Biochemical and Biophysical Research Communications **267,** 96–102 (2000)
doi:10.1006/bbrc.1999.1901, available online at http://www.idealibrary.com on IDEAL®

Involvement of the Membrane Distal Catalytic Domain in Pervanadate-Induced Tyrosine Phosphorylation of Receptor Protein–Tyrosine Phosphatase α

Arjan Buist, Christophe Blanchetot, and Jeroen den Hertog[1]
Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

Received November 12, 1999

Receptor protein-tyrosine phosphatase α, RPTPα, is a typical transmembrane protein–tyrosine phosphatase (PTP) with two cytoplasmic catalytic domains. RPTPα became strongly phosphorylated on tyrosine upon treatment of cells with the PTP inhibitor pervanadate. Surprisingly, mutation of the catalytic site Cys in the membrane distal PTP domain (D2), but not of the membrane proximal PTP domain (D1) that harbors the majority of the PTP activity, almost completely abolished pervanadate-induced tyrosine phosphorylation. Pervanadate-induced RPTPα tyrosine phosphorylation was not restricted to Tyr789, a known phosphorylation site. Cotransfection of wild-type RPTPα did not potentiate tyrosine phosphorylation of inactive RPTPα-C433SC723S, suggesting that RPTPα-mediated activation of kinase(s) does not underlie the observed effects. Mapping experiments indicated that pervanadate-induced tyrosine phosphorylation sites localized predominantly, but not exclusively, to the C-terminus. Our results demonstrate that RPTPα-D2 played a role in pervanadate-induced tyrosine phosphorylation of RPTPα, which may suggest that RPTPα-D2 is involved in protein–protein interactions.

Key Words: **protein-tyrosine phosphatase; RPTPα; D2; tyrosine phosphorylation.**

Many important eukaryotic cell signaling pathways are controlled by protein tyrosine phosphorylation. The level of phosphotyrosine (P.Tyr) in cellular proteins is regulated by the balance of protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs). PTPs catalyze the hydrolysis of phosphoryl groups on Tyr residues in proteins. PTP family members share a conserved PTP domain of approximately 240 amino acids characterized by the signature motif [I/V]HCxAGxxR[S/T]G (1). Comparison of the crystal structures of PTPs that have been solved thus far indicate that the tertiary structure of PTP domains is highly conserved (2–8).

The PTP family can be subdivided based on structural differences into receptor-like (RPTP) and cytosolic proteins (9). RPTPs, with CD45 as the founding member (10), consist of an extracellular domain, a single membrane spanning domain, and a cytoplasmic domain. Most RPTPs contain two tandemly repeated PTP domains in their cytoplasmic domain. For all RPTPs with two PTP domains, the majority of the catalytic activity resides within the membrane proximal PTP domain (D1) whereas the membrane distal domain, D2, displays little or no catalytic activity. The strongest indication for catalytic activity in D2 comes from work on RPTPα. RPTPα-D2 has been shown to have intrinsic activity when expressed in the absence of D1 (11, 12). Using artificial substrates, RPTPα-D2 was found to be only 10-fold less active than RPTPα-D1. However, the activity of RPTPα-D2 towards P.Tyr-containing peptides was shown to be several orders of magnitude lower than D1 (12). Inactivation of RPTPα-D1 is sufficient to abolish its biological activity (13). In addition, mutation of the critical Cys in CD45-D1, but not in D2, prevented restoration of CD45-mediated T cell signaling events, indicating that PTP activity in D1, but not D2, was essential for this function (14).

To elucidate the function of PTPs *in vivo* it is essential to know their natural substrates. Therefore, "substrate trapping" mutants have been designed. Mutation of the catalytic site Cys to Ala in YopH, a Yersinia PTP, resulted in a mutant that could still bind to substrates, but was no longer able to dephosphorylate them making this an excellent tool for identifying potential substrates (15). Other PTPs with catalytic site Cys to Ser or Ala mutations have been used successfully in substrate trapping experiments as well (16–19).

We set out to use substrate trapping mutants to identify potential substrates of RPTPα. To induce ty-

[1] To whom correspondence should be addressed. Fax: +31 30 2516464. E-mail: hertog@niob.knaw.nl.

0006-291X/00 $35.00

rosine phosphorylation of potential substrates, RPTPα expressing cells were treated with the PTP inhibitor pervanadate to increase overall P.Tyr content of all proteins in the cell. No potential substrates were co-immunoprecipitated with a substrate trapping mutant of RPTPα. However, we observed a strong pervanadate-induced tyrosine phosphorylation of RPTPα itself. Surprisingly, mutation of the catalytic site Cys in RPTPα-D2 almost completely abolished pervanadate-induced tyrosine phosphorylation, while mutation of the catalytic site Cys in D1 had only moderate effects, suggesting that RPTPα-D2 and not D1 is somehow involved in RPTPα hyperphosphorylation. RPTPα is phosphorylated on Tyr789 *in vivo* (20). Here, we demonstrate that pervanadate-induced tyrosine phosphorylation was not restricted to Tyr789. Since pervanadate treatment of cells by itself was sufficient to induce tyrosine phosphorylation of RPTPα, we conclude that the PTK responsible is constitutively active. Site-directed mutagenesis and partial digestion of hyperphosphorylated RPTPα suggested that pervanadate mainly, but not exclusively induced tyrosine phosphorylation in the C-terminus. From these results, we conclude that RPTPα-D2 is involved in pervanadate-induced tyrosine phosphorylation of RPTPα, which may be mediated by protein–protein interactions via its catalytic site.

