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Mutant Forms of the Protein Tyrosine Phosphatase α Show Differential Activities towards Intracellular Substrates

Reiner Lammers,[1] Niels Peter H. Møller,[2] and Axel Ullrich
Max-Planck-Institut für Biochemie, Am Klopferspitz 18A, D-82152 Martinsried, Munich, Germany

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BHK cells overexpressing five million IR (BHK-IR) respond to insulin with reduced growth and detachment from the dish surface. We have recently identified protein tyrosine phosphatase (PTP) α as a negative regulator of the insulin receptor (IR) tyrosine kinase that is able to rescue BHK-IR cells from the insulin effect. In this report we describe the effect of several point mutations in PTPα on the phosphatase activity and regulation of insulin signaling in BHK-IR cells. Analysis of total cellular phosphotyrosine protein revealed several molecules that were dephosphorylated when PTPα or a phosphatase active mutant was overexpressed. By contrast, some proteins were tyrosine phosphorylated as strong or to an even higher extent as in the parental line when PTPα Y798F was present. We conclude that mutation of the carboxyterminal tyrosine in PTPα uncovers a dual function of this phosphatase in BHK cells: reduction of the IR signal and activation of an endogenous kinase. © 1998 Academic Press

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Tyrosine phosphorylation is an important control mechanism for the catalytic activity of many proteins and also regulates the assemblage of protein complexes. Consequently, tyrosine kinases are found in different tissues and are involved in the regulation of proliferation, differentiation and metabolism (1). In the adult organism, insulin regulates glucose homeostasis (2). The insulin receptor (IR) is a heterotetrameric transmembrane protein and contains an intrinsic tyrosine kinase activity (3). The binding of insulin to the extracellular domain of the IR activates the intracellular tyrosine kinase, leading to autophosphorylation of several tyrosines including three closely positioned tyrosine residues around tyrosine 1150. In this state the kinase is activated and now phosphorylates substrate proteins including IRS-1,-2,-3 and-4, SIRP, SHP-2 and SHC (reviewed in 4). In turn, these proteins then serve as docking molecules for other proteins, for example, phosphorylated IRS binds the regulatory subunit of the PI3-kinase. This activates the PI3-kinase catalytic subunit and, e.g., eventually mediates an increased glucose transport into the cell. The integrity of this signaling process is of high importance for glucose homeostasis in the body, and disturbing its regulation can lead to diabetes mellitus (2).

The steady state of tyrosine phosphorylation of a protein is a consequence of the activity of the responsible kinase versus the activity of protein tyrosine phosphatases (PTPs). Phosphatases seem to be as diverse as kinases but much less about the regulation of phosphatase activity or the phosphatase substrates is known (5). They are located either at the cell surface as transmembrane proteins or are found in the cytoplasm. For transmembrane PTPs the extracellular domains can be rather small (PTPα or ϵ) or extremely large (PTPß) and then contain immunoglobulin or fibronectin homology domains. This is in contrast to the cytoplasmic part of these PTPs that is rather homologous and mostly consists of two PTP domains of which the aminoterminal is the most active. Most cytoplasmic PTPs contain only one PTP domain but carry different additional structural features like GLGF repeats (PTPH1, MEG1, BAS), SH2 (src homology 2; SHP-1,-2) or ezrin-like domains (PTPH1, D1, PEZ, MEG1, BAS). These motifs mediate the intracellular localization of the PTP and could thus contribute to their substrate specificity (reviewed in 6, 7).

Deregulation of the activity of PTPs impairs cellular signaling processes. This is demonstrated for erythropoietin signaling that is enhanced in mice lacking in-

[1] To whom correspondence should be addressed. Present address: Medizinische Klinik und Poliklinik, Eberhard-Karls-Universität, Otfried-Müller Str.10, D-72076 Tübingen. Fax: 49-7071-292784. E-mail:rrlammer@med.uni-tuebingen.de.
[2] Present address: Novo-Nordisk, Novo Alle, DK-2880, Bagsvaerd.

