The Dephosphorylation of Insulin and Epidermal Growth Factor Receptors

ROLE OF ENDOSOME-ASSOCIATED PHOSPHOTYROSINE PHOSPHATASE(S)*

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Robert Faure†, Gerry Baquiran, John J. M. Bergeron, and Barry I. Posner†

From the Polypeptide Hormone Laboratory and Department of Medicine, The Royal Victoria Hospital and the Department of Anatomy, McGill University, Montreal, Quebec H3A 1A1, Canada

The autophosphorylation, from [γ-32P]ATP, of insulin and epidermal growth factor receptors in rat liver endosomes peaked at 2–5 min and declined thereafter. When autophosphorylation from either [γ-32P]ATP or unlabeled ATP was stopped after 5 min by adding excess EDTA or ATP, the phosphotyrosine (PY) content of each receptor decreased at 37 °C with a t½ of 1.6 min. This was equally so whether the PY content of 32P-labeled receptors was analyzed by autoradiography of KOH-treated gels or by Western blotting with PY antibodies of immunoprecipitated receptors. The dephosphorylation reaction was strictly dependent on the presence of sulfhydryl, was unaffected by the addition of rat liver cytosol, and was temperature-dependent. The phosphotyrosine phosphatase(s) (PTPase(s)) appeared to be tightly anchored to the endosomal membrane, since the dephosphorylation reaction was unaffected by sodium carbonate and 0.6 M KCl treatments. However, treatment with Triton X-100 abolished dephosphorylation, implying an intimate association between the PTPase(s) and its substrate in an intact membrane environment. The powerful insulinominimetic agent pervanadate was the most potent inhibitor (50% inhibition at 1 μM). Increasing the dose of injected ligand augmented the rate of insulin and decreased that of EGF receptor dephosphorylation, respectively. Immunoblotting with specific antibodies failed to identify PTPase 1B or T-cell PTPase in ENs, whereas positive signals were seen in plasma membrane. These studies indicate that the phosphorylation state of receptor tyrosine kinases is dynamically regulated, with dephosphorylation, by closely associated PTPase(s), playing an important role.

Following their intravenous injection insulin, EGF and other ligands bind to their respective receptors in target cells and are rapidly translocated as ligand-receptor complexes into the endosomal apparatus (1). Subsequent studies have documented a corresponding dose-dependent activation of the insulin and EGF receptor tyrosine kinases and an accumulation of these activated kinases within rat liver endosomes (ENs) (2–5). Of interest were the observations that both the insulin receptor and EGF receptor kinases displayed greater autophosphorylation activity in ENs than seen in plasma membrane (PM) (3, 5). In more detailed recent studies of the insulin receptor, it was found that the endosomal receptor contained less phosphotyrosine (PY) per receptor than that in PM even though the former was a more active kinase (6). We inferred that an endosomal phosphotyrosine phosphatase (PTPase) may play a role to effect the partial dephosphorylation observed. In other studies, we measured the PTPase activity against artificial substrates in solubilized rat liver PM and EN cell fractions (7). These studies indicated equal PTPase activity in PM and ENs in both control rats and those receiving a pharmacologic dose (150 μg/100 g, body weight) of insulin. Solubilization would be expected to disrupt the spatial relationship between a receptor and receptor-associated PTPase(s). Furthermore, receptor-associated PTPase(s) might show considerably different activity toward artificial substrates than toward the receptor as substrate. Given these considerations, we set out to develop a method for assaying receptor-associated PTPase in intact EN membranes in order to evaluate its functional characteristics in situ.

MATERIALS AND METHODS

Animals—Female Sprague-Dawley rats (140–160 g, body weight) were purchased from Charles River Ltd. (St. Constant, Quebec) and were fasted overnight prior to killing. Rats were anesthetized with ether, and hormones were injected via the portal vein. They were killed by decapitation at 2 min postinjection of insulin and at 15 min postinjection of EGF, corresponding to the respective peak times of receptor kinase stimulation (2, 4, 5). Livers were rapidly excised, placed in ice-cold homogenizing buffer (0.25 M sucrose, 25 mM KCl, 5 mM Hepes, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, pH 7.4) and minced before homogenization.

