Reversible Inactivation of Protein-tyrosine Phosphatase 1B in A431 Cells Stimulated with Epidermal Growth Factor*

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Stimulation of various cells with growth factors results in a transient increase in the intracellular concentration of H$_2$O$_2$ that is required for growth factor-induced protein tyrosine phosphorylation. The effect of H$_2$O$_2$ produced in response to epidermal growth factor (EGF) on the activity of protein-tyrosine phosphatase 1B (PTP1B) was investigated in A431 human epidermoid carcinoma cells. H$_2$O$_2$ inactivated recombinant PTP1B in vitro by oxidizing its catalytic site cysteine, most likely to sulfenic acid. The oxidized enzyme was reactivated more effectively by thioredoxin than by glutaredoxin or glutathione at their physiological concentrations. Oxidation by H$_2$O$_2$ prevented modification of the catalytic cysteine of PTP1B by iodoacetic acid, suggesting that it should be possible to monitor the oxidation state of PTP1B in cells by measuring the incorporation of radioactivity into the enzyme after lysis of the cells in the presence of radiolabeled iodoacetic acid. The amount of such radioactivity associated with PTP1B immunoprecipitated from A431 cells that had been stimulated with EGF for 10 min was 27% less than that associated with PTP1B from unstimulated cells. The amount of iodoacetic acid-derived radioactivity associated with PTP1B reached a minimum 10 min after stimulation of cells with EGF and returned to baseline values by 40 min, suggesting that the oxidation of PTP1B is reversible in cells. These results indicate that the activation of a receptor tyrosine kinase by binding of the corresponding growth factor may not be sufficient to increase the steady state level of protein tyrosine phosphorylation in cells and that concurrent inhibition of protein-tyrosine phosphatases by H$_2$O$_2$ might also be required.

Ligation of a variety of cell surface receptors, including those for growth factors and cytokines, induces a transient increase in the intracellular concentration of H$_2$O$_2$ in mammalian cells (1–3). Inhibition of this effect blocks receptor-mediated signal transduction. For example, inhibition of the accumulation of H$_2$O$_2$ by introducing catalase into NIH 3T3 or A431 cells prevented the induction of tyrosine phosphorylation by platelet-derived growth factor or epidermal growth factor (EGF) (2, 3). Direct exposure of cells to H$_2$O$_2$ also increases protein tyrosine phosphorylation and activates signal transduction pathways (2–5). Because the extent of protein tyrosine phosphorylation in a cell reflects an equilibrium between the actions of protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs), either stimulation of PTKs or inhibition of PTPs would be expected to shift the equilibrium toward phosphorylation. PTP activity in crude cell extracts can be inactivated by various oxidants, including H$_2$O$_2$, and this inactivation can be reversed by incubation with thiolic compounds such as dithiothreitol (DTT) and GSH (6, 7). These observations suggest that PTPs might undergo H$_2$O$_2$-dependent inactivation in cells, resulting in a shift in the equilibrium with PTKs toward phosphorylation.

PTPs constitute a diverse family of enzymes that can be divided into several subgroups, including receptor PTPs and nonreceptor PTPs (8–11). All PTPs contain an essential cysteine residue in the signature active site motif, HCXXC(s/T). The PTP active site cysteines exhibit low $pK_a$ values (5.4 for mammalian PTP1 (12), 5.6 for human dual specific PTP (13), and 4.7 for Yersinia PTP (14)) and are readily ionized at neutral pH, whereas the $pK_a$ of a typical cysteine residue is 8.5. The ionized essential sulfhydryl group (thiolate anion) contributes to the formation of a thiol-phosphate intermediate in the catalytic mechanism of PTPs (15). In addition, the essential cysteine is the target of specific modification by various sulf-hydryl-alkylating reagents (12–14, 16, 17).

