s medium without insulin at a density of 1 × 10⁶ cells/dish. The cells were cultured for 48 h, trypsinized, and seeded at a density of 200,000 cells/cm² containing 4 ml of medium. After a further 24 h, the cells were washed once in Dulbecco’s modified Eagle’s medium containing 0.5% fetal calf serum, glutamine, methotrexate, and antibiotics, and starved in the same medium for 22–24 h. Insulin was added at appropriate time intervals, and the cells were lysed directly in SDS loading buffer (20% (v/v) glycerol, 3% (w/v) SDS, 3% (v/v) 2-mercaptoethanol, 10 mM EDTA, 0.05% (w/v) bromphenol blue), boiled for 5 min, and analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting by loading lysates corresponding to the same number of cells in all lanes. To assess the cell numbers, four 6-cm dishes from each of the cell lines were grown in parallel with those used for immunoblotting. These dishes were trypsinized to obtain the cell number using a Coulter counter. The enhanced chemiluminescence system (Amersham Corp.) was used for detection of phosphorysorine and protein antigens on immunoblots.

RESULTS

Insulin-mediated Growth Inhibition of BHk-IR Cells—With the objective of identifying PTPs that negatively regulate the insulin receptor activity we developed a novel insulin-based selection strategy. A previously established BHk-IR cell line (14) was found to possess two essential properties for an efficient dominant selection strategy. First, like its parental line BHK tk-ts13 it can be transfected very efficiently with DNA (18). Second, it exhibits high levels of IR expression and responds to insulin stimulation with growth inhibition, rounding, and detachment from the substratum of cell culture dishes (Fig. 1A). These phenotypic changes were specific for BHk-IR cells and could not be evoked in the parental line or transfected BHk control cells overexpressing ELAM1 (Fig. 1B). We reasoned that these responses could be used to establish conditions where insulin would completely inhibit the growth of adherent cells. Thence, molecules that negatively control the insulin signal could be identified by virtue of their rescuing capacity upon transfection of corresponding cDNA expression plasmids into BHk-IR cells.

An Insulin-based Selection Method—To first establish conditions for the selection procedure we determined the optimal hormone concentration (Fig. 2A) and time of stimulation for achievement of a measurable cell response (Fig. 2B). Half maximal effect was observed at a concentration of approximately 1 nm, with an optimal concentration at about 100 nm. Rounding of cells started quickly after addition of insulin, with detachment being detectable as early as 1 h after insulin stimulation. A significant growth arrest could be observed after 24 h (Fig. 2B). Therefore, the growth of adherent BHk-IR cells could be fully inhibited by insulin treatment in analogy to conventional dominant selection systems, e.g. methotrexate (Fig. 2C).

We then tested whether expression of PTPs in BHk-IR cells could counteract the insulin-induced effects. Twelve different PTP cDNAs inserted into cytomegalovirus promoter-based plasmids were transfected into BHk-IR cells (20) which were subsequently kept under selection pressure with insulin for 14 days. This time allowed for the growth of individual insulin resistant cell clones that were stained and counted (Fig. 2D; Table 1). Two closely related receptor-type PTPs, PTPα (15) and PTPc (16), and with a lower efficiency, the intracellular TC-PTP (21) were most efficient in suppressing cell detachment. Very few clones were obtained with CD45 (22), PTP1C (23), PTPH1 (24), and PTP1B (25), whereas LAR (26), PTPα (27), PTPα (28), PTP1D (29), PTP1D (30) did not have any effect on the restoration of cell attachment. Stable clones that were obtained in parallel assays were found to exhibit IR expression levels comparable to the parental BHk-IR line (Fig. 3). PTP overexpression was additionally verified in these clones by immunoblotting with specific antibodies (Fig. 4). It has recently been shown that PTPα, when transiently expressed in COS-1 cells, gives rise to two proteins of about 100 and 130 kDa (31). The 100-kDa species exhibits N-linked glycosylation only and is a precursor of the 130-kDa protein which in addition contains O-linked carbohydrates. We have also observed these two molecular species when PTPα is transiently expressed in human embryonic kidney fibroblast 293 cells and BHk-IR cells (not shown). In contrast, the fully glycosylated 130-kDa protein is the predominant form in the rescued BHk-IR cells (Fig. 4). Transient expression of PTPH1 results in a protein of about 105 kDa (not shown), which is in agreement with its predicted molecular mass (24). Interestingly, Yang and Tonks (24) found that reticulocyte lysate expression of PTPH1 yielded a protein...
of about 120 kDa, which is in complete agreement with the molecular mass of PTPH1 observed in BHK-IR/PTPH1 cells (Fig. 4). The molecular basis for this difference needs to be investigated further. For those PTPs that were not able to restore cell growth, transient expression in transfected

![Image of a graph showing cell number over insulin concentration and time course.](http://www.jbc.org/)