EXPERIMENTAL PROCEDURES

Cells and transfections. SK-N-MC neuroepitheloma cells were cultured in a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 (DF) medium supplemented with 10% fetal calf serum (Gibco, Paisley, UK). Transient transfection of SK-N-MC was done using calcium phosphate precipitation, as described (13).

Plasmids and site-directed mutagenesis. SV40 driven expression vectors, based on pSG-5 (21), for expression of RPTPα or mutants have been described (20). A hemagglutinin epitope tag was cloned at the N-terminal site of RPTPα as described (22). Site-directed mutagenesis was done on pSG-RPTPα or on pSG-HA-RPTPα. Mutations were verified by sequencing. The oligonucleotides that were used for site-directed mutagenesis were (numbering of residues in RPTPα according to (23)): RPTPα-C433S, ACC TGC ACT GGA GTG GAC CAC; RPTPα-C723S, CCC GGC ACT GGA GTG GTG CAC AGT; and RPTPα-Y789F, GCC TTT TCA GAT TTT GCC AAC TTC AAG.

Pervanadate treatment, immunoprecipitation and immunoblotting. Nearly confluent cells were treated with 1 mM orthovanadate and 1 mM H_2O_2, directly into the medium, generating pervanadate. The cells were treated for 30 min and lysed in CLB (50 mM HEPES pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 10 U/ml aprotinin, 1 μM PMSF, 200 μM sodium orthovanadate). Immunoprecipitation was done by incubation with anti-haemagglutinin epitope tag antibody (MAb 12CA5) and protein A-Sepharose (Pharmacia, Uppsala, Sweden) for 3 h at 4°C. The beads were washed four times with HNTG (20 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 10 U/ml aprotinin, 1 μM PMSF), resuspended in Laemmli sample buffer and boiled for 5 min, and the samples were loaded onto 10% SDS-polyacrylamide gels. For immunoblotting analysis the material on the polyacrylamide gels was transferred to Immobilon (Millipore, Bedford, MA). Blots were probed with anti-P.Tyr antibody PY-20 (Transduction Laboratories, Lexington, KY), 12CA5 antibody, affinity-purified anti-RPTPα antiserum 5478, raised against the entire cytoplasmic domain, or affinity-purified anti-RPTPα antiserum 5476, raised against a C-terminal peptide (CYKVVQEYIDAFSDYANFK). The primary antibodies were detected with horseradish peroxidase (HRP)-conjugated anti-mouse antibody or HRP-conjugated protein A (Transduction Laboratories, Lexington, KY) and enhanced chemiluminiscence (ECL). Before reprobing with different primary antibodies, the blots were stripped by incubation in stripping buffer (62.5 mM Tris, pH 6.8, 2% SDS, 100 mM β-mercaptoethanol) for 45 min at 70°C.

Proteolytic analysis. SK-N-MC cells, transiently transfected with wild type pSG-HA-RPTPα or pSG-HA-RPTPα-Y789F, were incubated with 1 mM orthovanadate (Sigma, St Louis, MO), and 1 mM H_2O_2. The cells were washed two times with PBS and lysed in CLB. Immunoprecipitation with anti-hemagglutinin epitope tag antibody (MAb 12CA5) was done as described above. The samples were loaded onto SDS-polyacrylamide gels. RPTPα was cut out and eluted from the gel, precipitated with trichloroacetic acid, and resuspended in V8 digestion buffer (125 mM Tris, pH 6.8, 1 mM EDTA, 0.1% SDS). The isolated RPTPα protein was digested with limited amounts of *Staphylococcus aureus* strain V8 protease (Sigma, St Louis, MO) at 37°C for 30 min. Following digestion, the samples were mixed with an equal volume of 2× Laemmli sample buffer, boiled for 5 min and loaded onto a 20% SDS-polyacrylamide gel. After electrophoresis, the gels were immunoblotted as described above.