We now demonstrate that H$_2$O$_2$, either added extracellularly or generated intracellularly in response to EGF, can cause reversible inactivation of PTPs in cells, and we identify the most plausible electron donor responsible for the reactivation of such inactivated PTPs. PTP1B, the widely expressed cytosolic enzyme originally purified from human placenta (18), was chosen as the target enzyme and was studied in A431 human epidermoid carcinoma cells.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium, fetal bovine serum, penicillin, and streptomycin were from Life Technologies, Inc. Rabbit polyclonal antibodies to PTP1B were kindly provided by B. G. Neel (Harvard University). A monoclonal antibody to PTP1B was obtained from Oncogene Science. Horseradish peroxidase-conjugated antibodies to mouse or to rabbit immunoglobulin G were from Amersham Pharmacia Biotech. Yeast glutathione reductase (GR) and bovine catalase were from Boehringer Mannheim. The synthetic peptide Raytide and the kinase p43abl were from Oncogene Science. [γ-32P]ATP was from

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† The abbreviations used are: EGF, epidermal growth factor; PTK, protein-tyrosine kinase; PTP, protein-tyrosine phosphatase; PTP1, protein-tyrosine phosphatase 1; PTP1B, protein-tyrosine phosphatase 1B; DTT, dithiothreitol; GR, glutathione reductase; PAG, polyacrylamide gel electrophoresis; Trx, thioredoxin; Grx, glutaredoxin; TR, thioredoxin reductase; HPLC, high performance liquid chromatography; nNPP, p-nitrophenyl phosphate; Bis-Tris, [bis(2-hydroxyethyl)amino]-tris(hydroxymethyl)methane; DTNB, 5,5’-dithiobis-(2-nitrobenzoic acid).
Redox Regulation of Protein-tyrosine Phosphatase 1B

Iod acoustic Acid Labeling and Immunoprecipitation of PTP1B—A431 cells, maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 units/ml), were allowed to reach 80–90% confluence in 150-mm dishes. The cells were then deprived of serum for 16 h and subsequently treated with 25 mM H2O2 for 0.5 h. The cells were rinsed and then exposed in an anaerobic chamber to 1 ml of O2-free lysis buffer (50 mM Bis-Tris-HCl (pH 6.5), 0.5% Triton X-100, 0.05% deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, leupeptin (0.5 μg/ml), aprotinin (0.5 μg/ml), and 0.1 mM iodoacetamide)-benzenesulfonyl fluoride hydrochloride containing 2 mM [14C]iodoacetic acid and briefly sonicated. As a control, serum-deprived cells that were not stimulated with EGFR or H2O2, were likewise lysed and labeled. After 30 min at room temperature in the dark, the labeling reaction was stopped by adding 0.2 ml of 200 mM cold iodoacetic acid in 0.8 M Tris-HCl (pH 7.5) and followed by the addition of 0.1 ml of 1 M DTT. The reaction mixtures were then centrifuged at 10,000 × g for 20 min. The resulting supernatants were subjected to a G-25 gel filtration chromatography to remove excess radioactivity. Protein-containing fractions were pooled, and protein concentrations were measured. The pooled samples were preclayed by incubating for 4 h at 4°C with 40 μl of goat anti-mouse immunoglobulin-coated immunobeads (25% slurry) (Sigma) and centrifuging at 3000 × g for 30 s. Afterward, 40 μl of goat anti-mouse immunoglobulin immunobeads (25% slurry) that had been absorbed with an excess of monoclonal antibody to PTP1B were added to the resulting supernatants, and incubation was continued for an additional 4 h. The beads were separated; extensively washed once in ice-cold O2-free lysis buffer, twice in O2-free 50 mM Tris-HCl buffer (pH 7.5), and twice in O2-free phosphate-buffered saline (pH 7.4); and subjected either to SDS-PAGE in order to measure the amount of radioactivity incorporated into PTP1B or to assay the amount of PTP activity regenerated by treatment with DTT.