Hormones, Antibodies, and Other Reagents—Porcine insulin (26.8 units/mg) was a gift from Lilly. Mouse EGF was purchased from...
Collaborative Research (Waltham, MA). Anti-insulin receptor antibodies were purified from a patient’s serum as previously described (3). The EGF receptor was immunoprecipitated with monoclonal antibody IgG 151, BH6 (5). Antiphosphotyrosine antibody (α-PTY) was used in Western blots using phosphotyrosine coupled to keyhole limpet hemocyanin as antigen as described in detail elsewhere (6). The antibody was subsequently purified by affinity chromatography on a phosphotyrosine Affi-Gel column (8). Antibodies against a synthetic peptide (ENLTTQETELIHFFYTT) corresponding to residues 161–175 in the human placental PTPase (PTP-1B), which are conserved in rat brain PTPase, were prepared, as previously described (9), and kindly provided by Dr. D. L. Brautigan (Brown University). Antibody to the T-cell PTPase (PTP-TC) was generated by immunizing rabbits with the protein expressed in Escherichia coli and was provided by the laboratory of Dr. S.-H. Shen (Biotechnology Institute, Montreal, Canada). The BamHindIII fragment of the cDNA encoding the human PTP-TC (10) was inserted between the Smal and HindIII sites of the expression vector pWR-590 generating the plasmid pWR-PTP-TC. This construct encoded a fusion protein of the tyrosine phosphatase with 590 amino acids of E. coli β-galactosidase. To purify the fusion protein DH5, transformed with pWR-PTP-TC were induced with IPTG (1 mM) for 18 h and lysed in Laemmli buffer containing 5 M urea. After boiling, the sample was subjected to SDS-PAGE using 7.5% gels (11). The fusion protein was excised from the gel, and the polyacylamide gel slice was homogenized and lyophilized. The dried material was emulsified with an equal volume of complete Freund’s adjuvant for the first injection and with incomplete Freund’s adjuvant for subsequent injections. Each rabbit received subcutaneously five injections (50 μg/injection) at 3-week intervals. Animals were bled 2 weeks after the last injection. IgG was purchased from Sigma and radioiodinated to a specific activity of 6 × 10⁹ dpm/μg of protein using the chloramine-T procedure (12). Protein A-Sepharose and wheat germ agglutinin-Sepharose 6 MB were from Pharmacia Fine Chemicals (Dorval, Quebec). [-p3'P]ATP (32 pCi/nmol) was purchased from New England Nuclear Radiochemicals (Lachine, Quebec). Reagents for SDS-PAGE were obtained from Bio-Rad. Sodium orthovanadate, phenylarsine oxide, sodium molybdate, and sodium tungstate were obtained from Sigma. All other chemicals were of analytical grade and purchased from Fisher or Boeringer Mannheim (Montreal, Quebec). Vanadate solutions were prepared as previously described in order to avoid changes in pH leading to the generation of decavanadate (orange) or vanadyl ions (V02+, blue) (13). Peroxide(s) of vanadate (pV) was denoted by the vanadate concentration (14). The concentration of pV generated is denoted by the vanadate concentration expressed as micromoles of vanadyl ions (V02+, blue) (13). Peroxide(s) of vanadate (pV) was generated in a buffer mimicking the intracellular milieu (15) and in the presence of [γ-32P]ATP. Fig. 1 depicts the time course of alkaliresistant 32P incorporation from [γ-32P]ATP into insulin (panel A) and EGF (panel B) receptors. In each case, 32P incorporation reached a maximum (insulin, 5 min; EGF, 2 min) and subsequently declined to 50% of the maximal value by 15 min of incubation. In the presence of 100 μM pV, the extent of 32P incorporation was augmented to a maximum of 2-fold above that of control incubations, and the decrease in insulin receptor labeling was completely prevented. The decline in ATP concentration by 40% after 10 min and 90% after 30 min of incubation (Table I) was the same in the absence or presence of pV. This demonstrated that pV did not act through the inhibition of an endosomal ATPase and was consistent with the inhibition by pV of endosomal PS phosphatase(s), as indicated in a previous study (14). These data suggested that under the conditions employed for studying autophosphorylation, a dynamic process involving receptor phosphorohaplation/dephosphorylation was occurring.
Receptor Dephosphorylation in Intact Endosomes

