Dimerization In Vivo and Inhibition of the Nonreceptor Form of Protein Tyrosine Phosphatase Epsilon

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cyt-PTPε is a naturally occurring nonreceptor form of the receptor-type protein tyrosine phosphatase (PTP) epsilon. As such, cyt-PTPε enables analysis of phosphatase regulation in the absence of extracellular domains, which participate in dimerization and inactivation of the receptor-type phosphatases receptor-type protein tyrosine phosphatase alpha (RPTPα) and CD45. Using immunoprecipitation and gel filtration, we show that cyt-PTPε forms dimers and higher-order associations in vivo, the first such demonstration among nonreceptor phosphatases. Although cyt-PTPε readily dimerizes in the absence of exogenous stabilization, dimerization is increased by oxidative stress. Epidermal growth factor receptor stimulation can affect cyt-PTPε dimerization and tyrosine phosphorylation in either direction, suggesting that cell surface receptors can relay extracellular signals to cyt-PTPε, which lacks extracellular domains of its own. The inactive, membrane-distal (D2) phosphatase domain of cyt-PTPε is a major contributor to intermolecular binding and strongly interacts in a homotypic manner; the presence of D2 and the interactions that it mediates inhibit cyt-PTPε activity. Intermolecular binding is inhibited by the extreme C and N termini of D2. cyt-PTPε lacking these regions constitutively dimerizes, and its activities in vitro towards para-nitrophenylphosphate and in vivo towards the Kv2.1 potassium channel are markedly reduced. We conclude that physiological signals can regulate dimerization and phosphorylation of cyt-PTPε in the absence of direct interaction between the PTP and extracellular molecules. Furthermore, dimerization can be mediated by the D2 domain and does not strictly require the presence of PTP extracellular domains.

Phosphorylation of tyrosine residues in proteins is a central mechanism for protein regulation and is well established as a master regulator of physiological processes. Tyrosine phosphorylation is reversible and is controlled by the generically opposing activities of protein tyrosine kinases and protein tyrosine phosphatases (PTPs) (25). Receptor-type PTPs (RPTPs), which are a major structural subfamily of the PTP superfamily, are integral membrane proteins which possess extracellular domains of various lengths and typically two cytosolic phosphatase domains (2, 12, 49). Although their importance in regulating biological processes is well established, relatively little is known about how activities of RPTPs are regulated.

Extracellular molecules have been suggested to bind RPTPs and to influence PTP activity or function, much as binding by pleiotrophin inhibits activity of PTPβ/ζ (36). However, little is known about how such binding affects signal transduction across cell membranes or RPTP activity (5, 41, 42, 53). Structural studies have suggested that ligand-induced dimerization inhibits activity of RPTPs by stabilizing homodimeric structures, in which the helix-turn-helix "wedge" domain of one molecule prevents access of substrates to the catalytic domain of its dimerization partner (6, 34, 51). Dimerization by native RPTPs has been difficult to observe and in several cases could be examined only after additional stabilization. For example, dimerization of RPTPα by a disulfide link introduced into its

extracellular domain or of an epidermal growth factor receptor (EGFR)-CD45 chimeric protein following EGF treatment reduced phosphatase activity (13, 27, 35). In parallel, destabilization of the wedge domain increased activity of CD45 in vivo (35). The critical role of extracellular domains is also evident in that isoforms of CD45, which differ in the structure and glycosylation of their extracellular domains, homodimerize and are inhibited to different extents (52). Yet, recent data have suggested that interactions between cytosolic regions of RPTPα or of CD45 are not limited to the wedge domain (7, 8, 17, 23, 26) and that homodimerization may not occur among all RPTPs (24, 40). These findings raise the question of whether intermolecular interactions among RPTPs depend on input from membrane-spanning and extracellular domains.

Prominent candidates for mediating intermolecular interactions are the membrane-distal (D2) PTP domains present in most RPTPs. Although typically inactive, structures and sequences of D2 domains are often very similar to those of active, membrane-proximal (D1) domains to the point where activities of the D2 domains of RPTPε, RPTPα, or LAR could be significantly increased by a small number of specific point mutations (10, 31–33, 40). Evidence for a regulatory role for D2 domains has come from studies in which D2 of CD45 bound D1 of the phosphatase (17, 23) and the D2 domains of RPTPα, RPTPσ, LAR, RPTPδ, and RPTPμ bound RPTPα (7). The effect of D2-D1 binding is context dependent—it may inhibit PTP activity, as in the case of PTPσ (50), or can help achieve optimal enzymatic activity, as in CD45 (18). Binding by the RPTPα D2 domain was increased by oxidative stress, UV irradiation, or heat shock but not by tetradecanoyl phorbol

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acetate, serum, or EGF treatment (8), suggesting that distinct physiological signals can affect dimerization. In all, the high degree of conservation of D2 domains and their participation in molecular interactions strongly indicate that these domains have significant regulatory roles, although many details of how this is achieved are missing.

PTP epsilon (PTPε) exists as a small group of four proteins produced by the single PTPε gene. The two most prevalent are the receptor-type (RPTPε) and non-receptor-type (cyt-PTPε) forms, each the product of a distinct PTPε mRNA species. p67 and p65 PTPε are shorter molecules that are expressed together with either RPTPε or cyt-PTPε and whose production is regulated at the levels of translation and posttranslational processing (16, 20, 21, 29, 39, 45). RPTPε supports the transformed phenotype of Neu-induced mammary epithelial tumor cells, most likely by activating Src (14, 15, 19). The same PTPε isoform can also down-regulate insulin receptor signaling in BHK cells (1, 38). cyt-PTPε dephosphorylates and inactivates the Kv1.5 and Kv2.1 voltage-gated potassium channels in Schwann cells in vivo, in correlation with reduced myelination of sciatic nerves in young PTPε-deficient mice (43). Other roles for PTPε include inhibition of JAK-STAT signaling in M1 leukemia cells (46, 47), suppression of endothelial cell proliferation (48), and ensuring proper functioning of mouse macrophages (44).

Structural differences between RPTPε and cyt-PTPε are limited to their N termini; the extracellular and transmembranal domains of RPTPε are replaced in cyt-PTPε by a stretch of 12 hydrophilic amino acid residues. Downstream sequences, including the two tandem PTP domains, are identical in both molecules. As a result RPTPε is an integral membrane protein and cyt-PTPε is predominantly cytosolic, although some cyt-PTPε molecules can be found in association with the cell membrane and in the nucleus (16, 21, 28). cyt-PTPε is therefore an unusual naturally occurring non-receptor-type PTP with two catalytic domains. As such it enables us to address the question of whether membrane-spanning and extracellular domains and direct input from extracellular events are in fact required for dimerization in vivo, in the context of a naturally occurring molecule.

In this study we use coimmunoprecipitation and gel filtration to show that cyt-PTPε very readily forms homotypic dimers and higher-order associations in vivo. This significant basal self-association can be further affected by EGFR activation or increased oxidative stress, indicating that dimerization can be up- or down-regulated by physiological events. These findings also indicate that extracellular events can influence cyt-PTPε dimerization indirectly, by mediation of cell surface molecules such as the EGFR. We demonstrate that the inactive cyt-PTPε D2 domain is crucial in mediating interactions between cyt-PTPε molecules and that this domain strongly interacts in a homotypic manner and inhibits cyt-PTPε activity. We also show that, in the context of the entire cyt-PTPε molecule, the extreme N and C termini of the D2 domain act to inhibit dimerization. Accordingly, cyt-PTPε lacking either or both D2 termini constitutively dimerizes and exhibits significantly reduced activity in vitro towards *para*-nitrophenylphosphate (PNPP) and in vivo towards the Kv2.1 potassium channel in cells. We conclude that physiological events can regulate dimerization and phosphorylation of cyt-PTPε by a mechanism involving D2 and without requiring direct input from extracellular molecules.