RESULTS

PTP1B was originally isolated from human placenta as a soluble 37-kDa protein (18). It was subsequently shown that the full-length (50-kDa) PTP1B protein contains 345 amino acids and that the 37-kDa protein corresponds to the NH2-terminal 321 residues (26). The COOH-terminal 114 residues of the full-length protein contain a sequence responsible for localization of PTP1B in the endoplasmic reticulum (23). The 37-kDa PTP1B contains six cysteine residues, which do not appear to form disulfide bonds on the basis of the crystal structure of the protein (27). Both Cys121 and Cys215 of PTP1B, which are conserved among all members of the PTP family (28). Cys121, which has a pKa value of 5.4, is the essential cysteine residue located at the active site. However, mutation of Cys121 in PTP1B, the rat homolog of PTP1B, markedly reduces PTP activity (15). The active site cysteines of various PTPs, including Cys215 of PTP1B, are specifically targeted by the sulfhydryl-modifying reagent iodoacetic acid (12–14, 16, 17). The same active site cysteine residues have also been implicated as the site of oxidation by various oxidants (2, 3, 6, 7, 29), in which case one should be able to monitor the extent of H2O2-induced inactivation of PTP1B in cells by measuring the amount of radiolabeled protein incorporated into the enzyme by cell lysis in the presence of radiolabeled iodoacetic acid. With this in mind, we expressed the 37-kDa PTP1B in E. coli, purified the recombinant protein, and subjected it to modification by H2O2 and iodoacetic acid.

Identification of the H2O2-sensitive Cysteine in PTP1B—Incubation with H2O2 resulted in the inactivation of the purified recombinant 37-kDa PTP1B in a manner dependent on time and H2O2 concentration (Fig. 1, A and B). When the enzyme samples from the experiment shown in Fig. 1B (which had been inactivated to various extents by incubation with different concentrations of H2O2) were subjected to labeling with [14C] iodoacetic acid at pH 6.5, the extent of labeling decreased in proportion to the extent of inactivation (Fig. 1C), indicating that the site of oxidation by H2O2 is the same as the site of labeling by iodoacetic acid. The data shown in Fig. 1 were

Amersham Pharmacia Biotech, and 14C- or 3H-labeled iodoacetic acid was from NEN Life Science Products.

Recombinant PTP1B—Complementary DNA corresponding to the 37-kDa form (NH2-terminal 321 residues) of PTP1B was obtained by the polymerase chain reaction, cloned downstream of the phase T7 RNA polymerase promoter at the NcoI site of PET-14b (Novagen) (thus providing a histidine tag attached to the NH2 terminus of PTP1B by a thrombin-sensitive sequence), and expressed in Escherichia coli strain BL21 (DE3) by standard procedures. The histidine-tagged PTP1B fusion protein was purified from Escherichia coli extract with the use of an immobilized nickel resin (Novagen). The purity of the PTP1B preparation was determined spectrophotometrically, and the histidine-tagged PTP1B and Trx were determined spectrophotometrically, with the 280 values of 0.1% solutions were 1.231 and 0.738, respectively. The concentrations of other proteins were determined with the BCA protein assay reagent (Pierce), with bovine serum albumin as a standard.

Preparation of Thioredoxin (Trx), Glutaredoxin (Grx), and Trx Reductase (TR)—Rat Trx cDNA (19) was obtained by the polymerase chain reaction, cloned into the pET-17b expression vector, and expressed in E. coli by standard procedures. Recombinant Trx was purified from the cytosolic fraction of E. coli by heat treatment at 65°C followed by sequential chromatography on Sephacryl S-100 HR gel filtration (Amersham Pharmacia Biotech) and high performance liquid chromatography (HPLC) DEAE-5PW ion exchange columns. TR and Grx were purified from rat liver as described (20–22).

Determination of Protein Concentration—The concentrations of recombinant PTP1B and Trx were determined spectrophotometrically, and the A280 values of 0.1% solutions were 1.231 and 0.738, respectively. The concentrations of other proteins were determined with the BCA protein assay reagent (Pierce), with bovine serum albumin as a standard.

Assay of PTP1B Activity—Two different methods were used to assay PTP1B activity. The activity of recombinant PTP1B was measured spectrophotometrically with p-nitrophenyl phosphate (pNPP) as a substrate in a reaction containing 40 mM Bis-Tris-HCl (pH 7.0), 2 mM EDTA, 50 mM NaCl, and 10 mM pNPP (13). The initial velocity of p-nitrophenol formation was measured by monitoring the change in A405. An assay of PTP1B activity in immune complexes was performed with 32P-phosphorylated Raytide as a substrate (23). Assay mixtures (50 μl) containing the immune complex, 20 mM Tris-HCl (pH 7.5), bovine serum albumin (0.1 mg/ml), leupeptin (0.1 mg/ml), and 10 mM EDTA, 10 mM DTT, 0.1 mM 32P-phosphorylated Raytide were incubated at 30°C for 30 min, after which the reaction was terminated by the addition of 10 μl of glacial acetic acid, and the radioactivity associated with the peptide was measured as described (24).