Injected via the portal vein with insulin (1.5 μg/100 g, body weight) or EGF (1 μg/100 g, body weight). After 2 min (insulin) or 15 min (EGF) postinjection, livers were excised and ENs were isolated. ENs (25 μg of protein) were suspended in the assay buffer and subjected to phosphorization in the presence of 55 μM [γ-32P]ATP at 37 °C for the indicated times. At the noted times, ENs were solubilized, receptors were immunoprecipitated, and the immunoprecipitates were subjected to SDS-PAGE as described under “Materials and Methods.” After development, the spot corresponding to ATP was located under “Materials and Methods” in the absence and presence of EDTA, which chelates Mg2+ and abrogates phosphorylation. Receptor PY content was subjected to thin layer chromatography as described under “Materials and Methods.” At the noted times following EDTA addition, ENs were solubilized, the receptors were immunoprecipitated, and the immunoprecipitates were subjected to SDS-PAGE and immunoblotting with antiphosphotyrosine antibodies to determine their relative PY content as described in the text.

**TABLE I**

**ATP degradation during autophosphorylation**

Autophosphorylation was carried out on ENs prepared from rats injected with insulin (1.5 μg/100 g, body weight) as described under “Materials and Methods” in the absence and presence of 100 μM pV. At the noted times, the reaction was terminated and, after removal of ENs by centrifugation a volume of supernatant (1 ml), was subjected to thin layer chromatography as described under “Materials and Methods.” After development, the spot corresponding to ATP was located under UV light, excised, and counted (Cerenkov). The results reflect the mean ± S.E. of three separate experiments.

| Incubation time (min) | Control | 32P|ATP |
|-----------------------|---------|-----|-----|
| 0                     | 181,472 ± 1,600 (100%) | 184,880 ± 3,200 (100%) |
| 5                     | 87,710 ± 2,150 (48%) | 114,392 ± 740 (62%) |
| 10                    | 69,468 ± 610 (38%) | 69,909 ± 1,799 (38%) |
| 15                    | 41,315 ± 2,320 (23%) | 43,778 ± 605 (24%) |
| 30                    | 20,578 ± 760 (11%) | 24,587 ± 930 (13%) |

**Fig. 1.** Time course at 37 °C of insulin (A) and EGF (B) receptor 32P autophosphorylation in endosomes. Rats were injected via the portal vein with insulin (1.5 μg/100 g, body weight) or EGF (1 μg/100 g, body weight). After 2 min (insulin) or 15 min (EGF) postinjection, livers were excised and ENs were isolated. ENs (25 μg of protein) were suspended in the assay buffer and subjected to phosphorization in the presence of 55 μM [γ-32P]ATP at 37 °C for the indicated times. At the noted times, ENs were solubilized, receptors were immunoprecipitated, and the immunoprecipitates were subjected to SDS-PAGE and immunoblotting with antiphosphotyrosine antibodies to determine their relative PY content as described in the text.

**Fig. 2.** Time course of loss of phosphotyrosine content of autophosphorylated insulin (A) and EGF (B) receptors. ENs, isolated at 2 min (insulin, 1.5 μg/100 g, body weight) or 15 min (EGF, 1 μg/100 g, body weight) following ligand injection were autophosphorylated with 1 mM ATP before adding EDTA (zero time) to terminate phosphorylation and initiate the dephosphorylation assay as described under “Materials and Methods.” At the noted times following EDTA addition, ENs were solubilized, the receptors were immunoprecipitated, and the immunoprecipitates were subjected to SDS-PAGE and immunoblotting with antiphosphotyrosine antibodies to determine their relative PY content as described in the text.

**Time Course of Loss of PY from Endosomal Insulin and EGF Receptors**

To study the dephosphorylation step, the autophosphorylation reactions for insulin and EGF receptors were inhibited at their respective times of maximal phosphorylation (insulin, 5 min; EGF, 2 min) by adding excess EDTA, which chelates Mg2+ and abrogates phosphorylation. Fig. 2 depicts the rate of decline in insulin (panel A) and EGF (panel B) receptor PY content following the termination of autophosphorylation with EDTA. Receptor PY content was assessed by immunoblotting with α-PY (Fig. 2, upper panels), followed by densitometric analyses to determine relative signal intensity (Fig. 2, lower panels). In each case, there was a rapid loss of receptor PY content at a t1/2 of 1.6-1.7 min. We thus conclude that a rapid receptor phosphotyrosine dephosphorylation reaction was occurring in ENs.