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tact SHP-1, the phosphatase responsible for downregulation of the erythropoietin signal (8). The signaling of the insulin receptor (IR) is also influenced by PTPs. The addition of a stearylated sulfo-tyrosyl peptide corresponding to the main autophosphorylation site of the IR to cells enhanced insulin signaling, probably by inhibition of a phosphatase specific for the IR (9). When in rat hepatoma cells the expression of PTPLAR was reduced using antisense oligonucleotides, insulin signaling was increased (10). In an extension of this experiment, overexpression of PTPLAR attenuated insulin signaling (11). Also, the IR and PTPLAR were found to be associated in a cell line overexpressing both proteins (12). By contrast, using a functional assay with a BHK cell line overexpressing 5 million IR, we did not find an effect of PTPLAR but PTPα on insulin signaling (13). In this cell line insulin addition had a growth inhibiting effect and cells detached from the dish. Transfection of different PTPs into BHK-IR cells revealed that PTPα had the highest capacity to rescue cells. The interaction of PTPα and the IR was further confirmed in a transient expression experiment where PTPα dephosphorylated the ß-subunit of the mature IR (14). In the present study, we investigated the effect of several mutations in PTPα on substrate dephosphorylation and its capability to rescue BHK-IR cells from insulin treatment.

MATERIALS AND METHODS

DNAs, antibodies and cell lines. All cDNAs were cloned into a cytomegalovirus early promoter based expression plasmid and have been described earlier (15, 13). PTPα mutants were generated using standard cloning procedures. Polyclonal antisera were generated in rabbits using KLH coupled carboxyterminal peptides of the IR, PTPα and SHP-2 and a GST-fusion protein including the carboxyterminal 200 amino acids for SHC. Monoclonal antibody D2 is directed against an unknown epitope of the intracellular domain of PTPα and does not interfere with binding of grb2 to the phosphorylated Tyr798. Anti-phosphotyrosine antibody 4G10 was obtained from UBI, antibody 5E.2 is described in (16). The generation of BHK cell lines overexpressing the IR and PTPα has been described by (13). BHK-IR cells were grown in DMEM with 10% fetal calf serum and 2 mM L-glutamine, 293 cells in F13/DMEM, 50:50 with 10% fetal calf serum, 2 mM L-glutamine.

Transient expression experiments and protein analysis. Transient expression experiments were performed as described earlier (15). Briefly, 2 μg of plasmid DNA encoding the insulin receptor or the phosphatase were transfected into 3×10^5 cells/10-cm^2 well according to the protocol of (17). Eighteen hours after the addition of DNA precipitate, cells were washed once, supplied with fresh medium containing 0.5 % serum and harvested the next day.

BHK and 293 cells were stimulated with 100 ng/ml insulin for 10 min and then lysed in 200 μl lysis buffer per 10 cm^2 dish (50 mM HEPES, pH 7.2, 150 mM NaCl, 1.5 mM $MgC1_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 100 mM NaF, 10 mM sodium pyrophosphate and 1 mM Na-orthovanadate). The lysate was centrifuged for 2 min at 12500 g, 30 μl of the supernatant taken, sample buffer added, proteins boiled for 10 min and analyzed by SDS-PAGE and immunoblotting. Alternatively, antibody and ProteinA-Sepharose were added to the lysate supernatant, incubated for several hours at 4°C on a turning wheel, the Sepharose beads collected, washed and subjected to immunoanalysis as described above.

RESULTS

Generation of Mutants and Evaluation of Activity

To investigate more closely the role of PTPα in rescuing BHK-IR cells we generated several point mutants leading to the following amino acid exchanges: Cys442 or Cys732 to Ser (inactivation of the catalytic centers of PTPα phosphatase domains I and II, respectively); Tyr 781 to Phe (possible target for the