MATERIALS AND METHODS

DNA constructs. cDNAs of cyt-PTPε, RPTPε, Kv2.1, and Y527F Src in the pcDNA3 plasmid (Invitrogen) were described previously (21, 43). The following cDNAs were derived from mouse cyt-PTPε by PCR and site-directed mutagenesis and cloned into pcDNA3: D1 + 12 (amino acid residues 1 to 394, numbering as in the sequence with accession number U36758), D2 + 12 (residues 1 to 12 linked to residues 360 to 642), D2 (residues 360 to 642), D2-N (residues 381 to 642), D2-C (residues 360 to 620), D2-N,C (residues 381 to 620), ΔN (full-length cyt-PTPε [residues 1 to 642] from which residues 360 to 380 had been deleted), ΔC (residues 1 to 620), and ΔN,C (residues 1 to 620, from which residues 360 to 380 had been deleted). Constructs were tagged with either FLAG or hemagglutinin (HA) at their C termini and were sequenced prior to use. EGFR cDNA was a generous gift of Yosef Yarden.

Cell culture. NIH 3T3 and 293 human embryonic kidney cells were grown in Dulbecco modified Eagle medium (Gibco-BRL), supplemented with 10% fetal calf serum (Gibco-BRL), 2 mM glutamine, 50 U of penicillin/ml, and 50 μg of streptomycin/ml. Transfections were done by the calcium phosphate technique (11) or with the Lipofectamine reagent (Invitrogen). 293 cells stably expressing Kv2.1 were generated by transfection of cells with Kv2.1 cDNA followed by selection in 1.5 mg of G-418/ml. Ras-transformed NIH 3T3 cells were produced by transfecting NIH 3T3 cells with EJ-Ras cDNA. Cells were maintained in medium containing 0.5% fetal calf serum until foci of transformed cells arose, which were then pooled. In some studies 293 cells were transfected with 1 μg of EGFR cDNA, grown in 0.1% serum for 24 h, and then stimulated with 100 ng of EGF (Sigma)/ml as indicated in Fig. 2. When used, 10 mM N-acetylcysteine was added to medium containing 0.1% serum during the last 12 to 14 h of incubation prior to EGF stimulation. In other cases cells in regular growth medium were treated with 1 mM H_2O_2 for 10 min and lysed and processed immediately.

Protein analysis. Cells were washed with phosphate-buffered saline and lysed in cold buffer A (50 mM Tris-Cl [pH 7.5], 100 mM NaCl, 1% Nonidet P-40), containing protease inhibitors [1 mM N-(α-aminoethyl)benzene-sulfonyl fluoride, 40 μM bestatin, 15 μM E64, 20 μM leupeptin, 15 μM pepstatin; Sigma]. For analysis after EGF and H_2O_2 treatments buffer contained also 0.5 mM sodium pervanadate. For immunoprecipitation 0.5 to 1 mg of total cell protein was mixed with anti-FLAG M2 affinity beads (Sigma) or with 3 μl of antiphosphotyrosine antibodies (Transduction Laboratories) coupled to protein A-Sepharose beads for 2 to 4 h at 4°C, followed by four extensive washes with radioimmunoprecipitation assay buffer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotting were performed as described previously (20). In control experiments where known, graded amounts of protein were subjected to SDS-PAGE and blotting, the intensities of signals obtained were proportional in a linear fashion to the different amounts of antigen loaded on the gel. Primary antibodies used in this study included polyclonal anti-PTPε (15), anti-Kv2.1 (Alomone), anti-HA (Santa Cruz), monoclonal anti-v-Src (Calbiochem), antiphosphotyrosine (Transduction Laboratories, clone PY20, or Santa Cruz, clone PY99), anti-FLAG (M2, Sigma), and anti-EGFR (Sigma).

Analysis of cyt-PTPε by gel filtration. Cells were lysed in buffer A, after which lysates were centrifuged for 10 min at 13,000 × g and for 60 min at 100,000 × g to remove all insoluble material. One milligram of protein was fractionated at 4°C on a Superdex 200 gel-filtration column (Pharmacia), previously equilibrated with running buffer (phosphate-buffered saline supplemented with 5% glycerol and protease inhibitors). The column was run at a constant flow rate of 0.5 ml/min, and fractions of 0.3 ml were collected until all loaded protein had flowed through. Equal portions of each fraction were resolved by SDS-PAGE, blotted, and probed with anti-PTPε antibodies to determine the distribution of cyt-PTPε among the fractions. Molecular mass standards (listed in the legend to Fig. 3) were fractionated under the same conditions, enabling estimation of molecular mass of the various fractions by semilogarithmic curve fitting.

Purification of PTPε and activity assay. FLAG-tagged cyt-PTPε was expressed in 293 cells. Following anti-FLAG precipitation, beads underwent three extensive washes in buffer B (20 mM HEPES [pH 7.6], 100 mM KCl, 0.5 mM EDTA, 0.4% Nonidet P-40, 20% [vol/vol] glycerol) and two rinses in buffer 54K (50 mM Tris-Cl [pH 7.9], 150 mM NaCl, 0.5% Triton X-100). Bound material was specifically eluted by incubating the beads at 30°C for 3 min in an equal volume of TBS buffer (20 mM Tris-Cl [pH 7.35], 150 mM NaCl) containing 1 mg of FLAG peptide (Sigma)/ml and 0.1 mM EGTA. Phosphatase activity of the eluted material was assayed in 96-well plates in reaction mixtures containing equal amounts of PTPε in 5 to 15 μl of eluate and 200 μl of assay buffer (50 mM morpholineethanesulfonic acid [pH 7.0], 0.5 mg of bovine serum albumin/ml, 10

mM PNPP). Activity was measured by monitoring the increase in absorption at 405 nm for an hour at room temperature, during which absorption was linear with time. Negative controls included reaction mixtures containing eluates prepared from mock-transfected cells or reaction mixtures containing cyt-PTPε to which 0.5 mM sodium pervanadate had been added; phosphatase activity in these controls was negligible. Activity measured was normalized to the relative amount of PTPε actually present in each eluate as determined by protein blotting.

RESULTS

RPTPε and cyt-PTPε form homotypic interactions in vivo. In order to determine whether RPTPε or cyt-PTPε can form homotypic interactions in vivo, we expressed HA- and FLAG-tagged versions of either protein in 293 cells. These and other constructs used throughout this study are shown schematically in Fig. 1A. Following immune precipitation of FLAG-tagged molecules, the presence of coprecipitated HA-tagged molecules was determined by blotting the precipitate with anti-HA antibodies. As seen in Fig. 1B, HA-RPTPε readily coprecipitated with FLAG-RPTPε, and similar results were obtained with FLAG- and HA-tagged cyt-PTPε. This result indicates that the divergent N termini of both molecules do not prevent intermolecular homotypic associations from being formed. Similar results were obtained when the antibodies were reversed, i.e., by immunoprecipitation with HA and probing with FLAG antibodies (data not shown). RPTPε and cyt-PTPε could be coprecipitated with each other (data not shown), indicating that formation of heterotypic interactions among PTPε molecules is not dependent upon the two molecules having identical N termini. This last result is primarily of mechanistic importance, since the expression patterns of RPTPε and cyt-PTPε in cells and tissues rarely overlap (16). In separate experiments comparison of the fraction of HA-cyt-PTPε molecules that coprecipitated with a given fraction of precipitated FLAG-cyt-PTPε molecules indicated that 12 to 25% of cyt-PTPε molecules were involved in homotypic associations in unstimulated cells. In contrast with RPTPα (8, 27), PTPε coimmunoprecipitation was detected with ease; chemical cross-linkers or engineered disulfide links were not required throughout these studies.