**Time Course of Receptor Dephosphorylation**

To characterize this process, we designed an alternative assay that was more rapid and convenient. Using [γ-32P]ATP endosomal labeling, receptor was inhibited at maximal phosphorylation times with EDTA and a 20-fold excess of unlabeled ATP, following which the incubation was continued at 37 °C. Fig. 3 depicts the level of alkali-resistant 32P in endosomal insulin (panel A) and EGF (panel B) receptors as a function of time following the inhibition of phosphorylation. In each case, there was a rapid loss of receptor 32P content at a t1/2 of 1.6-1.7 min, which is entirely comparable with the results observed in the α-PY immunoblotting studies. The omission of DTT from the incubation resulted in the complete inhibition of dephosphorylation. Though 100 μM pV completely inhibited dephosphorylation, a comparable concentration of vandate had no such effect. Finally, the addition of hepatic cytosol to ENs did not influence the rate of dephosphorylation.

**Characterization of Endosomal PTPase Activity Toward the Insulin Receptor**

As expected for an enzyme-mediated process, the endosomal dephosphorylation activity was temperature-dependent (Fig. 4A). Dephosphorylation was rapid at 37 °C, with a t1/2 of 1.5-2.0 min, whereas at 25 °C, the t1/2 was 8-10 min. After 10 min at 4 °C, the reaction was barely perceptible.

The failure of cytosol to accelerate the rate of insulin receptor dephosphorylation (Fig. 3) suggested that the observed endosomal PTPase activity was not due to associated cytosolic enzyme(s). This was confirmed by a series of experiments in which we attempted to "strip" away the PTPase activity by procedures that remove peripheral proteins. Thus, pretreatment of ENs with 0.6 M KCl or with 0.1 M Na2CO3 (pH 11.0) (23) did not result in the removal of any endosomal PTPase activity (Fig. 4B). We conclude that endosomal PTPase(s) are either intrinsic membrane proteins or cytosolic-like enzymes tightly coupled to the membrane.

Most of the assays designed to measure PTPase activity
FIG. 3. Time course of dephosphorylation of $^{32}$P-labeled insulin (A) and EGF (B) endosomal receptors. ENs, isolated as in the legend of Fig. 2, were subjected to autophosphorylation with 25 mM $\gamma$-[$^{32}$P]ATP before adding EDTA/ATP (zero time) to terminate $^{32}$P labeling and initiate the dephosphorylation assay as described under Materials and Methods. At the noted times, ENs were solubilized, and receptors were immunoprecipitated and subjected to SDS-PAGE, alkali treatment, and autoradiography (top panels), followed by densitometric analyses (bottom panels) as described in the text. Each point is the mean (±S.D.) of three separate experiments.

FIG. 4. The effect of incubation temperature (A), salt "stripping" (B), and Triton X-100 (C) on dephosphorylation of the $^{32}$P-labeled endosomal insulin receptor. $^{32}$P-Labeling and dephosphorylation of endosomal insulin receptors were carried out as described in the legend of Fig. 3. A, temperature dependence was evaluated by performing the dephosphorylation assay at 37, 25, or 4°C. B, ENs were subjected to autophosphorylation followed by the extraction procedures for removing peripheral membrane proteins. The autophosphorylation reaction was stopped by adding ice-cold Na$_2$CO$_3$ to a final concentration of 0.1 M, pH 11.0, or by adding 0.6 M KCl and 5 mM H$_2$O$_2$ to a total volume of 4 ml. After 30 min of gentle shaking at 4°C, 2 volumes of ice-cold distilled water were added and the suspension was centrifuged at 100,000 g for 60 min. The pellet was resuspended in prewarmed (37°C) assay buffer minus DTT, and dephosphorylation was initiated by adding DTT to a final concentration of 1 mM. C, dephosphorylation was initiated by adding ATP/EDTA stopping solution containing Triton X-100 in order to obtain a final concentration of 0.0, 0.01, 0.1, and 1.0% (v/v) during the incubation.