**Fig. 2.** An insulin-based selection principle for establishment of stable BHK cell lines overexpressing the insulin receptor and PTPs. Insulin dose-response (A) and time course (B): open bars, adherent cells; solid bars, nonadherent cells. Bar graphs indicate mean ± S.D. (triplicate). Similar results were obtained in three independent experiments. C, growth curves of BHK-IR with (■—■) and without (□—□) insulin. The graph shows the mean of the number of adherent cells in three wells per time point. Similar results were obtained in three independent experiments. D, representative examples of BHK-IR clones rescued by transfection with PTPα, PTPε, and TC-PTP cDNA inserted into cytomegalovirus promoter-based expression plasmids.

| Phosphatase | Transfection 1 | Transfection 2 |
|-------------|----------------|----------------|
| Receptor-like PTPs | | |
| PTPα | >200 | >200 |
| PTPε | >100 | >100 |
| LAR | 0 | 0 |
| PTPγ | 1a | 0 |
| PTPe | 1b | 0 |
| CD45 | n.d. | 6 |
| Intracellular PTPs | | |
| TC-PTP | 77 | 46 |
| PTP1B | 3 | 4 |
| PTP1C | 2 | 3 |
| PTP1D | 0 | 0 |
| PTPH1 | 3 | 2 |
| PTPD1 | 0 | 0 |
| Control | 0 | 0 |

a No IR expression.  
b Low IR expression.

**Table 1** Number of BHK-IR clones rescued with different PTP cDNAs

Fifty thousand BHK-IR cells were seeded per 6-cm Petri dish. After 24 h, the cells were transfected with 6 μg of cytomegalovirus-based vectors containing cDNA encoding the indicated PTPs. Eighteen hours later the cells were washed once and fresh medium added. For the next 12 days the cells were treated daily with insulin (100 nM final concentration) and nonadherent cells were removed. The rescued clones were then visualized by staining in 0.5% crystal violet (w/v) in 30% (v/v) methanol. Two insulin-resistant clones were observed in a total of 15 control transfections with pCMV vector. Both clones had lost IR expression.

**Fig. 3.** Analysis of phosphotyrosine-containing proteins in BHK-IR cells stably overexpressing PTPs. a, an immunoblot with anti-phosphotyrosine antibody of cells untreated or treated for 10 and 60 min with 100 nM insulin. The M, of marker proteins, IR precursor (P), IR β-subunit (β), and pp35 are indicated. b and c, same as a, but a shorter exposure showing IR β-subunit from cells treated with 100 nM and 2 nM, respectively; d, control of the expression levels of IR by blotting with a polyclonal anti-IR antibody.
BHK-IR cells was monitored to confirm the capability of the BHK cells to express these phosphatases (not shown). Further, an inactive mutant of PTPα was unable to restore cell attachment despite the fact that it exhibited even higher levels of expression than the intact enzyme when transiently expressed in BHK cells (not shown). These results suggest that PTPs can specifically interfere with insulin-dependent signaling.

PTPα and PTPε Dephosphorylate the Insulin Receptor—To investigate the mechanism underlying the PTP-mediated rescue and to identify putative PTPs with direct activity toward the IRTK, we compared the pattern of tyrosine-phosphorylated proteins in selected BHK-IR/PTP cell clones. Pallen and Tong (32) recently found that the membrane tyrosine-phosphatase activity of Swiss 3T3 cells is about 8-fold higher in confluent cells than in cells harvested at low or medium density. In accordance with this we found that the insulin-mediated growth arrest as well as the overall tyrosine phosphorylation level of BHK-IR cells depend on the cell density (not shown). Therefore, we employed a strict and standardized scheme for passage of the cells before analysis as described under “Materials and Methods.”

After insulin stimulation several proteins were phosphorylated, among which the IR β-subunit and bands at 190, 75–80, 63, and 35 kDa were most prominent (Fig. 3). The PTPs that most potently restore cell growth of insulin-treated BHK-IR cells, i.e. PTPα and PTPε, also appeared to be the most effective in reducing tyrosine phosphorylation of the IR β-subunit and the unidentified 75–80 kDa kinase substrates, whereas intracellular PTPs showed little, if any, activity. Interestingly, CD45, which is normally expressed only in cells of the immune system, had a similar influence on the overall tyrosine phosphorylation of the IR β-subunit in BHK-IR cells when compared with PTPs α and ε but was less effective on pp35 and very inefficient in preventing cell detachment (Fig. 3, Table I). This suggests to us that CD45 is not directed toward the regulatory tyrosine 1150 domain, but phosphorylated tyrosine residues that reside outside this region and therefore are not involved in the activation of the IRTK.

Several studies have addressed the question of PTP specificity using synthetic peptides (39–42). These investigations have provided important insight with respect to primary structural sequence requirements for substrate recognition. However, an obvious limitation of this approach is the lack of defined three-dimensional structure of the peptides. Likewise, the PTPs utilized for these analyses are removed from their natural environment. Since at least part of the PTP specificity seems to be conveyed by a defined subcellular localization (43), it is essential that PTP activity toward cellular substrates is tested in intact cells. We believe that the present method with its functional coupling and presumed correct localization of the key elements to specific subcellular compartments provide the necessary tools for analysis of PTP specificity toward IRTK.