cyt-PTPε dimerization is affected by EGFR signaling and oxidative stress. In order to examine whether cellular signaling processes can affect the degree of dimerization of cyt-PTPε, we examined cyt-PTPε following EGF stimulation in 293 cells. Basal levels of cyt-PTPε dimers—evident as coprecipitation of HA- and FLAG-tagged cyt-PTPε—were observed in the cells prior to EGF stimulation (Fig. 2A). Massive autophosphorylation of the EGFR was evident within the first 5 min following stimulation, attesting to activation of the receptor by EGF (Fig. 2A). In parallel, a clear and reproducible 39% decrease in cyt-PTPε dimerization was noted 5 min following EGF treatment; dimerization levels rose to prestimulation values 10 min following application of EGF and rose to 310% of control values at 60 min poststimulation (Fig. 2A and B). Similar results were also obtained in NIH 3T3 cells, in which exogenous cyt-PTPε was expressed at lower levels (Fig. 2C). Decrease in dimerization at 5 min and its increase at 60 min were detected also in 293 cells that had been pretreated with the reactive oxygen species scavenger N-acetylcysteine, suggesting that effects of EGFR stimulation in this system are most likely not mediated by reactive oxygen species (Fig. 2E). Interest-
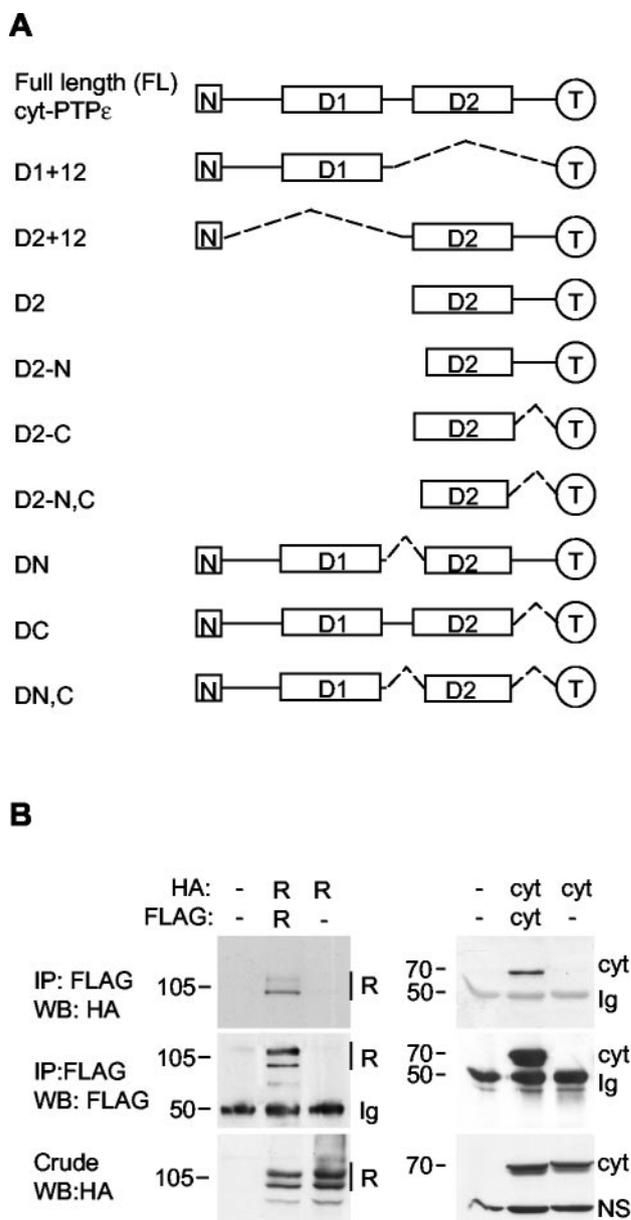
FIG. 1. cyt-PTPε and RPTPε form homodimers in vivo. (A) Schematic diagrams of PTPε proteins used in this study. Indicated are the 12 N-terminal residues unique to cyt-PTPε (N), the D1 and D2 PTP domains, and the site of HA or FLAG tag addition (T). Dashed lines denote deleted sequences. (B) 293 cells were transfected with HA- and FLAG-tagged RPTPε (R), after which FLAG-tagged RPTPε was precipitated. (Left) (top) Coprecipitated HA-RPTPε; (middle) precipitated FLAG-RPTPε; (bottom) HA-RPTPε present in cell lysates. Right panels show similar experiments for cyt-PTPε (cyt). NS, nonspecific bands; Ig, immunoglobulin. Size markers are in kilodaltons.

ingly, stimulation of the EGFR also affected cyt-PTPε phosphorylation (Fig. 2A). Phosphorylation was virtually undetectable before stimulation; 5 min after application of EGF, when dimerization was at its lowest, cyt-PTPε phosphorylation was significantly increased. At 60 min, when dimerization was maximal, phosphorylation levels had significantly decreased although they were still slightly higher than baseline levels. We conclude that extracellular events can indirectly influence cyt-
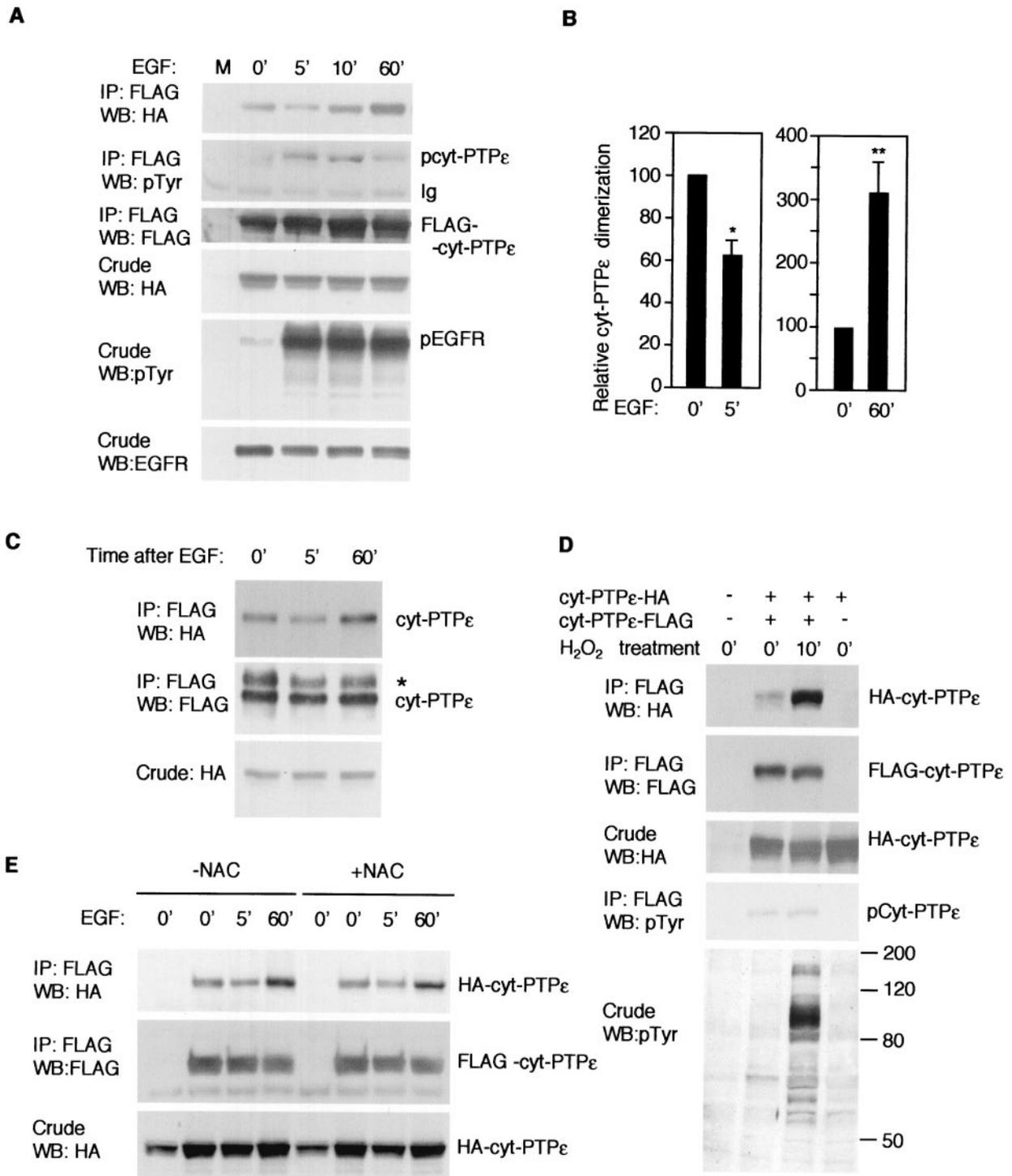
FIG. 2. EGFR signaling and oxidative stress affect cyt-PTPε dimerization. (A) EGFR stimulation affects cyt-PTPε phosphorylation and dimerization. 293 cells transfected with the EGFR and cyt-PTPε were treated with EGF for the times indicated. (Top) Dimerization of cyt-PTPε following EGF treatment, assayed as for Fig. 1B; (second from top) cyt-PTPε tyrosine phosphorylation; (third and fourth from top) immunopre-cipitated FLAG-cyt-PTPε and expression of HA-cyt-PTPε, respectively; (fifth from top) phosphorylation of cell proteins—mostly EGFR auto-phosphorylation—following EGF treatment; (bottom) expression levels of the EGFR in transfected cells. M, mock-transfected cells. (B) Bar diagram showing dimerization of cyt-PTPε at 5 and 60 min following application of EGF, relative to 0 min; results are from four and six independent experiments, respectively. Coprecipitated HA-cyt-PTPε amounts were normalized to amounts of FLAG-cyt-PTPε actually precipi-tated. *, $P = 0.0286$, and **, $P = 0.0022$, both by the Mann-Whitney test. (C) EGF treatment affects cyt-PTPε dimerization in NIH 3T3 cells. The experiment was performed as described for panel A with NIH 3T3 cells transiently expressing cyt-PTPε. The asterisk denotes a nonspecific band. Note reduced dimerization at 5 min and increased dimerization at 60 min. (D) Dimerization and tyrosine phosphorylation of cyt-PTPε following treatment of 293 cells with 1 mM H_2O_2. Subpanels shown are similar to those for panel A. Qualitatively similar phosphorylation results were obtained in cells expressing only FLAG-tagged cyt-PTPε (data not shown). Size markers are in kilodaltons. (E) Effect of EGFR stimulation on cyt-PTPε is not mediated by H_2O_2. The experiment presented in panel A was repeated with cells exposed to 10 mM N-acetylcysteine (NAC) for 12 to 14 h immediately preceding EGF stimulation. A reduction in dimerization at 5 min and an increase at 60 min post-EGF stimulation were detected in this experiment as well. Shown is an experiment representative of two performed.