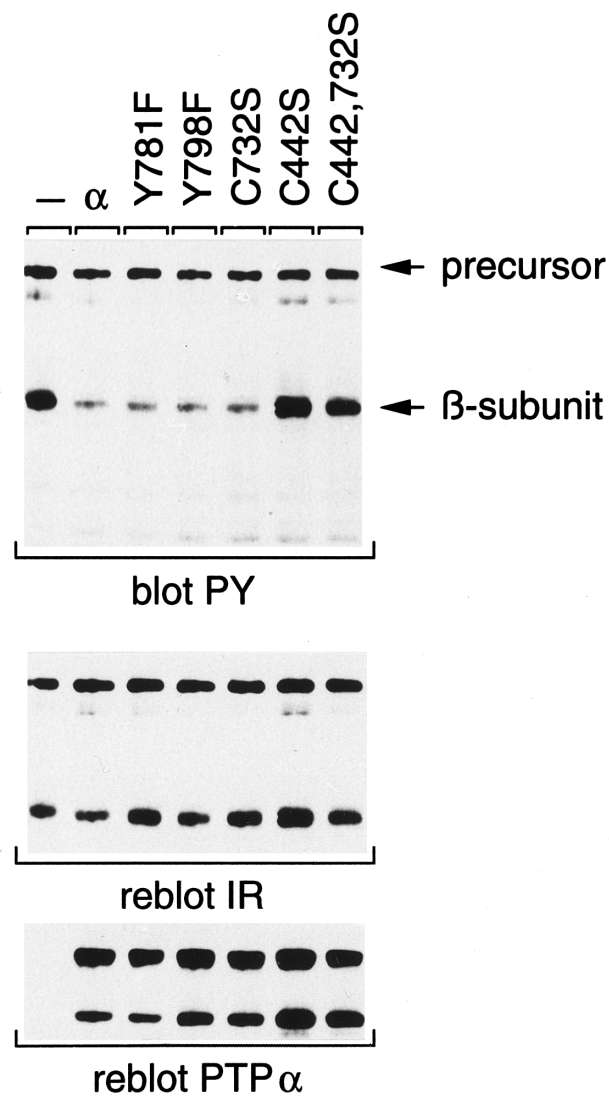

FIG. 1. Dephosphorylation of the IR by PTPα and PTPα mutants. The IR was transiently overexpressed in 293 cells either alone or together with PTPα or PTPα mutants. After stimulation with insulin for 5 min cells were lysed and an aliquot of the total lysate analysed by SDS-PAGE and immunoblot with anti-phosphotyrosine antibody 5E.2. Subsequently, expression of the IR and PTPα were verified by reblotting with the appropriate antibodies.