RESULTS

Pervanadate-induced hyperphosphorylation of RPTPα involves D2. The identification of substrates for several non-receptor PTPs has been done successfully in the past using catalytic site Cys to Ala or Ser mutations of PTPs (15–19). We used this approach to identify physiological substrates of RPTPα. RPTPα substrate trapping mutants were generated in which the active site Cys of one or both PTP domains was mutated to Ser (RPTPα-C433S, RPTPα-C723S, and RPTPα-C433SC723S). A hemagglutinin epitope tag (HA-tag) was cloned just downstream of the signal sequence in the extracellular part of RPTPα for efficient immunoprecipitation. SK-N-MC cells were transiently transfected with pSG-HA-RPTPα, C433S, C723S, C433SC723S, and treated with pervanadate for 30 min to increase P.Tyr levels of potential substrates, or left unstimulated. HA-RPTPα proteins were immunoprecipitated with 12CA5 and potential co-immunoprecipitating proteins were analyzed by immunoblotting, using anti-P.Tyr antibody (PY20) (Fig. 1, top panel). No obvious P.Tyr containing proteins co-immunoprecipitated with the substrate trapping mutant HA-RPTPα-C433SC723S. Surprisingly, pervanadate treatment of the cells induced high levels of Tyr phosphorylation of wild type RPTPα. Time courses of pervanadate treatment demonstrated that tyrosine phosphorylation of RPTPα occured within 10 min and reached a plateau within 30 min (data not shown). In contrast, pervanadate induced only a very small increase in tyrosine phosphorylation of RPTPα-C723S and of RPTPα-C433SC723S (Fig. 1). Only a small, but significant decrease of tyrosine phosphorylation compared to wild type was observed for RPTPα-C433S

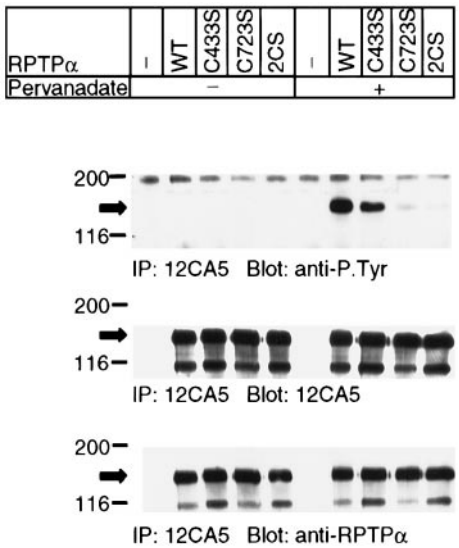

FIG. 1. D2 is involved in pervanadate-induced phosphorylation of RPTPα. SK-N-MC cells were transiently transfected with the expression vector pSG-5 (−), pSG-HA-RPTPα (WT), pSG-HA-RPTPα-C433S (C433S), pSG-HA-RPTPα-C723S (C723S), or pSG-HA-RPTPα-C433SC723S (2CS). The cells were treated with 1 mM orthovanadate and 1 mM H_2O_2 for 30 min, or were left unstimulated. The cells were lysed and HA-RPTPα proteins were immunoprecipitated with 12CA5 and analyzed by immunoblotting using anti-P.Tyr (PY20). The blot was stripped and reprobed with 12CA5 and anti-RPTPα antibodies, respectively. The molecular weights (in kDa) of marker proteins are indicated on the left. The position of RPTPα is indicated with an arrow.

(Fig. 1). The same results were obtained in stably transfected NIH 3T3 cells and transiently transfected COS-7 and HEK-293 cells, demonstrating that the observed effects were not cell type dependent (data not shown). These data show that RPTPα-D1 activity was not required for pervanadate-induced tyrosine phosphorylation, while D2 was somehow involved.

Pervanadate-induced tyrosine phosphorylation of RPTPα is not limited to Tyr789. Previously, we have demonstrated that RPTPα is phosphorylated on Tyr789 (20). In order to determine whether the effect of pervanadate treatment was limited to Tyr789, Tyr789 was mutated to Phe in wild type HA-RPTPα and C433SC723S (HA-RPTPα-Y789F and HA-RPTPα-C433SC723SY789F). SK-N-MC cells were transiently transfected with constructs as indicated and treated with pervanadate for 30 min or left untreated (Fig. 2). Pervanadate induced tyrosine phosphorylation of HA-RPTPα-Y789F to comparable levels as for wild type HA-RPTPα, indicating that pervanadate-induced tyrosine phosphorylation of RPTPα was not limited to Tyr789. The minor tyrosine phosphorylation of RPTPα-C433SC723S induced by pervanadate was completely abolished in the Y789F mutant background indicating that the minor phosphorylation of the Cys mutant is solely on Tyr789. Taken together, these data indicate that the observed pervanadate-induced tyrosine phosphorylation of RPTPα was not limited to Tyr789.