PTPε dimerization through mediation of receptor-type molecules, such as the EGFR. Furthermore, EGFR stimulation can affect both dimerization and phosphorylation of cyt-PTPε; analysis of functional interactions between cyt-PTPε and the EGFR is beyond the scope of the present study and will be presented elsewhere.

Previous studies have shown that oxidative stress promotes dimerization of the related receptor-type RPTPα by inducing a switch in the conformation of the molecule, and coimmunoprecipitation experiments with RPTPα were unsuccessful without prior treatment of cells with H_2O_2 (8). As noted above, dimers of cyt-PTPε are readily detected in the absence of H_2O_2. Nonetheless, treatment of cells expressing PTPε with H_2O_2 significantly increased interactions between PTPε molecules (Fig. 2D). Interestingly, while H_2O_2 treatment significantly increased overall cellular tyrosine phosphorylation, a parallel increase in tyrosine phosphorylation of cyt-PTPε was not detected (Fig. 2D). This finding is in agreement with the different trends for phosphorylation versus dimerization observed following EGFR stimulation. In separate experiments lysing cells in the presence of dithiothreitol or performing SDS-PAGE analysis of precipitated material or of crude cell lysates in the absence of β-mercaptoethanol did not change the ability of cyt-PTPε to dimerize (data not shown). This indicates that intermolecular disulfide bonds are not major stabilizers of cyt-PTPε dimerization, although it does not strictly rule out participation of intramolecular disulfide bonds in this process. In all, we conclude that cyt-PTPε molecules can form dimers without relying on the presence of membrane-spanning and extracellular domains and that cellular signaling events and the degree of oxidative stress to which cells are exposed can affect the degree of dimerization.

Analysis of cyt-PTPε complexes by gel filtration. We next examined interactions of cyt-PTPε by gel filtration. This approach enabled us to examine complexes that include endogenous, untagged cyt-PTPε from untransfected cells, as well as to confirm immunoprecipitation results by a second, unrelated experimental technique. Lysates of Ras-transformed NIH 3T3 cells, which express significant amounts of endogenous cyt-PTPε (H. Toledano-Katchalski, J. Kraut, and A. Elson, unpublished data), were fractionated on a Superdex-200 fast protein liquid chromatography (FPLC) system. Cells were fractionated either with or without prior treatment with H_2O_2, and molecular masses of the various column fractions were estimated by separation of molecular mass standards on the same column. Amounts of cyt-PTPε in each fraction were estimated by protein blotting with anti-PTPε antibodies, which revealed the presence of full-length cyt-PTPε (~70-kDa) and of the smaller p67 and p65 PTPε proteins (21). cyt-PTPε showed a broad elution profile starting approximately at the mass of the cyt-PTPε monomer and extending towards higher molecular masses (Fig. 3A and B, nontreated samples). Most of the protein was found in a single peak (fractions 27 to 29), in agreement with the size of the cyt-PTPε monomer (70 kDa). About a third as much PTPε fractionated as a peak of higher mass (fraction 24), in agreement with the expected size of cyt-PTPε dimers (~140 kDa). Some cyt-PTPε protein was recovered in the range of ~1,200 kDa (fractions 7 and 8), beyond the optimal range of separation by the column system used. Low levels of cyt-PTPε were detected also in fractions of in-

termediate mass. To rule out the possibility that this broad separation profile resulted from nonspecific interactions with the column matrix, we separated similar cell extracts on a glycerol gradient and obtained a very similar separation pattern (data not shown). The separation profile therefore most likely reflects the dynamic nature of homo- or heterotypic interactions in which cyt-PTPε participates.

Treatment of cells with H_2O_2 prior to lysis and fractionation caused several changes in the separation profile. Peaks at ~130 to 140 kDa (fractions 24 to 26) and at ~1,200 kDa (fractions 5 to 8) were significantly increased in intensity at the expense of the cyt-PTPε monomer peak, which nearly disappeared (fractions 27 to 29, ~70 kDa) (Fig. 3A and B, H_2O_2-treated samples). This result indicated that H_2O_2 treatment significantly increased intermolecular interactions involving cyt-PTPε, in agreement with the increase in coimmunoprecipitation of tagged cyt-PTPε molecules observed in Fig. 2D.

The presence of cyt-PTPε in fractions of higher molecular mass may indicate its participation in homotypic and/or heterotypic interactions. In order to determine whether the separation profile of cyt-PTPε was due at least in part to homotypic interactions, we transfected 293 cells with FLAG- and with HA-tagged cyt-PTPε and fractionated lysates of these cells by FPLC. Column separation profiles in these experiments were very similar to those of endogenous cyt-PTPε from NIH 3T3 cells, and a similar shift to larger molecular masses was encountered following H_2O_2 treatment in these experiments as well (data not shown). In order to determine whether the various column fractions contained cyt-PTPε molecules bound to one another, coimmunoprecipitation experiments were carried out in fractions 5 and 6 for high-molecular-mass complexes, fractions 23 to 26 for putative dimers, and fractions 27 and 28 for cyt-PTPε monomers (Fig. 3C). Coprecipitation of HA-tagged cyt-PTPε was detected in fractions 24 to 26, indicating that cyt-PTPε dimers were indeed present in these fractions (Fig. 3C). As expected, amounts of coprecipitating HA-cyt-PTPε were significantly reduced in fractions 27 and 28, which included molecules similar in mass to monomeric cyt-PTPε, despite high levels of precipitating FLAG-tagged cyt-PTPε in these fractions. Importantly, this last result indicated that coprecipitation of HA- and FLAG-tagged cyt-PTPε in fractions 24 to 26 was due to interactions present in cells and did not reflect associations formed in column fractions following separation. Very little HA-cyt-PTPε was coprecipitated from fractions 5 and 6, probably due in part to the small amounts of FLAG-cyt-PTPε present in these fractions; large amounts of HA-cyt-PTPε were coprecipitated from these fractions from cells treated with H_2O_2 (data not shown). In all, these results independently confirm that cyt-PTPε dimers exist in cells in vivo and that increased oxidative stress within cells can induce significant association between cyt-PTPε molecules.