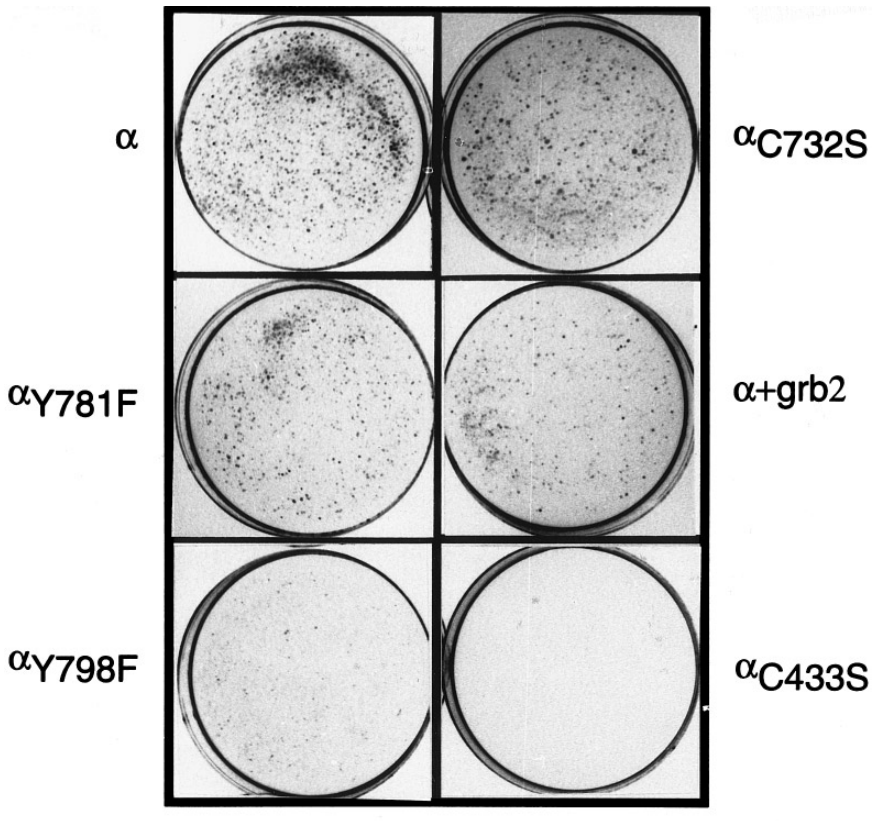

FIG. 2. Capability of PTPα mutants to rescue BHK-IR cells. BHK-IR cells were transfected with the indicated forms of PTPα or PTPα plus grb2. After 36 hours insulin was added to the medium and thereafter the medium changed daily with two washes. Fourteen days later, the cells were stained with crystal violett.

kinase CSK in analogy to CD45 (18)); and Tyr798 to Phe (constitutively phosphorylated in intact cells and binding site for the adapter protein grb2). The numbering of amino acids is according to (19). Like the native PTPα, the mutant cDNAs were cloned into an expression vector under the control of a cytomegalovirus immediate early promoter. Transfection of 293 cells yielded transiently expressed PTPs of the expected size of 130 kD and the incompletely glycosylated form of 100 kD. Cell lysates overexpressing PTPα or the mutant were tested for phosphatase activity *in vitro* with para-nitrophenyl phosphate as a substrate. No significant differences in activity were found for PTPα versus Y781F, Y798F, C732S or when PTPα was coexpressed with grb2 (data not shown). PTPα C442S, as expected, was not significantly active, confirming that the major activity of PTPα resides in the aminoterminal PTP domain (20). We next tested the activity of these mutants in intact cells. IR transiently overexpressed in 293 cells either alone or together with PTPα or the PTPα mutants was stimulated with insulin, lysed and IR phosphotyrosine content determined by immunoanalysis of total cell lysate with phosphotyrosine antibody. As shown in Fig.1 the phosphotyrosine content of the IR was clearly reduced upon coexpression with PTPα, tyrosine mutants of PTPα and PTPα carrying an inactivated carboxyterminal phosphatase domain. Completely inactivated PTPα C442, 732S or PTPα with an inactivated aminoterminal domain were not capable to downregulate IR tyrosine phosphorylation. The expression level of the overexpressed proteins was verified by reblotting with polyclonal antibodies directed against the IR or the PTPα carboxyterminus. This experiment confirms the data from the *in vitro* assay. It also shows that mutation of the carboxyterminal tyrosines as well as inactivation of the carboxyterminal phosphatase domain has no effect on the phosphatase activity against the IR.

Upon transfection of BHK-IR cells with a PTPα expression plasmid the cells become resistant to the growth inhibitory effect of insulin. They do not detach from the dish and form colonies while cells that do not take up DNA are washed away during the course of the experiment. The colonies can then be stained with crystal violett (13). Evaluation of the activity of PTPα versus the mutants in this system yielded similar results as determined for the *in vitro* and *in vivo* PTPα activity (Fig.2). No significant differences in the number of colonies were found when plasmids encoding catalytically active PTPα