Determination of Free SH Groups—PTP1B (100 μg) that had been treated with DTT and then dialyzed against a DTT-free buffer under anaerobic conditions was incubated with 266 μM iodoacetic acid (pH 7.2) and 100 μM 2-monobromoisobutyric acid (pH 7.0) for 20 min at room temperature, and the oxidation reaction was stopped by adding 1 μl of catalase. The resulting oxidized and control unoxidized enzymes (100 μg in 100 μl) were separately mixed with 300 μl of a solution containing 266 μM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), 6 μg guanidine hydrochloride (pH 9.0). The concentration of thionitrosobenzene acid released was determined spectrophotometrically with a molar extinction coefficient of 13,700 at 412 nm (25).

Identification of the H2O2-sensitive Residue in PTP1B—PTP1B (64 μg) that had been incubated with 100 μM H2O2 for 10 min and then treated with catalase as described above was incubated with 2 μM [3H]Idoic acid in a total volume of 100 μl of 40 mM Bis-Tris-HCl (pH 6.5) containing 0.1 mM EDTA. As a control, unoxidized PTP1B (64 μg) was likewise treated with [3H]Idoic acid. After 10 min at room temperature, the reaction was stopped by adding 100 μl of 2-mercaptoethanol and the reaction mixtures were subjected to gel filtration chromatography on a Sephadex G-25 column to remove unreacted iodoacetic acid. The PTP1B-containing fractions were pooled and incubated with 2.5 μg of endotoxin LPS-C in a total volume of 400 μl. The resulting digestion products were analyzed by HPLC on a C18 column with a linear gradient (0–60% buffer A) of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min over 60 min. Fractions corresponding to each peptide peak were collected manually, and a portion (10%) of each fraction was analyzed for 3H radioactivity.
before and after oxidation by H$_2$O$_2$ (100 μM for 20 min) with the use of Ellman’s reagent (DTNB). Data from five independent experiments revealed the presence of 5.81 ± 0.19 and 4.98 ± 0.17 (means ± S.E.) sulphydryl groups per PTP1B molecule for the fully reduced and H$_2$O$_2$-oxidized enzymes, respectively, suggesting that only one cysteine residue per molecule is affected by H$_2$O$_2$.

To identify the H$_2$O$_2$-sensitive cysteine, we exposed PTP1B oxidized with H$_2$O$_2$ (100 μM for 10 min to achieve 80% inactivation) as well as unoxidized control enzyme to $^3$H]iodoacetic acid. The labeled enzymes were then cleaved with endoproteinase Lys-C, the resulting peptides were separated by HPLC on a C$_{18}$ column to yield 25 identifiable peaks, and the amount of $^3$H radioactivity associated with each peak was measured (data not shown). The peptides from oxidized and unoxidized PTP1B enzymes produced virtually identical elution profiles. For unoxidized PTP1B, ~80% of total radioactivity was associated with a major peak that eluted at 36.4 min, whereas the remaining 20% of radioactivity was distributed among several other peaks. However, for the H$_2$O$_2$-treated enzyme, although the peak that eluted at 36.4 min contained the most radioactivity, this amount was only 23% of that associated with the corresponding peak for the unoxidized enzyme. Edman degradation of the major radioactive peptide derived from the unoxidized enzyme yielded a complete sequence of VRESGISLPEHGPVVHIXSAGIRSGTFXLADTXLLMDK, which matches exactly the sequence of amino acids 198–237 of PTP1B with the exception that the residue corresponding to position 215 was identified as carboxymethylated cysteine and the residues corresponding to positions 226 and 231 were unknown. The PTP1B sequence between residues 198 and 237 contains three cysteines at positions 215, 226, and 231. When the Edman degradation products after each cycle were collected and measured for radioactivity, most of the $^3$H was detected at the 18th cycle (Cys$^{215}$), with smaller amounts also present for the next several cycles. No radioactivity was detected at the cycles corresponding to Cys$^{226}$ and Cys$^{231}$. Together, these results suggest that the active site Cys$^{215}$ is the major site of alkylation by iodoacetic acid as well as the site of oxidation by H$_2$O$_2$. Thus, it should be possible to monitor the extent of PTP1B inactivation by H$_2$O$_2$ by measuring the amount of radioactive iodoacetic acid incorporated into the enzyme at pH 6.5.