The D2 domain is an important mediator of binding between cyt-PTPε molecules. In order to identify sequences that mediate homotypic binding, we examined cyt-PTPε molecules in which either the D2 or the D1 PTP domain was removed (D1 + 12 and D2 + 12 proteins, respectively, "+12" indicating the presence of the 12 N-terminal-most residues unique to cyt-PTPε; Fig. 1A). Binding of each of these proteins to itself, to the other deletion mutant, or to full-length cyt-PTPε was examined by coprecipitation (Fig. 4). The D2 + 12 protein
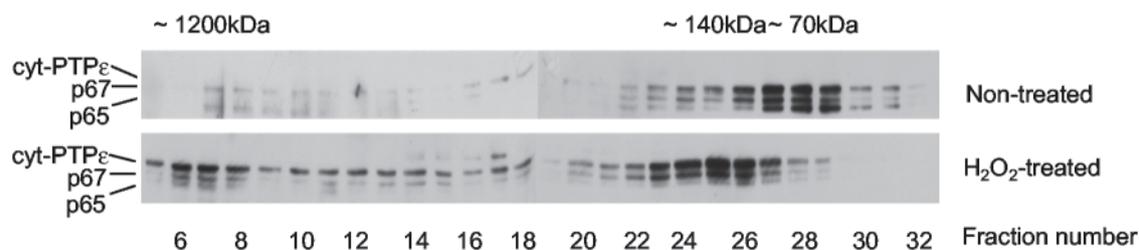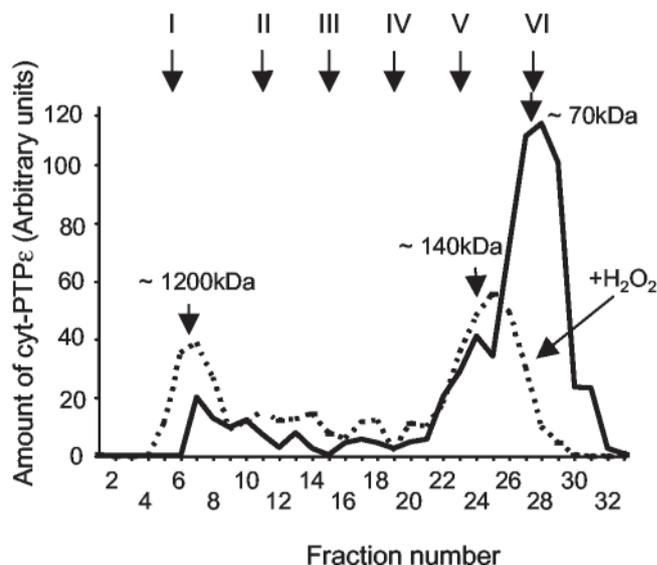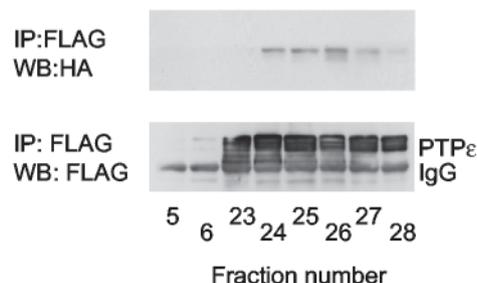
FIG. 3. Analysis of complexes formed by cyt-PTPε by gel filtration. (A) Ras-transformed NIH 3T3 cells were lysed and fractionated on a Superdex 200 FPLC system. Shown is the distribution of endogenous cyt-PTPε among column fractions in untreated cells (top) or in cells exposed to 1 mM H_2O_2 for 10 min (bottom). Fraction 1 immediately follows the column void volume. (B) Graph depicting relative amounts of cyt-PTPε in fractions of panel A. Arrows denote elution of molecular mass standards. Standards (Sigma) used were the following: dextran blue (I), bovine thyroglobulin (II), horse spleen apoferritin (III), β-amylase (IV), alcohol dehydrogenase (V), bovine serum albumin (VI), and carbonic anhydrase (not shown), at 2,000, 669, 443, 200, 150, 66, and 29 kDa, respectively. (C) cyt-PTPε forms homodimers in vivo. 293 cells were transfected with HA- and FLAG-tagged cyt-PTPε, and following gel filtration, FLAG-cyt-PTPε was precipitated. (Top) Coprecipitated HA-cyt-PTPε; (bottom) precipitated FLAG-cyt-PTPε.

bound its counterparts more strongly than did the D1 + 12 protein; in particular, D2 + 12 molecules were capable of strong homotypic binding, while only very weak binding of D1 + 12 molecules to each other was detected. Binding between D1 and D2 domains was also detected in these experiments (Fig. 4).

Further analysis revealed that the interaction between D2 + 12 molecules could not be further enhanced by treatment with H_2O_2 (Fig. 5A). In contrast, binding between D2 domains lacking the 12 N-terminal residues was observed only in cells that had been treated with H_2O_2 (Fig. 5A). Interestingly, replacing the 12 N-terminal residues of D2 + 12 with the D1 domain of PTPε did not reduce strong constitutive homotypic binding (data not shown), indicating that the precise sequence of the protein segment added to the N terminus of the D2 domain is not critical. Together, these results indicated that the potential for homotypic binding exists in D2 domains but is

apparently masked by intramolecular interactions within D2. Presumably, conformational changes in D2 caused by oxidation by H_2O_2, as in the case of RPTPα (8), or by addition of N-terminal residues, as done here, unmask the binding ability of D2 domains. As the full-length cyt-PTPε molecule contains sequences N-terminal to the D2 domain, these data strongly suggest that the D2 domain is not "closed" and is capable of binding in the context of full-length cyt-PTPε.

Sequences at the termini of D2 control intermolecular binding and activity of cyt-PTPε. In order to identify subdomains in D2 that regulate intermolecular binding, we examined the effects of deleting sequences at the N or C terminus of this domain on its binding ability. Sequences deleted were the 21 N-terminal or the 22 C-terminal residues of the D2 domain (residues 360 to 380 and 621 to 642 in full-length cyt-PTPε, respectively). Isolated D2 proteins lacking one or both of these regions were examined in coprecipitation experiments together
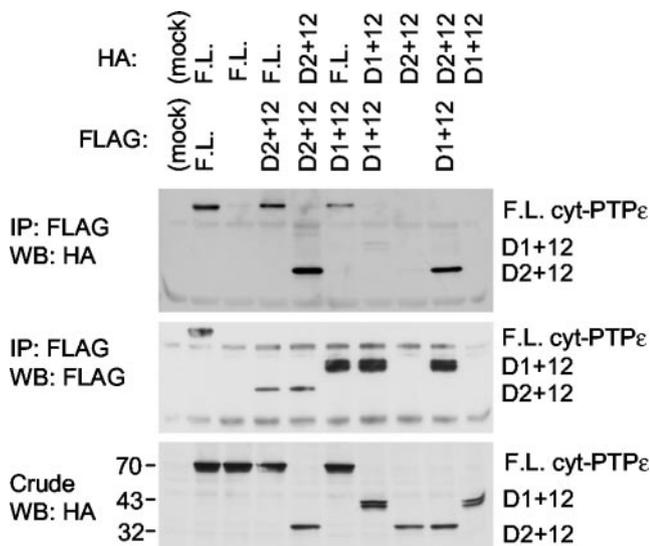
FIG. 4. Strong homotypic binding of the D2 domain. 293 cells were transfected with HA- and FLAG-tagged cyt-PTPε constructs as indicated, after which FLAG-PTPε was precipitated. (Top) Coprecipitated HA-PTPε; (middle) precipitated FLAG-PTPε; (bottom) HA-PTPε present in cell lysates. Size markers are in kilodaltons.