FIG. 5. Relative potency of different inhibitors on endosomal insulin (A) and EGF (B) receptor-associated PTPase activities. Intact ENs were prepared from insulin or EGF-injected rats and subjected to autophosphorylation (insulin, 5 min; EGF, 2 min) and subsequent dephosphorylation, as described under Materials and Methods, except that inhibitors were present in the ATP/EDTA stopping solution in order to achieve the final concentrations indicated. The shaded area delineates agents that were active in the millimolar range. A, dephosphorylation of the 94-kDa subunit of the insulin receptor. B, dephosphorylation of the EGF receptor. Each point is the mean (±S.D.) of three or four independent experiments.
of injected insulin (0.15–15 µg/100 g, body weight), the rate of dephosphorylation was substantially the same. However, at a pharmacologic dose of insulin (150 µg/100 g, body weight), dephosphorylation was significantly accelerated. The half-times of dephosphorylation following the 0.15 and 150 µg/100 g, body weight, doses were 2.32 ± 0.45 and 1.48 ± 0.10 (mean ± S.D., n = 3), respectively, and these values were significantly different at p < 0.035. In contrast, increasing the dose of injected EGF (0.1 to 10 µg) significantly retarded the rate of EGF receptor dephosphorylation. The half-times of dephosphorylation following the 0.1 and 10 µg/100 g, body weight, doses were 1.43 ± 0.20 and 2.23 ± 0.10 (mean ± S.D., n = 3), respectively, and these values were significantly different at p < 0.004. Thus, ligand dose did influence dephosphorylation rates but in opposite directions for the insulin and EGF receptors.

Compartmentalization of PTPase 1B and the T-cell PTPase—Attempts were made to assay receptor PTPase activities in PM cell fractions in order to compare these activities with those in ENs. As noted previously (3) the extent of in vitro autophosphorylation of either insulin or EGF PM receptors was substantially less than that seen for EN receptors. The reason for this difference remains unclear, but studies of PM in a manner comparable with EN receptors were not possible. As part of the attempt to compare PTPase activity in PM and EN cell fractions, we examined these cell fractions for their content of PTP-1B- and PTP-TC-like enzymes using antibodies raised against these enzymes, as described under "Materials and Methods." With each antibody, Western blots of PM fractions showed a clear signal of the appropriate molecular size, whereas no signal was seen in ENs (Fig. 6). The absence of an endosomal signal on Western blotting persisted in endosomes prepared from rats injected with 1.5 µg of insulin/100 g, body weight. These observations indicate compartmentalization of these enzymes in rat liver.

**DISCUSSION**

On examining the time course of insulin receptor autophosphorylation in intact ENs, it was evident that, at 37 °C, peak labeling occurred at 5 min (Fig. 1A), only to be followed by a loss of label thereafter. At the point of maximal autophosphorylation, the intensity of the signal was twice as high in the presence of pV, indicating that maximal autophosphorylation was not attained in controls. This limitation did not reflect higher [32P]ATP levels in pV-treated samples, since the rates of ATP degradation were the same in the absence or presence of pV. The results were consistent with the occurrence of an endosomal receptor dephosphorylation reaction and a capacity of pV to inhibit phosphotyrosine phosphatase (PTPase) activity as previously observed (14). The progressive decline in receptor autophosphorylation observed during prolonged incubation in the absence of pV was due to the progressive loss of ATP from the reaction mixture, with the consequence that the dephosphorylation reaction was progressively favored. Thus, under control conditions, the level of receptor autophosphorylation attained in intact ENs reflects the balance existing between phosphorylation and dephosphorylation reactions.

The dephosphorylation reaction could be accurately measured by stopping kinase activity. Under such conditions, the dephosphorylation of both insulin and EGF receptors was rapid and occurred at a comparable rate, with a t1/2 of 1.6–1.7 min. Similar results were observed when receptors were autophosphorylated with unlabeled ATP or [γ-32P]ATP. In the former case, kinase activity was stopped by chelating divalent cations with EDTA and following receptor phosphotyrosine content by immunoblotting with antiphosphotyrosine antibodies. In the latter case, the rate of loss of 32P was the same when EDTA alone was added or when excess unlabeled ATP was added to dilute out [32P]ATP as a substrate. Taken together, these data indicate that the loss of 32P receptor content was not the result of an exchange reaction (34).