Pervanadate-induced tyrosine phosphorylation of RPTPα is independent of RPTPα activity. One possible explanation why pervanadate induced tyrosine phosphorylation of wild type RPTPα, but not of the catalytically inactive mutant is that RPTPα might activate a kinase that in turn phosphorylates RPTPα. To test this hypothesis, SK-N-MC cells were transiently transfected with wild type HA-RPTPα or the HA-RPTPα-C433SC723S mutant in combination with untagged wild type RPTPα or the RPTPα-C433SC723S mutant and treated with pervanadate for 30 min. The HA-RPTPα proteins were immunoprecipitated with antibodies against the tag (12CA5) and the P.Tyr content was analyzed by immunoblotting using anti-P.Tyr antibodies (PY20) (Fig. 3, top panel). Pervanadate treatment induced tyrosine phosphorylation of wild type HA-RPTPα and this was not affected by coexpression of untagged wild type RPTPα or the RPTPα-C433SC723S mutant, indicating that RPTPα-C433SC723S does not exhibit dominant negative effects. In addition, tyrosine phosphorylation of HA-RPTPα-C433SC723S was not influenced by coexpression of untagged wild type RPTPα or the RPTPα-C433SC723S mutant. Apparently, the activity of wild type RPTPα did not induce hyperphosphorylation of RPTPα-C433SC723S. Taken together, it is un-

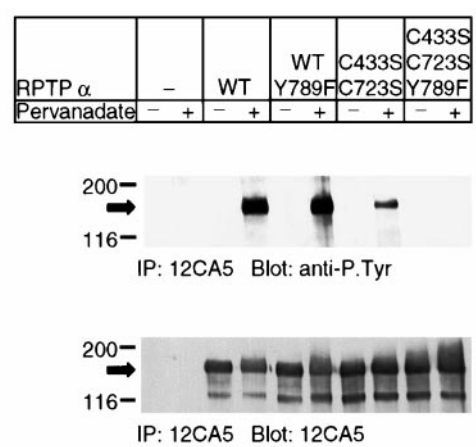

FIG. 2. Pervanadate-induced tyrosine phosphorylation of RPTPα is not limited to Tyr789. SK-N-MC cells were transiently transfected with 15 μg pSG-5 (−), pSG-HA-RPTPα (WT), pSG-HA-RPTPα-Y789F (WT Y789), pSG-HA-RPTPα-C433SC723S (C433S C723S), or pSG-HA-RPTPα-C433SC723SY789F (C433S C723S Y789F). The cells were treated with pervanadate for 30 min or left untreated, lysed and HA-RPTPα proteins were immunoprecipitated with 12CA5 and analyzed by immunoblotting using anti-P.Tyr (PY20). The blot was stripped and reprobed with MAb 12CA5. The molecular weights (in kDa) of marker proteins are indicated on the left. The position of RPTPα is indicated with an arrow.

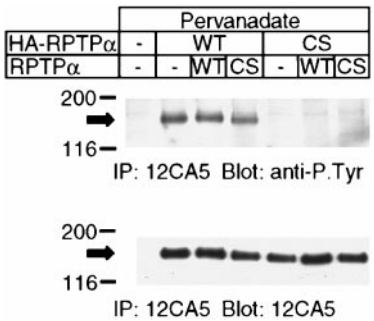

FIG. 3. RPTPα hyperphosphorylation is not an enzymatic effect. SK-N-MC cells were transiently transfected with 4 μg pSG-5 (−), HA-tagged pSG-HA-RPTPα (WT) or pSG-HA-RPTPα-C433SC723S (CS) in combination with 12 μg pSG-5 (−), untagged pSG-RPTPα (WT), or pSG-RPTPα-C433SC723S (CS) and treated with pervanadate for 30 min. The cells were lysed and HA-RPTPα proteins were immunoprecipitated with 12CA5 and analyzed by immunoblotting using anti-P.Tyr MAb (PY20). The blot was stripped and reprobed with MAb 12CA5. The molecular weights (in kDa) of marker proteins are indicated on the left. The position of RPTPα is indicated with an arrow.

likely that RPTPα activates a kinase that in turn phosphorylates RPTPα.

Another approach to test whether PTP activity is necessary for pervanadate-induced tyrosine phosphorylation is the use of other mutants that lack PTP activity. Mutation of Arg221 to Lys in PTP1B completely abolished PTP activity, rendering an inert protein (17). We mutated Arg in the signature motifs of RPTPα-D1 and D2 to Lys (R438K or R729K, respectively), which completely inactivated all PTP activity in these domains *in vitro* (data not shown). Despite the complete lack of activity, pervanadate still induced strong tyrosine phosphorylation of RPTPα-R438K and R729K, comparable to the levels seen for wild type RPTPα (data not shown). Taken together, these data suggest that RPTPα activity is not involved in pervanadate-induced tyrosine phosphorylation.