Oxidative Inactivation of PTP1B in A431 Cells—A431 cells were incubated for various times with EGF and then lysed in an anaerobic chamber by exposure to a pH 6.5 buffer containing Triton X-100 and $[^{14}C]$iodoacetic acid. PTP1B was then immunoprecipitated from the cell lysate with a highly specific monoclonal antibody, and the radioactivity associated with the precipitated 50-kDa PTP1B protein was measured after SDS-PAGE. The extent of incorporation of $[^{14}C]$iodoacetic acid into PTP1B decreased with time of incubation of the cells with EGF, reaching a minimum at 10 min, and returned to the basal value by 40 min (Fig. 2).

Data from six independent experiments revealed that the extent of the decrease in $[^{14}C]$iodoacetic acid incorporation into the PTP1B immunoprecipitate measured after stimulation of cells for 10 min with EGF was 26.8 ± 6.4% (Fig. 3A). Stimulation of A431 cells for 10 min with H$_2$O$_2$ at concentrations of 1 or 3 mM reduced the extent of radioactivity incorporated into the PTP1B immunoprecipitate by 33.9 ± 7.4 and 42.3 ± 6.1%, respectively (Fig. 3A). These results suggest that PTP1B is oxidized at the active site cysteine in response to stimulation of cells with EGF or H$_2$O$_2$.

Additional evidence for the oxidation of the active site cysteine of PTP1B was provided by reaction experiments. Cys$^{215}$ of PTP1B is likely oxidized to sulfenic acid (Cys-SOH) by...
H$_2$O$_2$ (see “Discussion”). Because sulfenic acid is readily reduced by a thiol, incubation of the immunoprecipitates from the experiments shown in Fig. 3A with DTT would be expected to reactivate the PTP1B molecules oxidized by H$_2$O$_2$ but not those modified by iodoacetic acid. To test this prediction, we subjected the iodoacetic acid-labeled immunoprecipitates from the EGF-treated and H$_2$O$_2$-treated cells to DTT treatment and subsequent assay for PTP activity. As expected, the PTP activity of immunoprecipitates from EGF-treated (0.56 ± 0.09 pmol/30 min) or H$_2$O$_2$-treated (0.60 ± 0.13 and 0.69 ± 0.12 pmol/30 min for 1 and 3 mM H$_2$O$_2$, respectively) cells was greater than that for immunoprecipitates from unstimulated A431 cells (0.29 ± 0.08 pmol per 30 min). These results thus support the notion that PTP1B is oxidized by H$_2$O$_2$ and that the modified enzyme can be reduced to its original state by DTT.