with full-length cyt-PTPε (Fig. 5B). Consistent with results of Fig. 5A, the D2 protein did not precipitate full-length cyt-PTPε (Fig. 5B). However, removal of the N-terminal region caused significant binding to occur between this protein (D2-N) and full-length cyt-PTPε, implying that the N-terminal region of D2 had prevented this domain from binding cyt-PTPε. In contrast, the D2 C terminus appeared to affect binding by D2 mainly positively. Removal of the 22 C-terminal-most residues from D2 domains already lacking their N terminus (construct D2-N,C) significantly reduced binding to cyt-PTPε, suggesting that the C-terminal region of D2 assists binding in *trans* to other cyt-PTPε molecules. In agreement, D2 lacking only the C-terminal region (D2-C) bound cyt-PTPε significantly more weakly than did D2-N (Fig. 5B). However, binding of D2-C was still stronger than that of D2; this implies that the C terminus of D2 also has a role in inhibiting binding in the context of the D2 molecule. Removal of the 21 N-terminal residues from D2 or from the entire cyt-PTPε molecule significantly altered their electrophoretic mobility (Fig. 5A and 6A). The cause for this is not clear but may be related to the high proportion of charged residues (9 of 21) in this region.

We next examined the consequences of removing the N and C termini of D2 from full-length cyt-PTPε. Removal of either or both of these sequences resulted in similarly strong constitutive binding (Fig. 6A), which was not increased further by H₂O₂ (Fig. 6B). We next examined activities of cyt-PTPε molecules purified from cells towards PNPP. Activities of cyt-PTPε molecules lacking either or both termini were significantly reduced, despite the fact that the sequence of the D1 domain, which is the only active PTP domain in PTPε (32), was not altered (Fig. 6C). Similar results were obtained when activities of these mutant proteins towards the voltage-gated potassium channel Kv2.1, a physiological substrate of cyt-PTPε (43), were examined in cells. In agreement with previous results, Kv2.1 was clearly tyrosine phosphorylated when expressed together

with activated Y527F Src; Kv2.1 phosphorylation was reduced by 94% when cyt-PTPε was coexpressed (Fig. 6D) (43). In contrast, expression of cyt-PTPε molecules lacking either or both the N and C termini of D2 reduced Kv2.1 phosphorylation only mildly (Fig. 6D), indicating that all three deletion mutants were significantly less active than full-length cyt-PTPε towards Kv2.1. Importantly, confocal microscopy revealed that

A
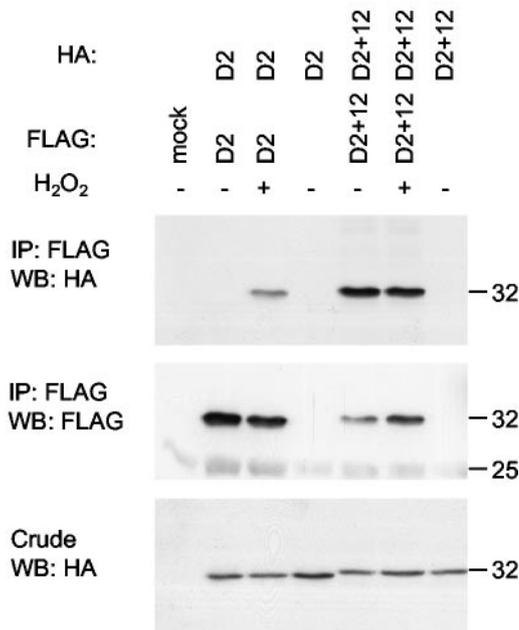
B
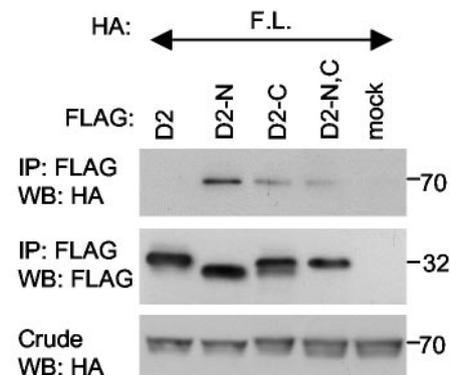
FIG. 5. Regulation of D2-mediated binding. (A) Addition of H₂O₂ or of N-terminal sequences unmasks the binding ability of the D2 domain. 293 cells were transfected with HA- and FLAG-tagged cyt-PTPε constructs as indicated. Some cell cultures were treated with 1 mM H₂O₂ immediately prior to lysis, after which FLAG-PTPε was precipitated. (Top) Coprecipitated HA-PTPε; (middle) precipitated FLAG-PTPε; (bottom) HA-PTPε present in cell lysates. (B) Regulation of D2-mediated binding by the C- and N-terminal regions of D2. 293 cells were transfected with full-length HA-cyt-PTPε and with FLAG-tagged D2 constructs as indicated, after which FLAG-PTPε was precipitated. (Top) Coprecipitated HA-PTPε; (middle) precipitated FLAG-D2; (bottom) HA-cyt-PTPε present in cell lysates. Note the higher mobility of D2-N. Size markers are in kilodaltons.
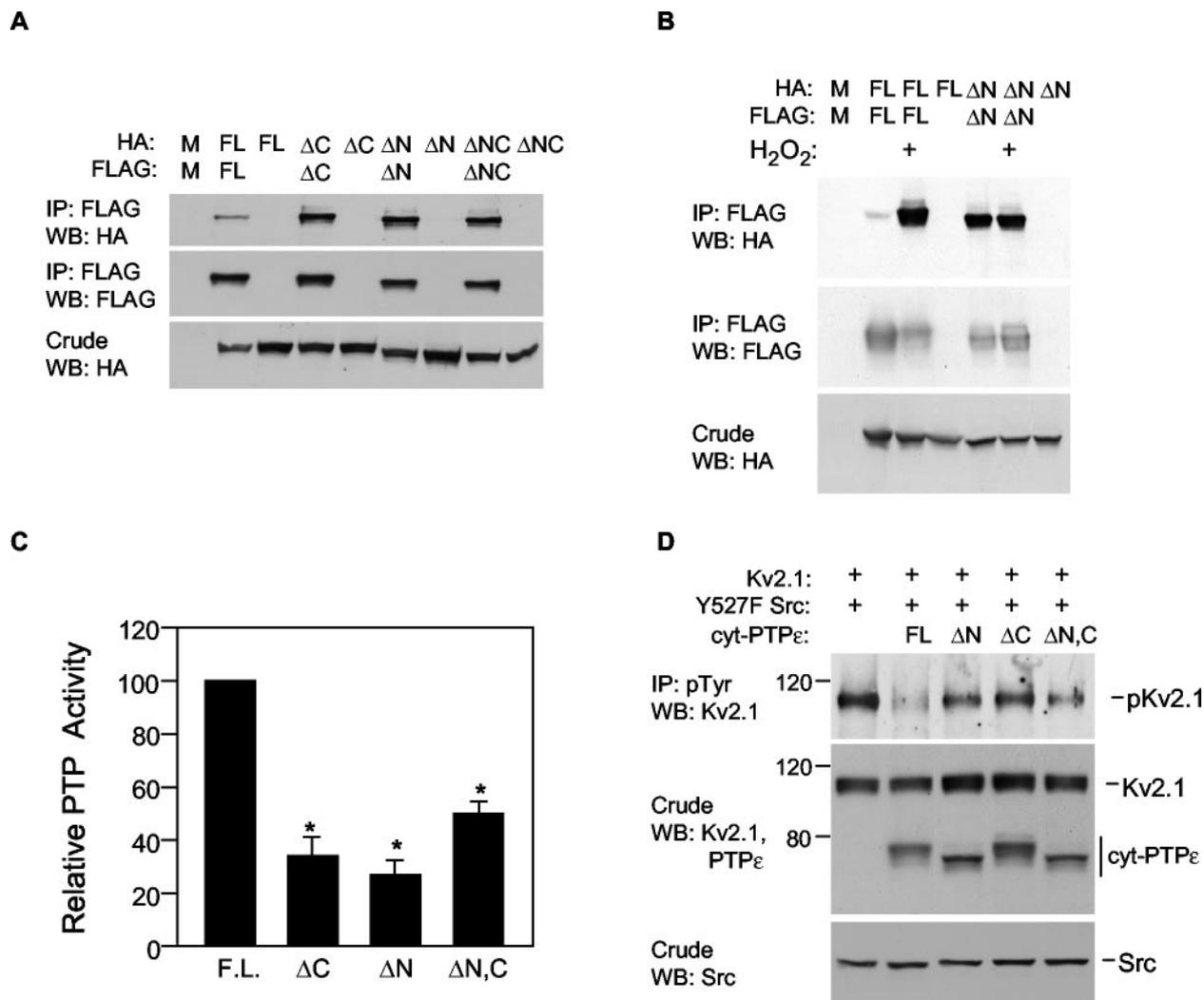
FIG. 6. Deletion of the D2 domain N- and/or C-terminal residues from full-length cyt-PTPε induces homotypic binding and inhibits activity of cyt-PTPε. (A) 293 cells were transfected with HA- and FLAG-tagged versions of full-length cyt-PTPε (FL) or mutants lacking the C terminus (ΔC), N terminus (ΔN), or both termini (ΔNC) of D2 as indicated, followed by anti-FLAG immunoprecipitation. (Top) Coprecipitated HA-PTPε; (middle) precipitated FLAG-PTPε; (bottom) HA-PTPε present in cell lysates. (B) Homotypic binding of mutants is not further increased by exposure to H_2O_2. Cells indicated were treated with 1 mM H_2O_2 for 10 min prior to lysis. Shown are results obtained for the ΔN mutant. (C) In vitro PTP activity of full-length cyt-PTPε and of the three deletion mutants. cyt-PTPε proteins were purified from 293 cells by immunoprecipitation and elution, and activities of equal amounts of protein towards PNPP were measured and normalized as detailed in Materials and Methods. Results (means ± standard errors) are from one experiment (in triplicate) and are representative of three similar experiments performed. *, $P < 0.0001$ by Student's t test. (D) Deletion mutants of PTPε are severely impaired in their ability to dephosphorylate the Kv2.1 potassium channel in cells. 293 cells stably expressing Kv2.1 were transfected with constitutively active Y527F Src and with full-length (FL) or ΔN, ΔC, or ΔN,C cyt-PTPε as indicated. (Top) Precipitation of the cells by tyrosine-phosphorylated Kv2.1; (middle and bottom) expression levels of Kv2.1 and PTPε and of Src in the lysates used, respectively. Shown is one experiment representative of two performed. Note the increased mobility of cyt-PTPε lacking the D2 N terminus. Size markers are in kilodaltons.