Pervanadate-induced tyrosine phosphorylation of RPTPα is located in the C-terminus. Finally, we mapped the pervanadate-induced tyrosine phosphorylation sites in RPTPα. To this end, SK-N-MC cells were transiently transfected with HA-RPTPα-Y789F and treated with pervanadate for 30 min. HA-RPTPα-Y789F mutant was used to exclude phosphorylation of Tyr789. Purified HA-RPTPα-Y789F protein was digested with various amounts of V8-protease and the P.Tyr content and identity of the fragments was analyzed by immunoblotting (Figs. 4A–4D), using anti-P.Tyr antibody (PY20), anti-HA-tag antibody (12CA5), an antibody against the C-terminus of RPTPα, or an antibody against the entire cytoplasmic domain of RPTPα (Fig. 4). The epitopes that are recognized by these antibodies are indicated in Fig. 4E. Treatment of RPTPα with increasing amounts of V8 protease resulted only in a few major fragments containing P.Tyr. The largest fragment, a fragment of approximately 110 kDa, lacks the C-terminus, since this fragment was not recognized by the RPTPα antibody directed against the C-terminus (Fig. 4C), while the N-terminal HA-tag was present (Fig. 4B). Compared to the starting material, the 110 kDa fragment contained only very little P.Tyr, suggesting that the majority of the P.Tyr lies in the C-terminus. The 20 kDa fragment that was cleaved off of RPTPα to yield the 110 kDa fragment was rapidly degraded further, since it was not detected by the antibody against the RPTPα C-terminus (Fig. 4C) or the polyclonal RPTPα antibody (Fig. 4D). Three other major tyrosine phosphorylated fragments were observed: two fragments of approximately 64 kDa and a 30 kDa fragment. The two fragments of approximately 64 kDa were detected with PY20, the antibody against the RPTPα C-terminus, and the RPTPα polyclonal antibody (Fig. 4A, C, D), but not with 12CA5 (Fig. 4B) indicating that these fragments still contain the very C-terminal end but miss the N-terminus. The 30 kDa fragment lacks both C-terminus and N-terminus since it is only recognized by the RPTPα polyclonal antibody. Based on these data we conclude that pervanadate induced tyrosine phosphorylation throughout RPTPα, but mostly in the C-terminal 20 kDa of RPTPα. The observed tyrosine phosphorylation was not on Tyr789, since Tyr789 was mutated to Phe in Fig. 4. It is noteworthy that similar results were obtained when wild type RPTPα was used instead of the Y789F mutant. Mutation of the four most C-terminal tyrosine residues, Tyr772, Tyr776, Tyr782 and Tyr789, individually, had little or no effect on the pervanadate-induced tyrosine phosphorylation of RPTPα. However, mutation of all four C-terminal tyrosine residues reduced, but did not abolish pervanadate-induced tyrosine phosphorylation (data not shown). In conclusion, pervanadate predominantly induced tyrosine phosphorylation of the C-terminal 20 kDa in RPTPα on multiple sites.

DISCUSSION

Pervanadate treatment of cells induced strong tyrosine phosphorylation of RPTPα, which required intact RPTPα-D2, but not RPTPα-D1. Pervanadate-induced tyrosine phosphorylation of RPTPα was not restricted to Tyr789, and the kinase, responsible for pervanadate-induced tyrosine phosphorylation remains to be identified. Pervanadate-induced hyperphosphorylation localized predominantly to the C-terminus, although many other Tyr residues throughout RPTPα may be phosphorylated as well.

Inactivation of RPTPα by mutation by itself was not sufficient to induce tyrosine phosphorylation. RPTPα has autodephosphorylation activity, in that RPTPα dephosphorylated itself *in vitro,* and Tyr789 phosphorylation was enhanced in catalytically inactive RPTPα-