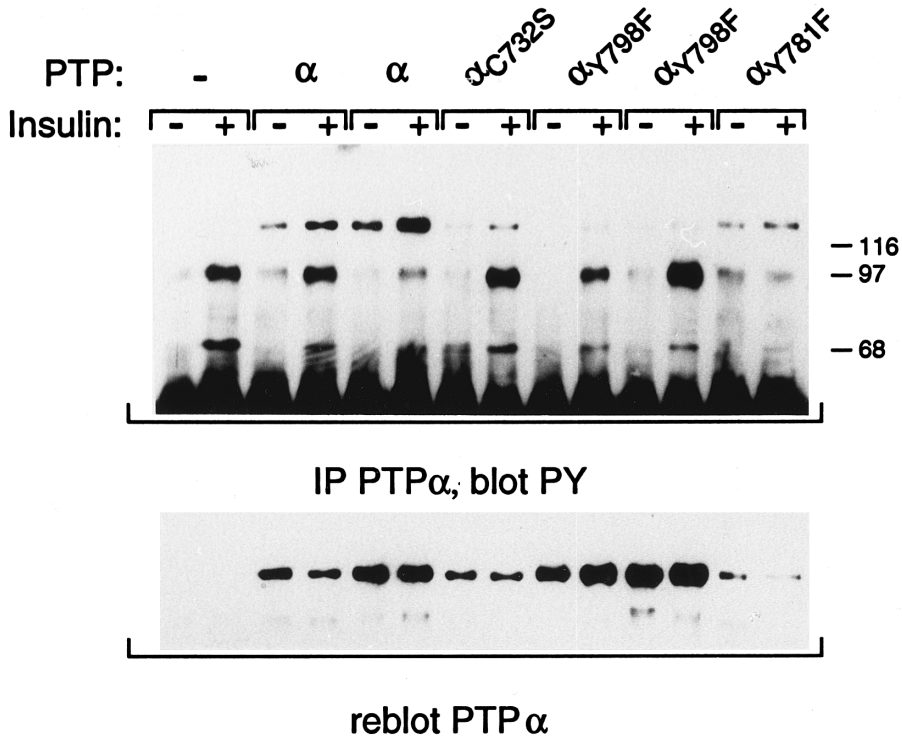

FIG. 3. Tyrosine phosphorylation of PTPα or PTPα mutants in BHK-IR cells. Individual colonies from a BHK-IR rescue experiment were isolated and the cells expanded. Serum starved cells were treated with insulin for 5 min as indicated, lysed and PTPα immunoprecipitated with antibody D2. Immunoprecipitates were analysed by SDS-PAGE and immunoblot with anti-phosphotyrosine antibody 4G10. The amount of PTPα was subsequently verified by reblotting with antibody D2.

forms were transfected. Transfections of the plasmid encoding PTPα C442S did not yield any colonies, thus documenting the lack of phosphatase activity in this mutant and proving the specificity of the experiment. To investigate the consequences of a parallel increase in expression of PTPα and grb2, the corresponding expression plasmids were cotransfected. Again, as for the *in vitro* determination of activity, no effect on the rescuing of BHK-IR cells was found. Interestingly, transfections with the PTPα Y798F plasmid repeatedly showed a significantly lower degree of staining. Microscopic inspection of stained dishes revealed that the number of colonies was similar to that achieved by transfection with native PTPα expression plasmid but that the morphology was different. Cells were not forming a close contact and therefore not staining well. When growing these cells part of them was rounding up and had a tendency to lift off the plate even in the absence of insulin (data not shown). Thus, the different forms of active PTPα show a similar phosphatase activity, but the mutation of the carboxyterminal tyrosine has an additional effect in cells overexpressing this protein.

Tyrosine Phosphorylation of PTPα Is Increased upon Insulin Treatment

From experiments similar to the one described insulin resistant colonies were isolated from dishes transfected with PTPα or PTPα mutant expression plasmids, cells expanded and characterized more closely. First, the phosphorylation and expression of PTPα was tested (Fig.3). The parental cell line, two clones each overexpressing PTPα or Y798F and one clone each overexpressing PTPα C732 or PTPα Y781F were stimulated with insulin as indicated. After lysis, PTPα was immunoprecipitated with monoclonal antibody D2 that is directed against the intracellular domain. Analysis of the phosphotyrosine content of the immunoprecipitate revealed a constitutive tyrosine phosphorylation of the 130 kD PTPα proteins that was significantly increased by insulin treatment. PTPα Y798F lacks the main tyrosine phosphorylation site and therefore was only weakly phosphorylated. Proteins of 97 and 70 kD were immunoprecipitated unspecifically since they are also seen in the parental cell line not overexpressing PTPα. A cross reaction of the secondary antibody with the immunoglobulin heavy chain of the immunoprecipitating antibody can be seen at the bottom of this part of the figure.