This study indicates that receptor-like PTPs play a significant role in regulating the IRTK, whereas intracellular PTPs have little, if any, activity toward the insulin receptor. Even though TC-PTP efficiently prevents insulin-induced detachment of BHK-IR cells, the effect is not as pronounced as with PTPα and PTPε. While it appears that the target of the negative regulatory activity of PTPs α and ε is the receptor itself, the down-modulating effect of the intracellular TC-PTP seems to be due to a downstream function in the IR-activated signal resulting in cell detachment. We speculate that pp35 could be a direct target of TC-PTP. Although PTP1B and TC-PTP are closely related, PTP1B is only weakly active in the present selection system and has only little influence on the phosphorylation pattern of insulin-treated BHK-IR cells. Both PTPs have distinct structural features that determine their subcel-
ular localization and thereby their access to defined cellular substrates (44, 45). Therefore, the lack of activity of PTP1B and TC-PTP toward the IRTK may, at least in part, be explained by the fact that they do not co-localize with the activated insulin receptor. In support of this view, PTP1B and TC-PTP have been excluded as candidates for the IR-associated PTPs in hepatocytes based on subcellular localization studies (10).

It was recently found that the ubiquitously expressed SH2 domain containing protein tyrosine phosphatase, PTP1D (29), associates with and dephosphorylates IRS-1, but apparently not the IR itself (46, 47). Further, PTP1D seems to act as a positive mediator in insulin-stimulated Ras activation (48) and of growth factor-induced mitogenic signal transduction (49). It is in agreement with these studies that PTP1D does not exhibit any rescuing capacity in our selection system which is designed to identify negative regulators only. In contrast, the structurally related SH2-containing phosphatase, PTP1C, which is predominantly expressed in hematopoietic cells, seems to act as a negative regulator of growth factor-stimulated proliferation (50–52). In accordance with this we have observed that PTP1C can act as a weak, negative regulator of the insulin response in BHK-IR cells (Table I). However, even though PTP1C is heavily phosphorylated after insulin treatment of BHK-IR cells, it has only weak, if any, activity toward the IRTK (Fig. 3) (48).

Two intracellular PTPs with ezrin-like N-terminal domains, PTPD1 (30) and PTPH1 (24), were also analyzed for their capacity to negatively regulate the insulin signal in BHK-IR cells. Both PTPs have been proposed to localize to the junction of the cytoskeleton and the plasma membrane. We have previously shown PTPD1 to be phosphorylated by and associated with c-Src in vitro and hypothesized it to be involved in the regulation of phosphorylation of focal adhesions (30). PTPD1 is thus an unlikely candidate as regulator of the IRTK. In agreement with this, PTPD1 did not show any effects at all, whereas PTPH1 gave rise to very few stable cell clones, but did not lead to a change in the phosphorylation pattern of BHK-IR cells.

With regard to the molecular basis of the BHK-IR cell detachment response, several observations point to a phosphoprotein of 35 kDa as an important mediator (Fig. 3; Table I: not shown): (i) the rescuing capacity of PTPs inversely parallels the degree of pp35 phosphorylation in BHK-IR/PTP cell lines; (ii) confluent BHK-IR cells respond poorly to insulin with respect to cell detachment and show relatively low levels of pp35 phosphorylation; (iii) transient expression of v-Src in BHK-IR results in massive rounding and detachment of the cells with a concomitant intense phosphorylation of pp35; (iv) time course studies show that p35 phosphorylation increases even further several hours after insulin stimulation, i.e. corresponding to the occurrence of rounding of the cells. Interestingly, in A431 cells the epidermal growth factor induces phenotypic changes similar to insulin in BHK-IR cells and also causes tyrosine phosphorylation of a 35-kDa polypeptide (53, 54). This protein was identified as a member of the lipocortin/annexin family (54), which has previously been shown to include substrates of the IRTK in vitro and in intact hepatocytes after corticosteroid treatment (55). Whether pp35 in BHK-IR cells belongs to this class of proteins remains to be investigated.

PTPs or other proteins that would impede or block any of the signaling steps leading to the insulin-induced phenotypic changes of the BHK-IR cells could be identified by the method presented here. These changes are prerequisites for the selection principle and the establishment of stable cell lines with functionally coupled expression of IR and opposing PTPs. Some of the downstream elements involved in induction of growth inhibition and detachment of BHK-IR cells may not represent molecules that actually mediate the effects of insulin in vivo.

Nevertheless, since the changed phenotype of the BHK-IR cells is induced via normal activation of the insulin receptor our procedure allows, for the first time, identification of PTPs as regulators of insulin signaling at the level of the receptor itself.

While previous reports have suggested a role of PTPs in signal transduction through Src activation (56, 57) and interaction with GRB-2 (58, 59), our results suggest a function for this phosphatase and its close relative PTPs a and b as negative regulators of the insulin receptor. In view of recent findings indicating the crucial importance of signal duration and therefore negative regulation of tyrosine kinase signals (60, 61), PTPs a and b may be key elements in the definition of insulin action in different tissues and in the pathophysiology of non-insulin-dependent diabetes.

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