all three mutants exhibited subcellular localization patterns similar to those of full-length cyt-PTPε, indicating that their increased association and reduced activity were not caused by widespread aggregation or altered subcellular localization (data not shown). In all, these results indicate that in the context of the full-length cyt-PTPε molecule, the N- and C-terminal sequences of D2 negatively regulate intermolecular binding and that removal of these sequences results in constitutive dimerization and in reduction in enzyme activity.

Presence of D2 reduces cyt-PTPε catalytic activity. We next examined the effect of the presence of the D2 domain on

cyt-PTPε activity. It should be stressed that the D2 domain of cyt-PTPε is catalytically inactive (32) and did not result in detection of background PTP activity when expressed on its own (Fig. 7). Here, too, activity was measured in vitro by using purified PTPε molecules. Activity of full-length cyt-PTPε was readily detected in this system, while deletion of the D2 domain, as in the D1 + 12 protein, resulted in doubling of PTPε activity. On the other hand, adding similar amounts of inactive D2 + 12 protein to the active D1 + 12 protein significantly reduced activity of D1 + 12, although it was still higher than that of full-length cyt-PTPε (Fig. 7). Incomplete reduction of
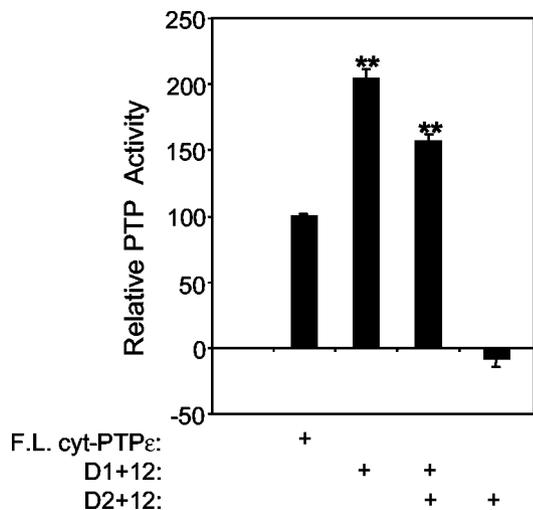
FIG. 7. D2 inhibits cyt-PTPε activity. Full-length (F.L.), D1 + 12, and D2 + 12 cyt-PTPε proteins were expressed in 293 cells as indicated. cyt-PTPε proteins were then purified by immunoprecipitation via an N-terminal FLAG tag followed by elution, and their activities in vitro towards PNPP were determined. Results (means ± standard errors) are from one experiment (in triplicate) and are representative of three similar experiments performed. **, $P < 0.0042$, versus full-length cyt-PTPε. Reduced activity of D1 + 12 in the presence of D2 + 12 protein is significant also versus D1 + 12 protein alone ($P = 0.0184$).

activity to cyt-PTPε levels is probably due to inhibition being caused in this case by a bimolecular reaction, rather than by a more efficient unimolecular reaction as in full-length cyt-PTPε. Taken together, these results indicate that the net effect of the D2 domain on cyt-PTPε activity is inhibitory. Both results are consistent with D2 mediating inhibitory interactions between cyt-PTPε molecules and with its ability to bind various domains of cyt-PTPε, as shown in several experiments with multiple PTPε proteins (Fig. 3 to 6). Note that inhibition by D2 was demonstrated without treatment with hydrogen peroxide, i.e., it reflects an inherent property of this domain. These results also indicate that D1 and D2 domains located on the same or on separate molecules can interact with each other.

DISCUSSION

cyt-PTPε is rather unique among PTPs since it is a naturally occurring, non-receptor-type isoform of RPTPε and, therefore, in a manner atypical of non-receptor-type PTPs, possesses two PTP domains; these domains are identical to those of the receptor-type form of PTPε. As such this molecule enables examination of the relative roles of various cytosolic domains in promoting self-association in the absence of extracellular domains. The present study elaborates and expands on results obtained previously with the related RPTPα (8). Points unique to this study include the demonstration that cyt-PTPε molecules can form dimers and possibly higher-order associations in vivo in a manner that is independent of membrane-spanning and extracellular domains. Furthermore, we show that associations of cyt-PTPε are readily detected, including those in cells that express endogenous cyt-PTPε, without the need for additional stabilization. The ability to form homotypic associations is therefore an inherent property of the cytosolic domains of

PTPε. We also establish that, despite lack of direct contact with extracellular molecules, cyt-PTPε can be influenced indirectly by extracellular events through mediation of other receptors, such as the EGFR. The significant basal interaction among cyt-PTPε molecules allowed us to demonstrate that physiological signaling can down-regulate cyt-PTPε dimerization, as seen during the first few minutes following stimulation of the EGFR. The data do not exclude the possibility that cell surface molecules, such as the EGFR, can serve as sensors of extracellular events also for RPTPs that have extracellular domains of their own. This possibility is in fact somewhat appealing in light of the ongoing difficulties in defining specific roles for such domains in direct transduction of signals across cell membranes. At the molecular level we identify a critical role for the D2 domain in mediating dimerization of cyt-PTPε. We show that binding by the D2 domain of cyt-PTPε is regulated by sequences at its ends and that removal of one or of both these sequences from full-length cyt-PTPε promotes dimerization and significantly reduces cyt-PTPε activity. We also show that the "closed," inactive conformation of D2 can be relaxed by addition of sequences at its N terminus, suggesting that D2 is somewhat relaxed and accessible for binding in the context of the full-length cyt-PTPε. Finally, we demonstrate that the net effect of D2 on cyt-PTPε activity is inhibitory. Inhibition is inherent to D2 and can be demonstrated in the absence of oxidation by H_2O_2. In all, while this study does not rule out a role for extracellular domains in RPTP dimer formation, it shows that other mechanisms for achieving this end exist.