Stripping the blot from phosphotyrosine antibody and probing with antibodies specific for PTPα confirmed expression in all clones tested but showed different expression levels. For native PTPα the clone with higher expression also showed an increased phosphotyrosine signal. The low level of tyrosine phosphoryla-

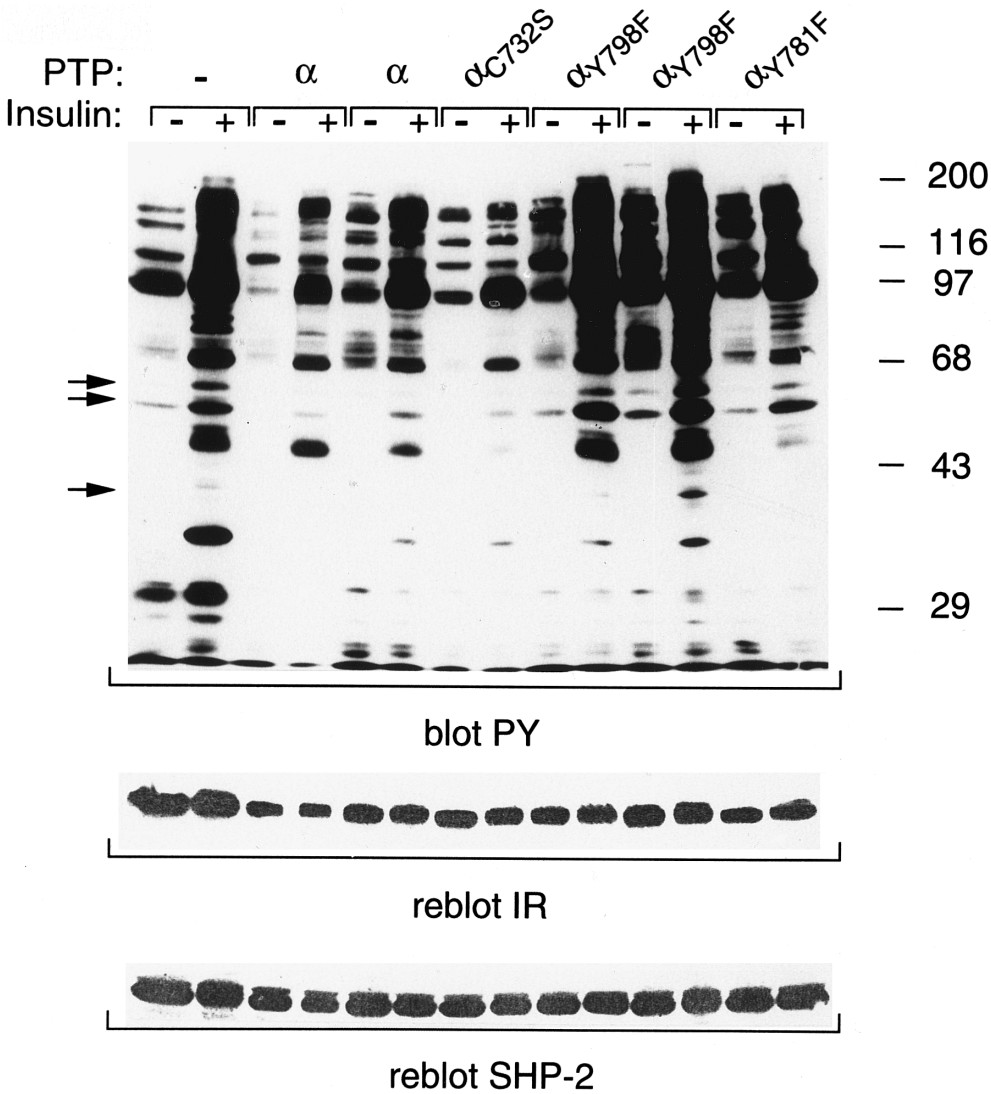

FIG. 4. Insulin induced tyrosine phosphorylation pattern in BHK-IR cells overexpressing PTPα or PTPα mutants. BHK-IR cells overexpressing PTPα or PTPα mutants were serum starved, stimulated with insulin for 5 min and lysed. An aliquot of the cell lysate was size separated by SDS-PAGE and phosphotyrosine determined in an immunoblot. Subsequent reblotting verified similar amounts of the overexpressed IR in these cell lines, while blotting with antibodies against SHP-2 verified the loading of similar amounts of protein.

tion of PTPα C732S and PTPα Y781F was due to their low expression level while PTPα Y798F. In conclusion, in BHK-IR cells insulin is capable of increasing the tyrosine phosphorylation on PTPα.

Effect of PTPα Mutants on the Pattern of Protein Tyrosine Phosphorylation

To detect possible substrates of PTPα we compared the insulin dependent protein tyrosine phosphorylation pattern in the different cell lines (Fig.4). After insulin stimulation many phosphotyrosine containing proteins were detected in the parental BHK-IR cells, most