To estimate the total activity of unlabeled PTP1B, immunoprecipitates were prepared from A431 cell lysates and subjected to DTT treatment and subsequent assay for PTP activity as described in the legend of Fig. 3, with the exception that iodoacetic acid was omitted from the lysis buffer. The total PTP activity thus assessed was 4.5 ± 0.09 pmol/30 min and was not affected by stimulation of cells with EGF or H$_2$O$_2$, suggesting that the total amount of PTP1B was not affected by EGF or H$_2$O$_2$. The PTP activities of DTT-treated immunoprecipitates derived from iodoacetic acid-labeled lysates were then expressed as a percentage of the total PTP activity (Fig. 3B). The observation that such immunoprecipitates from unstimulated cells exhibited substantial PTP activity (6.4% of total activity) suggests that PTP1B was not fully carboxymethylated by iodoacetic acid or that a small fraction of PTP1B in unstimulated cells exists in an oxidized state. The percentages of the total activity regenerated by DTT for the stimulated cells (12.4% for EGF-treated cells; 13.3 and 15.3% for cells treated with 1 or 3 mM H$_2$O$_2$, respectively) (Fig. 3B) were substantially lower than the corresponding percentage decreases in iodoacetic acid labeling (26.8, 33.9, and 42.3%, respectively) (Fig. 3A). The reason for this discrepancy is not clear. It is possible that exposure of immunoprecipitates to air during handling might have resulted in further oxidation of sulfenic acid to sulfinic acid (Cys-SOOH), which cannot be reduced by DTT.
**Fig. 4. Reactivation of H$_2$O$_2$-inactivated PTP1B by various electron donors.** Recombinant PTP1B (378 µg) was inactivated by incubation for 20 min on ice with 100 µM H$_2$O$_2$ in a total volume of 150 µl containing 40 mM Bis-Tris-HCl (pH 6.5) and 0.1 mM EDTA. After stopping the reaction by adding 1 µg of catalase, reactivation reactions were initiated at 25 °C by adding 20 µl of the inactivated enzyme to 80 µl of activation buffer (100 mM Tris-HCl (pH 7.5), 0.15% NaCl, 1 mM EDTA) in the absence (○) or presence of either the Trx system (3.8 µM Trx, 0.2 µM TR, 200 µM NADPH) (○), the Grx system (3.8 µM Grx, 4 mM GSH, 0.21 µM GR, 200 µM NADPH) (●), 4 mM GSH (△), or 4 mM DTT (□). At the indicated times, 15 µl of the reactivation mixture were removed and assayed for PTP activity with pNPP as a substrate. Data were corrected for background activity (1.4 nmol/min) due to incomplete inactivation by H$_2$O$_2$ and are means of two independent experiments.

**Fig. 5. NADPH oxidation coupled to the regeneration of PTP1B by the Trx, Grx, or GSH systems.** Recombinant PTP1B (7 µM) inactivated by H$_2$O$_2$ as described in Fig. 4 was placed in a cuvette containing 200 µl of activation buffer in the presence of either the Trx system (10 µM Trx, 0.2 µM TR, 200 µM NADPH) (○), the Grx system (10 µM Grx, 0.2 µM GR, 4 mM GSH, 200 µM NADPH) (△), or the GSH system (4 mM GSH, 0.2 µM GR, 200 µM NADPH) (□). Other cuvettes contained identical reaction mixtures with the exception that the fully reduced form of PTP1B was used. NADPH oxidation coupled to PTP1B reduction was measured spectrophotometrically by monitoring the difference in A$_{340}$ (ΔA$_{340}$) of the corresponding pairs of cuvettes. Data are from a representative experiment.

**DISCUSSION**

Given the recent observation that H$_2$O$_2$ is required for the growth factor-induced tyrosine phosphorylation of cellular proteins, we investigated whether H$_2$O$_2$ produced in response to EGF is capable of inactivating PTP1B in A431 cells.

First, with the use of the recombinant 37-kDa form of PTP1B, we demonstrated that the essential residue Cys$^{215}$ is the site of oxidation by H$_2$O$_2$. The oxidized products of cysteine include sulfenic acid, disulfide, sulfonic acid, and sulfonic acid (Cys-SO$_3$H). The disulfide intermediate can be excluded as the H$_2$O$_2$-modified form of PTP1B on the basis of our observation that only one out of six DTNB-sensitive residues was lost after H$_2$O$_2$ oxidation, and the sulfenic and sulfonic acid intermediates can be excluded on the basis of the observation that the oxidized PTP1B can be reduced back to its original state by DTT. Nevertheless, PTP1B was shown to form sulfenic and sulfonic acid intermediates when oxidized in the presence of osteoporosis drug alendronate (32) and pervanadate (33), respectively. Cysteine sulfenic acid is highly unstable and readily undergoes condensation with a thiol. However, the sulfenic acid intermediate of PTP1B is probably stabilized by the fact that, according to the x-ray structure of the 37-kDa form of PTP1B (27), no cysteine residues are located near Cys$^{215}$. Furthermore, the sulfenate anion (Cys-SO$_3^-$) is also probably stabilized by a salt bridge to Arg$^{232}$, which was shown to stabilize the thiolate anion of Cys$^{215}$ and consequently to reduce its pK$_a$.