Experiments presented here establish a link between a mainstream mechanism of cellular regulation, namely EGFR stimulation, and dimerization of cyt-PTPε. The effects of EGFR stimulation on cyt-PTPε are complex. In the first few minutes following application of EGF, dimerization of cyt-PTPε is reduced and its tyrosine phosphorylation is increased. These changes in cyt-PTPε are then among the first events which follow EGFR activation, leading one to speculate that EGFR activation leads directly or indirectly to cyt-PTPε phosphorylation, resulting in reduced dimerization of the PTP. Further studies are required to determine whether cyt-PTPε is a direct target of the EGFR and whether phosphorylation and dimerization of cyt-PTPε affect each other. With time cyt-PTPε phosphorylation is reduced and dimerization is increased, suggesting that these changes are caused by events further downstream from the receptor itself. It is noteworthy that EGF stimulation did not result in detectable changes in RPTPα dimerization (8). Further EGF-induced reduction in the already low basal levels of RPTPα dimers may not have been detectable in the system used in that study.

Increased dimerization of cyt-PTPε following direct treatment of cells with H_2O_2 agrees with previous studies, in which addition of H_2O_2 to growth media at concentrations similar to those used here increased dimerization and inhibition of RPTPα (8) and resulted in oxidation of cellular PTPs in general (37). H_2O_2 treatment of RPTPα and PTEN resulted in direct oxidation of specific cysteine residues, which, in the case of RPTPα, mediated self-association (8, 30). We therefore believe that the effect of H_2O_2 seen in this study results from direct oxidation of cyt-PTPε molecules. Activation of growth factor receptors, such as those for EGF (3) or platelet-derived growth factor (4, 37), can generate H_2O_2. However, H_2O_2

A: Inter-molecular binding　　　　　　　　　　　　　　　　　　　　**B: Intra-molecular binding**
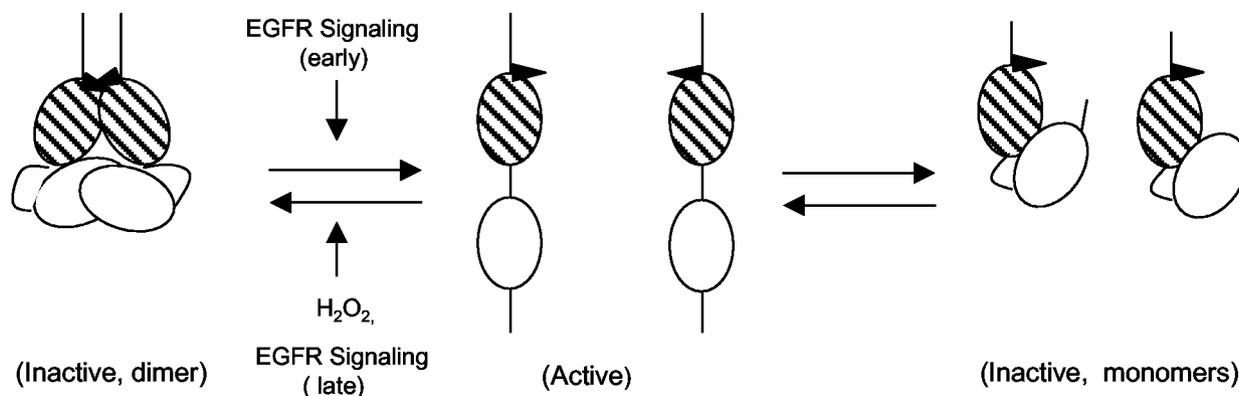
FIG. 8. Inter- and intramolecular associations of cyt-PTPε. (A) Intermolecular binding is mediated by interactions between D2 domains of adjacent molecules, which provide opportunity for the wedge domains (black triangles), located at the N termini of the D1 domains, to interact and inhibit PTPε activity. Binding between D1 (hatched) and D2 (open) domains within a single cyt-PTPε molecule is also consistent with this model. (B) The less favored possibility of intramolecular binding between D1 and D2 domains within a single cyt-PTPε molecule, which inhibits cyt-PTPε activity. Interactions can be affected by input from other signaling events, such as EGFR signaling, shown in this study.

produced by the EGFR probably does not affect the cyt-PTPε dimerization observed here since changes in cyt-PTPε dimerization and phosphorylation following EGF treatment were detected also in the presence of the reactive oxygen species scavenger *N*-acetylcysteine. This is in agreement with detection of reduced dimerization of cyt-PTPε 5 min after EGF stimulation, the opposite of what would have been expected if the stimulation effects were mediated by H_2O_2.

A major factor in the ability of cyt-PTPε molecules to bind each other is the D2 domain, which is capable of strong self-association and of binding D1 domains in vivo. Data shown here are consistent with the extreme N and C termini of D2 (residues 360 to 380 and 621 to 643 of cyt-PTPε, respectively) interacting with each other in a way that prevents the D2 domain from binding other PTPε molecules. Accordingly, removal of either or of both D2 termini from full-length cyt-PTPε increases dimerization and inhibits activity of cyt-PTPε. Binding of the N and C termini has been described previously in the case of RPTPα (8) and may occur in other PTPs as well as a mechanism for regulating the binding abilities of D2 domains. It is of interest to note that the N terminus of D2 contains a 4-amino-acid insertion in several RPTPs, which is missing from the analogous region of D1 domains of the same RPTPs (40). This insert, located in the loop between the α1' and α2' helices in the D2 domain as noted for the LAR protein (40), exists in PTPε, although its sequence is markedly divergent from that of LAR or of RPTPα. The roles of this insert and of the loop of which it is part are not known at present, although it is tempting to speculate that they may be related to the N-C binding within D2. The roles of the D2 C terminus differ somewhat in the more limited context of an isolated D2 domain, where it appears to play roles both in promoting and in inhibiting binding. Reduced abilities of the D2 C terminus to promote binding in the context of full-length cyt-PTPε are consistent with modification of the binding abilities of the D2

domain by addition of protein sequences at its N terminus (Fig. 5A).

Our data are consistent with D2-D2 and D1-D2 interactions stabilizing binding of cyt-PTPε molecules and producing inactive dimers (Fig. 8A). As each full-length cyt-PTPε molecule contains a single D2 domain, associations between D2 domains can occur only in dimers. An attractive feature of the dimer model of Fig. 8A is that it allows for the D1 wedge structures to block access to and inhibit activity of the D1 domains of their dimerization partner. Involvement of the wedge structure in regulation of RPTPα activity is believed to be significant (26, 27), and the residues of the RPTPα wedge domain are conserved in cyt-PTPε. Furthermore, the dimer model also accounts for the strong D2-D2 interactions and for dimerization of cyt-PTPε, both of which have been documented here. Although less consistent with our data, we cannot rule out the possibility of D1-D2 interactions occurring within the same molecule, in effect stabilizing it in a "closed," inactive conformation (Fig. 8B) or assisting in binding cyt-PTPε dimers (Fig. 8A). As immunoprecipitation and gel filtration techniques used in this study tend to focus on intermolecular interactions, further studies are required to clarify the existence and role of intramolecular interactions within cyt-PTPε.

How general are the results presented here? Our data raise the possibility of D2-D2 interactions occurring between molecules of other PTPs and promoting their homodimerization or heterodimerization, possibly acting as a general regulatory mechanism in PTPs that contain two PTP domains. Individual D2 domains have different abilities to bind D1 domains or full-length RPTP molecules (9, 50) and can affect PTP activities (e.g., reference 22). It is therefore reasonable to suggest that the D2 domain of a given RPTP can interact with D2 domains of other full-length RPTPs, but the strength of this effect and its effects on PTP activity should vary from case to case.

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