prominently a band at 97 kD that represents the IR ß-subunit. Different from our previous study (13), antibody 4G10 was used for phosphoprotein analysis and detected a distinct pattern of tyrosine phosphorylated proteins. When native PTPα was overexpressed the overall phosphotyrosine content of proteins was reduced in comparison to the parental line. This was also true for overexpression of the PTPα mutant proteins. However, proteins of 55 and 60 kD (upper arrows) were not affected when overexpressing PTPα Y798F. Despite the high level of Y798F expression a protein of 40 kD (lower arrow) was phosphorylated to an even higher extent than in the parental cells. Expression of similar levels of the IR in the different cell lines was confirmed by reblotting with antibodies directed against the IR while the loading of similar amounts of protein was verified by reblotting with antibodies

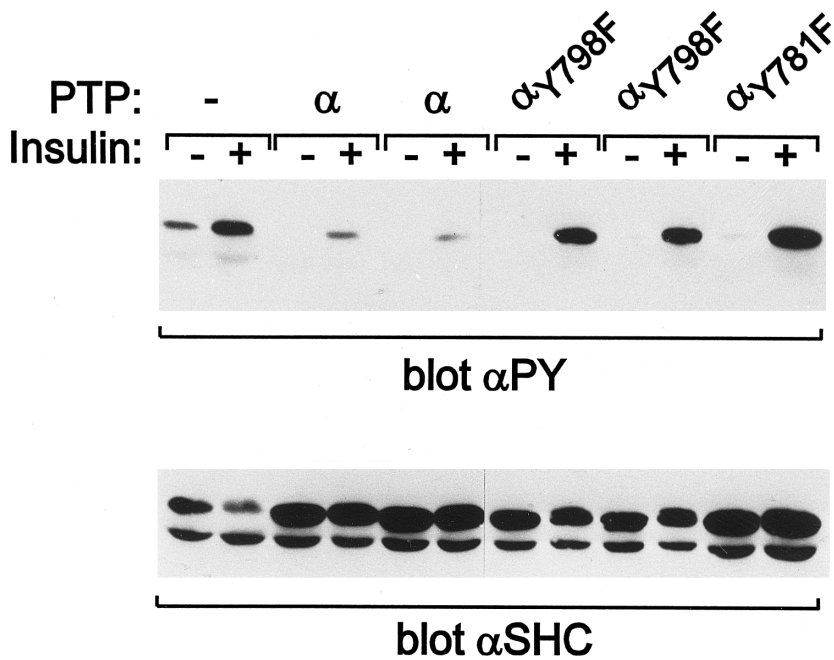

FIG. 5. Tyrosine phosphorylation of SHC in BHK-IR cells overexpressing PTPα or PTPα mutants. Cells were treated as described in Fig.4 and SHC immunoprecipitates analysed by blotting with anti-phosphotyrosine antibodies. The presence of similar amounts of SHC was verified by reblotting with anti-SHC antibodies.

directed against SHP-2. Taken together, the analysis of total protein tyrosine phosphorylation revealed distinct effects upon overexpression of the PTPα Y798F mutant.