Second, we measured the amount of radiolabeled iodoacetic acid incorporated into PTP1B as a means of monitoring changes in the oxidation state of the protein in A431 cells stimulated with EGF. This approach was based on our observations that iodoacetic acid reacts almost exclusively with Cys$^{215}$-SH of PTP1B at pH 6.5 and that the oxidation of this cysteine residue by H$_2$O$_2$ prevents its reaction with iodoacetic acid. Exposure of A431 cells to EGF resulted in a decrease in the extent of iodoacetic acid labeling of PTP1B, with the maximal (27%) decrease apparent 10 min after stimulation and the labeling returning to baseline values by 40 min. This reduced labeling is probably due to the oxidation of PTP1B by H$_2$O$_2$, with a reactivation was 0.15 µM.

Reactivation of PTP1B required all three components (Trx, TR, and NADPH); reactivation in the absence of one of the three components was negligible (data not shown). The half-maximal concentration of Trx required for reactivation was 0.15 µM. In agreement with the data shown in Fig. 4, the rank order for oxidation by including GR and NADPH in the reaction mixture. NADPH. PTP1B reduction by GSH was also coupled to NADPH. GSH system (Fig. 5). With the PTP1B concentration of 7 µM used in Fig. 5, the maximal change in A$_{340}$ is expected to be 0.042 absorbance units.

Reactivation of oxidized PTP1B by the Trx system required the absence of one of the three components was negligible (data not shown). The half-maximal concentration of Trx required for reactivation was 0.15 µM.
induced protein tyrosine phosphorylation requires H₂O₂ production and our current observation that growth factor-induced generation of H₂O₂ is sufficient to cause inactivation of PTP1B, we propose that the activation of a receptor PTK by interaction with a growth factor may not be sufficient to increase the steady state level of protein tyrosine phosphorylation in a cell; rather, concurrent inhibition of PTPs by H₂O₂ may also be required for this effect. The extent of tyrosine phosphorylation of receptor PTKs and their substrates would then return to basal values after degradation of H₂O₂ and the subsequent reactivation of PTPs by electron donors. Our in vitro data suggest that Trx might be a physiological electron donor for PTP1B. It remains to be determined whether other PTPs also form a sulfinic acid intermediate on oxidation with H₂O₂ and whether Trx reduces such oxidized intermediates. The low molecular weight PTP, which shows no apparent sequence similarity to other PTPs but which shares several common features in active site architecture (9, 39), forms a disulfide on oxidation with nitric oxide (40).

A scheme depicting the proposed roles of H₂O₂ and Trx in growth factor-induced protein tyrosine phosphorylation is shown in Fig. 6. This scheme is consistent with the following observations: 1) production of H₂O₂ via NADPH oxidase results in inhibition of the PTP activity of CD45 in neutrophils, 2) blocking the H₂O₂ production by treatment with N-acetylcysteine or diphenylene iodonium, an inhibitor of NADPH oxidase, restores its PTP activity (41, 42), and 3) removal of intracellular oxidants by pyrroline dithiocarbamate diminishes protein tyrosine phosphorylation (43). The scheme is also consistent with the suggestion that the ligand-independent basal activity of receptor PTKs might be sufficient to increase the extent of protein tyrosine phosphorylation in cells treated with thiol-alkylating agents, such as iodoacetic acid and iodoacetamide, or oxidants, such as ultraviolet light, that cause the inactivation of PTPs (29).

Previously proposed mechanisms for the regulation of non-receptor PTP activity include phosphorylation at serine and tyrosine residues (23, 44–48), anchoring via SRC homology 2 domains (49, 50), and proteolysis (51, 52). In contrast to these positive regulation mechanisms of PTP activity, oxidation by H₂O₂ provides a means for negative regulation of PTP activity. Negative modulation mediated by ligand-induced dimerization has also been proposed for the receptor PTP CD45 (53).

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Fig. 6. Roles of H₂O₂-dependent oxidation and Trx-dependent reduction of a PTP in growth factor-induced protein tyrosine phosphorylation. PY and YP, phosphotyrosine.

![Diagram](http://www.jbc.org/)
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