Several lines of evidence indicate that the dephosphorylation reaction reflected true PTPase activity. Thus, the presence of DTT in the reaction mixture was essential and fully in accord with previous studies documenting the strict dependence of PTPase activity on the presence of reducing agents (28, 29). In fact, recent mutational analyses support the idea that a highly conserved cysteine residue plays an essential role in the catalytic activity (35). Furthermore, it has recently been shown that a cysteine-phosphate intermediate forms during PTPase catalysis (36). In addition, dephosphorylation was inhibited by the classic PTPase inhibitors zinc (28, 29), vanadate, and molybdate (28, 29, 37). The fact that the observed PTPase activity resulted from the action of an intrinsic endosomal enzyme(s) was suggested by the observation that the rate of insulin receptor dephosphorylation was not accelerated by adding fresh hepatic cytosol. This was confirmed by drastic pretreatments well known to strip away intracellular proteins and loosely attached pe-
Based on their cDNA sequences, recently cloned PTPases can be divided into two subfamilies. One subfamily consists of transmembrane PTPases (38–42), whereas the second subfamily consists of cytosolic enzymes containing noncatalytic domains that appear to specify cellular distribution. Thus one recently cloned enzyme contains in its amino-terminal region SH2 domains that impart a capacity to bind to phosphotyrosine-containing proteins (43). Several others contain hydrophobic carboxyl termini and/or sites for lipid attachment (10, 24–26) that appear to specify a strong membrane attachment. In recent studies, both intact and carboxyl-terminal truncated PTP-TC were expressed in baby hamster cells and a baculovirus system (10, 26). A substantial fraction of the truncated enzyme was found in the cytosol, whereas the intact enzyme was entirely membrane-associated. From these considerations and our observations, we can ascertain whether the endosomal PTPase(s) is a transmembrane protein or a cytosolic-like enzyme with strong affinity for the lipid environment of membranes. We would interpret the effect of Triton X-100 to abolish insulin receptor dephosphorylation as indicating that within the environment of the intact membrane, intimate contact of the PTPase with its natural substrate occurred and was required for dephosphorylation to proceed. Our data do not permit us to distinguish between a model in which the phosphatase(s) is constitutively associated with the receptor and a model in which the phosphatase(s) is a membrane-diffusible protein whose association with the receptor is a regulated phenomenon.

Experiments with PTPase inhibitors were designed to meet three objectives: 1) to assess the potency of traditionally used inhibitors in this in situ assay, 2) to assess the potency of known insulin mimetic agents such as pV (14) and H2O2 (30), and 3) to assess their relative potency toward two different receptors. The results showed that among all the inhibitors tested, pV, the most powerful insulin mimetic agent (14), was 100–1000-fold more potent than the other inhibitors. It is tempting to relate the PTPase inhibitory effect of pV to its capacity to effect insulin receptor kinase activation and to mimic insulin (14). Of interest was the very comparable affinity for the lipid environment of membranes. We would tend to exclude the above as candidates for the receptor-associated PTPases characterized in the present work. When insulin dissociates from its receptor, the metabolic responses entrained by the hormone decay rapidly (22). In previous studies, in FA0 cells, pulse-chase experiments with 32P, labeling showed that label was lost from the receptor with a half-life of 20 min (22). This reflects a number of events in the intact cell, including the time taken to clear receptor-associated insulin, continuing autophosphorylation from an intracellular [32P]ATP pool, and dephosphorylation events. In digitonin-permeabilized adipocytes, dilution of the prelabeled intracellular ATP pool permitted a more precise measurement of the dephosphorylation rate that was found to be about 2 min (44), a rate similar to that observed in the present study.

We have recently observed that following insulin administration, PM insulin receptors contained more phosphorylase per β-subunit than did those in ENs (6); nevertheless, endosomal receptor kinase activity was augmented. It is thus possible that the consequence of a limited initial dephosphorylation is activation of the kinase, followed by later deactivation as dephosphorylation becomes more extensive and complete. It is noteworthy that in this latter study, the level of endosomal insulin receptor tyrosine phosphorylation was inversely related to the dose of injected insulin. In contrast, the level of endosomal EGF receptor tyrosine phosphorylation increased with increasing doses of injected ligand. Thus, the behavior of the endosomal receptor phosphorylation state in vivo paralleled the activities of the endosomal PTPase(s) regulating receptor dephosphorylation. The potentially complex regulatory role of the receptor-associated PTPase(s), as well as the possibility that they constitute the important target(s) for the pharmacologically potent peroxovanadium species makes their identification and full characterization important.

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