Based on these results we next wanted to identify specific proteins as different targets of PTPα and the PTPα Y798F mutant. However, we were not able to detect a differential dephosphorylation upon expression of the PTPα mutants for SHP-2 or IRS-1. This was different for the IR substrate SHC (Fig.5). Immunoprecipitated SHC proteins were first blotted with anti-phosphotyrosine antibody and then, after stripping off this antibody, reblotted with anti-SHC antiserum. Because the SHC antibody has a lower affinity for SHC that had been detected by a phosphotyrosine antibody the amount of the 52 kD form varies while the 46 kD form that was weaker phosphorylated is similar in all lanes and documents similar amounts of SHC protein in all lanes. Tyrosine phosphorylation of SHC was reduced in the PTPα expressing cells while in cells expressing the Tyr to Phe mutants of PTPα it was even stronger than in the parental cell line.

DISCUSSION

Overexpression of PTPα in murine embryonal carcinoma cells leads to neuronal differentiation upon retinoic acid treatment, probably mediated by an activation of the src kinase (21). In the same way, overexpression in rat embryo fibroblasts also activates the src kinase and leads to cell transformation (22). In contrast to this role in mitogenesis and differentiation, PTPα was identified as an effective down regulator of the IR kinase (13, 14). In the present study we have started to analyze the effect of PTPα on the IR signaling in BHK cells. Mutagenesis of amino acids that may be important for the function of PTPα could change the activity of the phosphatase and provide a hint on the interaction with the IR.

By separately inactivating the phosphatase domains we could show in the rescue experiment that in intact cells the capability of PTPα to dephosphorylate the IR was dependent only on the activity of the aminoterminal phosphatase domain of PTPα. Although the carboxyterminal domain does have an intrinsic activity (20), it either possesses a different substrate specificity, or its reduced activity is not sufficient to rescue the BHK-IR cells.

In intact cells PTPα is constitutively phosphorylated on its carboxyterminal tyrosine and binds the adaptor protein grb2 (23, 24). The meaning of this interaction is not clear since no change in phosphatase activity upon grb2 binding was found, and neuronal differentiation in P19 cells is also inducible when a PTPα mutant unable to bind grb2 is overexpressed (25). Cotransfection of grb2 and PTPα into BHK-IR cells did not change the number of rescued cells, indicating that in intact cells enhanced grb2 expression and, as a consequence, association with PTPα is not impairing the rescue capability.

On the other hand, mutation of the carboxyterminal tyrosines to phenylalanine did not lead to a detectable difference in activity of PTPα *in vitro* or in 293 cells against the IR, and both mutants were capable of rescuing BHK-IR cells. Interestingly, BHK-IR cells rescued by the PTPα Y798F mutant were growing as colonies where the cells were not attached closely to each other, and even in the absence of insulin part of the cells was rounded up. This different behaviour of PTPα Y798F expressing cells was also reflected in the pattern of tyrosine phosphorylation. In cells overexpressing PTPα several proteins were dephosphorylated while in cells overexpressing PTPα Y798F some of these proteins were phosphorylated as strong as in the parental cell line. When immunoprecipitating SHC the tyrosine phosphorylation was also reduced only in the presence of PTPα but not PTPα Y798F. Therefore, overexpression of this mutant must play a dual role: it is capable of suppressing some responses of IR activation while others may even be enhanced. One way this could be achieved is by adopting a different substrate specificity. However, this seems unlikely, since most substrates should be phosphorylated to a lower extent when the IR phosphorylation is reduced (as shown in Fig.1) and the kinase therefore is less active. An alternative explanation involves the src kinase that in some cell types is activated by PTPα overexpression (19, 20). If in BHK cells PTPα Y798F would activate src stronger than native PTPα src could be responsible for the increased tyrosine phosphorylation of some proteins while others are not affected. We are currently investigating this possibility.

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