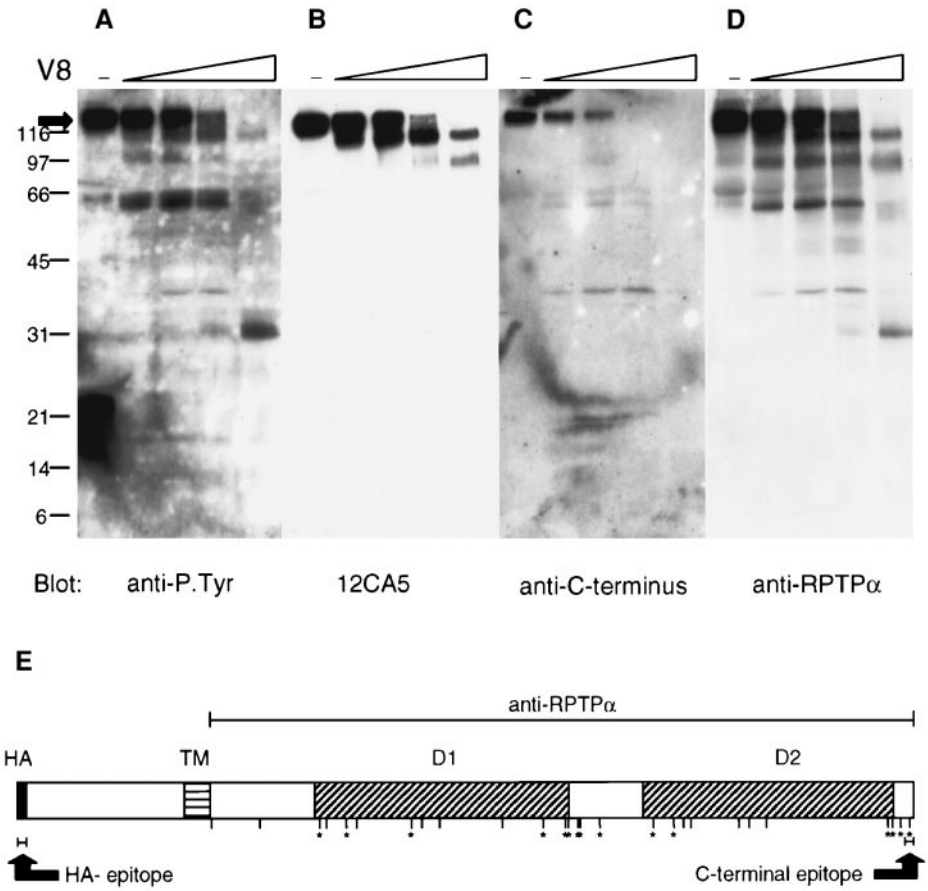

FIG. 4. Pervanadate-induced tyrosine phosphorylation of RPTPα predominantly in the C-terminal 20 kDa. SK-N-MC cells transiently transfected with 15 μg pSG-HA-RPTPα-Y789F were incubated with 1 mM orthovanadate and 1 mM H_2O_2. The cells were lysed and HA-RPTPα proteins were immunoprecipitated with 12CA5. After electrophoresis on a polyacrylamide gel, RPTPα was cut out of the gel, eluted from the gel, precipitated with trichloroacetic acid, and resuspended in 50 μl V8 digestion buffer with 0, 0.04, 0.1, 0.2, 0.5 units V8 protease and incubated at 37°C for 30 min. Samples were run on a 20% SDS polyacrylamide gel, and analyzed by immunoblotting as described under Experimental Procedures using sequentially: MAb PY20 (A), MAb 12CA5 (B), affinity-purified anti-RPTPα antiserum 5476, directed against the C-terminus (C), and affinity-purified anti-RPTPα antiserum 5478, directed against the entire cytoplasmic domain (D). The molecular weights (in kDa) of marker proteins are indicated on the left. The position of full length RPTPα is indicated with an arrow. A schematic representation of RPTPα is depicted in the lower panel (E). The HA-tag (HA), transmembrane domain (TM) and the two PTP domains (D1 and D2) are indicated. All tyrosines in the cytoplasmic domain are represented by a vertical line, and the ones that were mutated with an asterisk. The epitopes for 12CA5 (HA-epitope), the affinity-purified antibody 5476 directed against the C-terminus of RPTPα (C-terminal epitope), and the affinity-purified antibody 5478 directed against the full cytoplasmic domain of RPTPα (anti-RPTPα) are indicated.

C433AC723A (20). Since the PTP inhibitor pervanadate induced strong tyrosine phosphorylation of RPTPα, one might expect similar levels of tyrosine phosphorylation in catalytically inactive RPTPα in the case that RPTPα was solely responsible for its own dephosphorylation. Since RPTPα-C433S was clearly not highly phosphorylated, we conclude that RPTPα is not the major PTP responsible for keeping the basal tyrosine phosphorylation levels of RPTPα low.

A possible explanation why pervanadate induced tyrosine phosphorylation of wild type RPTPα, but not of RPTPα-C723S is that mutation of the catalytic site Cys may lead to conformational changes. Cys to Ser mutation is a single atom change, i.e., substitution of a sulfur for an oxygen. Therefore, it is unlikely that this mutation will significantly alter the structure of the protein. Detailed analysis, using circular dichroism and ultraviolet-spectra, of structural differences between the wild type *Yersinia* PTP, YopH and the catalytic site Cys to Ser mutant demonstrate that the overall secondary and tertiary structural elements are similar for both proteins. However, conformational differences in the active site were found involving the position of the WPD loop harboring the Asp that functions as general base/general acid in catalysis (24). In contrast, no obvious differences were observed between the crystal structures of the wild type PTP domain and the catalytic site Cys to Ser mutant of PTP1B (25). The structure of RPTPα-D2 shows that there are no Tyr residues in or near the pocket of the active site (A.

Bilwes and J. Noel, personal communication), making it unlikely that conformational changes in the active site due to mutation of the active site Cys to Ser will lead to "masking" of Tyr residues that are no longer accessible for kinases in the Cys to Ser mutant. Yet, a small conformational change in RPTPα-D2 due to the Cys to Ser mutation may still be responsible for the differences in pervanadate-induced tyrosine phosphorylation (see below).

Another possibility why pervanadate induced tyrosine phosphorylation of wild-type RPTPα, but not of the mutant, might be that wild-type RPTPα activates a kinase that in turn phosphorylates RPTPα. This explanation, however, is unlikely since RPTPα-D2 contains very little activity, especially towards phosphopeptide substrates (11, 12). It has been suggested that RPTPα-D2 may have a high activity towards a specific (unknown) substrate. However, two residues that are highly conserved in RPTP-D1s (Tyr262 and Asp401 in RPTPα) and PTP domains of cytoplasmic PTPs, are essential for catalysis, and are not conserved in RPTPα-D2 and other D2s (26). Mutation of these two residues in RPTPα-D2 to the corresponding residues in D1 turned D2 into a very potent PTP domain (26, 27). In addition, pervanadate still induced hyperphosphorylation of RPTPα-R729K with a mutation that abolishes all PTP activity in D2 (data not shown). Taken together, these data suggest that RPTPα activity is not involved in pervanadate-induced tyrosine phosphorylation.

RPTPα is phosphorylated constitutively on Tyr789 (20). Here we demonstrate that mutation of Tyr789 to Phe has no effect on pervanadate-induced tyrosine phosphorylation (Fig. 2). Partial digestion of hyperphosphorylated RPTPα with V8 suggested that pervanadate-induced tyrosine phosphorylation was mainly localized in the C-terminus (Fig. 4). The fragment that contained the majority of the pervanadate-induced P.Tyr is a fragment of the C-terminus of approximately 20 kDa. In this fragment there are 7 tyrosines, 5 of which have the OH-group at the surface, based on the crystal structure of RPTPα-D2 (A. Bilwes and J. Noel, personal communication), making them potential targets for tyrosine phosphorylation. Pervanadate-induced tyrosine phosphorylation of RPTPs is not unprecedented, since CD45 became phosphorylated on tyrosine upon pervanadate treatment of T cells. Mapping of the tyrosine phosphorylation site led to the identification of Tyr1193, to the C-terminal side in CD45-D2 (28). The corresponding Tyr in RPTPα, Tyr772, is completely buried in the structure, inaccessible for kinases. Therefore, it is unlikely that pervanadate induces phosphorylation of this Tyr in RPTPα. In fact, mutation of Tyr772 to Phe had no effect on RPTPα hyperphosphorylation. The corresponding Tyr in other PTP domains, like Tyr 482 in RPTPα-D1, is hidden in the structure as well (3). It will be interesting to see where Tyr1193 is located in the structure of CD45. We have mutated 15 out of the 27 Tyr residues in the cytoplasmic domain of RPTPα (Fig. 4E), but none of these single mutations had an effect on pervanadate-induced RPTPα tyrosine phosphorylation. Mutation of the four most C-terminal tyrosine residues reduced, but did not abolish pervanadate-induced P.Tyr levels, suggesting that pervanadate induced RPTPα phosphorylation on multiple sites.

We have shown that Src may be the kinase that phosphorylates RPTPα on Tyr789 (20). However, co-transfection of a constitutively active mutant of Src, SrcY529F together with RPTPα did not induce tyrosine phosphorylation of wild-type RPTPα or any of the mutants (data not shown) indicating that Src alone is not sufficient to induce hyperphosphorylation of RPTPα. The combination of co-transfection of SrcY529F and pervanadate treatment induced tyrosine phosphorylation of wild-type RPTPα, but not of the catalytic site Cys mutants, similar to pervanadate treatment alone (data not shown). In addition, co-transfection of inactive Src, SrcK297M, which may have dominant negative activity did not significantly lower pervanadate-induced tyrosine phosphorylation levels of RPTPα (data not shown). Therefore, the mechanism of pervanadate-induced tyrosine phosphorylation of wild-type RPTPα probably does not involve Src or any downstream targets of Src.

Several reports suggested that D2s may be involved in protein–protein interactions (6, 29, 30). The most likely explanation for the effect of the Cys to Ser mutation in RPTPα-D2 on pervanadate-induced tyrosine phosphorylation is that this mutation induced a small conformational change in the active site like YopH (24), thereby abolishing binding to a kinase, or a protein complex containing a kinase, that phosphorylated RPTPα on tyrosine. Presumably, this interaction was weak, since we have not been able to detect PTK activity co-immunoprecipitating with RPTPα. Moreover, yeast genetic two-hybrid screens, using RPTPα-D2 as a bait, did not lead to identification of any PTKs as interacting (prey) proteins (C.B., A.B., and J.d.H., unpublished results).

In conclusion, a constitutively active kinase phosphorylated RPTPα on tyrosine following treatment of cells with the non-selective PTP inhibitor pervanadate. Mutation of the catalytic site Cys to Ser in D2 abolished pervanadate-induced RPTPα tyrosine phosphorylation, demonstrating that RPTPα-D2 was somehow involved. It will be interesting to identify the kinase responsible for pervanadate-induced tyrosine phosphorylation of RPTPα and test whether the Cys to Ser mutation in RPTPα-D2 blocks the interaction with this kinase.

ACKNOWLEDGMENTS

We thank Alex Bilwes and Joe Noel (Structural Biology Laboratory, The Salk Institute, La Jolla, CA) for providing the coordinates of the crystal structure of RPTPα-D2 prior to publication. In addition, we thank John Overvoorde for technical assistance. This work was supported by the Research Council for Earth and Life Sciences (ALW) with financial aid from the Netherlands Organization for Scientific Research (NWO) (to A.B.), and a grant from the Dutch Cancer Society (to J.d.H.).

REFERENCES

1. Fischer, E. H., Charbonneau, H., and Tonks, N. K. (1991) *Science* **253,** 401–406.
2. Barford, D., Flint, A. J., and Tonks, N. K. (1994) *Science* **263,** 1397–1404.
3. Bilwes, A. M., den Hertog, J., Hunter, T., and Noel, J. P. (1996) *Nature* **382,** 555–559.
4. Hof, P., Pluskey, S., Dhe-Paganon, S., Eck, M. J., and Shoelson, S. E. (1998) *Cell* **92,** 441–450.
5. Hoffmann, K. M., Tonks, N. K., and Barford, D. (1997) *J. Biol. Chem.* **272,** 27505–27508.
6. Nam, H. J., Poy, F., Krueger, N. X., Saito, H., and Frederick, C. A. (1999) *Cell* **97,** 449–457.
7. Stuckey, J. A., Schubert, H. L., Fauman, E. B., Zhang, Z.-Y., Dixon, J. E., and Saper, M. A. (1994) *Nature* **370,** 571–575.
8. Yang, J., Liang, X., Niu, T., Meng, W., Zhao, Z., and Zhou, G. W. (1998) *J. Biol. Chem.* **273,** 28199–28207.
9. Neel, B. G., and Tonks, N. K. (1997) *Curr. Opin. Cell Biol.* **9,** 193–204.
10. Charbonneau, H., Tonks, N. K., Walsh, K. A., and Fischer, E. H. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 7182–7186.
11. Wang, Y., and Pallen, C. J. (1991) *EMBO J.* **10,** 3231–3237.
12. Wu, L., Buist, A., den Hertog, J., and Zhang, Z.-Y. (1997) *J. Biol. Chem.* **272,** 6994–7002.
13. den Hertog, J., Pals, C. E., Peppelenbosch, M. P., Tertoolen, L. G., de Laat, S. W., and Kruijer, W. (1993) *EMBO J.* **12,** 3789–3798.
14. Desai, D. M., Sap, J., Silvennoinen, O., Schlessinger, J., and Weiss, A. (1994) *EMBO J.* **13,** 4002–4010.
15. Bliska, J. B., Clemens, J. C., Dixon, J. E., and Falkow, S. (1992) *J. Exp. Med.* **176,** 1625–1630.
16. Chiarugi, P., Cirri, P., Raugei, G., Camici, G., Dolfi, F., Berti, A., and Ramponi, G. (1995) *FEBS Lett.* **372,** 49–53.
17. Flint, A. J., Tiganis, T., Barford, D., and Tonks, N. K. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 1680–1685.
18. Garton, A. J., Flint, A. J., and Tonks, N. K. (1996) *Mol. Cell Biol.* **16,** 6408–6418.
19. Sun, H., Charles, C. H., Lau, L. F., and Tonks, N. K. (1993) *Cell* **75,** 487–493.
20. den Hertog, J., Tracy, S., and Hunter, T. (1994) *EMBO J.* **13,** 3020–3032.
21. Green, S., Issemann, I., and Sheer, E. (1988) *Nucleic Acids Res.* **16,** 369.
22. den Hertog, J., and Hunter, T. (1996) *EMBO J.* **15,** 3016–3027.
23. Sap, J., D'Eustachio, P., Givol, D., and Schlessinger, J. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 6112–6116.
24. Zhang, Z.-Y., and Wu, L. (1997) *Biochemistry* **36,** 1362–1369.
25. Jia, Z., Barford, D., Flint, A. J., and Tonks, N. K. (1995) *Science* **268,** 1754–1758.
26. Buist, A., Zhang, Y.-L., Keng, Y.-F., Wu, L., Zhang, Z.-Y., and den Hertog, J. (1999) *Biochemistry* **38,** 914–922.
27. Lim, K. L., Kolatkar, P. R., Ng, K. P., Ng, C. H., and Pallen, C. J. (1998) *J. Biol. Chem.* **273,** 28986–28993.
28. Autero, M., Saharinen, J., Pessa-Morikawa, T., Soula-Rothhut, M., Oetken, C., Gassmann, M., Bergman, M., Alitalo, K., Burn, P., and Gahmberg, C. G. (1994) *Mol. Cell Biol.* **14,** 1308–1321.
29. Felberg, J., and Johnson, P. (1998) *J. Biol. Chem.* **273,** 17839–17845.
30. Wallace, M. J., Fladd, C., Batt, J., and Rotin, D. (1998) *Mol. Cell Biol.* **18,** 2608–2616.