We have employed transient co-overexpression of protein tyrosine phosphatases (PTPs) with a panel of receptor tyrosine kinases (RTKs) to investigate molecular parameters that regulate dephosphorylation activity and specificity in intact cells. Our results demonstrate clear differences in susceptibility of various forms of different RTKs to the action of PTP 1B, T-cell phosphatase (TC-PTP), and CD45, which suggests cellular compartmentalization as a major factor defining activity and overall function. TC-M PTP, a nonlocalized cytosolic mutant, is deregulated and is therefore able to efficiently suppress v-erbB- and v-fms-induced cell transformation, which is not observed with the intact TC-PTP or PTP 1B. The transmembrane PTP CD45 displays more selectivity but appears to be already active during transport to the cell surface. Dephosphorylation activity is also dependent on relative RTK/PTP expression levels and can be modulated by the SH2 domain-containing noncatalytic subunit of phosphatidylinositol 3'-kinase, p85. Overexpression of high affinity binding proteins could therefore contribute to RTK-induced cell transformation and cancer.

Protein tyrosine phosphorylation and dephosphorylation are essential mechanisms in the pathways by which cell proliferation and differentiation are regulated. While a great deal is known about the kinases that are responsible for tyrosine phosphorylation, much less information exists about the phosphatases, which are equally important regulators of these key cellular events. The first protein tyrosine phosphatases (PTPs) were only recently characterized in molecular detail, and subsequent studies have established PTPs as a family of proteins that are likely to be as large as the family of tyrosine kinases (Fischer et al., 1991; Saito and Streuli, 1991; Pot and Dixon, 1992). Like the PTK family, PTPs occur in two forms: cytoplasmic enzymes and the receptor-type transmembrane PTPs. The cytoplasmic PTPs include PTP 1B, T-cell phosphatase (TC), and STEP (Charbonneau et al., 1989; Cool et al., 1989; Lombroso et al., 1991). PTP 1B was recently shown to be localized to the surface of the endoplasmic reticulum (ER) by virtue of its 35 carboxyl-terminal most residues (Frangioni et al., 1992).

PTP H1, MEG, and PTP 1C are also cytoplasmic PTPs, but they are larger proteins and contain an additional structural motif (Gu et al., 1991; Yang and Tonks, 1991; Shen et al., 1991). PTP H1 and MEG show homology to several cytoskeleton-associated proteins, while PTP 1C contains two adjacent copies of a src homology domain (SH2). The second class of PTPs are membrane-spanning proteins and, in general, are larger in mass. The extracellular portion of this PTP class may contain immunoglobulin- or fibronectin III-like domains, which have been proposed to represent specific binding sites with SH2 domain-containing polypeptides. Moreover, several PTPs have been shown to be localized to the surfaces of lymphocytes, where they may be involved in antigen-induced activation and, as part of the T-cell receptor complex, may activate lck or fyn kinases by specifically dephosphorylating a regulatory phosphotyrosine residue within the COOH-terminal domain of the PTK (Koretzky et al., 1991). A hint that PTPs also play important roles in development came from studies in which PTP 1B injection into Xenopus oocytes led to a retardation in insulin-induced maturation (Tonks et al., 1990). In addition, the corkscrew gene product in the torso signaling pathway, which regulates the definition of terminal structures in the Drosophila embryo, has been shown to be an SH2 domain-containing PTP (Perkins et al., 1992). The more than 40 PTPs identified to date suggest that they may play distinct roles by acting in connection with specific targets. To test this possibility, we analyzed the dephosphorylation potential of three PTPs on eight different RTKs in intact cells. Our coexpression experiments demonstrate that PTPs are very selective in their choice of RTK substrates. This selectivity appears to be largely defined by cellular localization, and dephosphorylation efficiency may be modulated by competition for RTK binding sites with SH2 domain-containing polypeptides. Moreover,
we demonstrate that 3T3 cell transformation by \(-erbB\) or \(-fms\) oncogenes can be suppressed by T-cell phosphatase, but only after deregulation of its activity by removal of a localization signal.

**MATERIALS AND METHODS**

**Construction of Expression Plasmids for Transient Expression**—All cDNAs were cloned into a pCMV cytomegalovirus early promoter-based expression plasmid (Eaton et al., 1986). CD45 cDNA (Streuli et al., 1987) was cut with restriction enzymes \(Sph\) and \(HindIII\) to generate a 3801-bp fragment that was blunt-ended with mung bean nuclease and ligated into linearized pCMV vector. PTP 1B cDNA (Brown-Shimer et al., 1992), FTP TC, and PTP TC-M (Cool et al., 1989) containing ECOI sites at the cDNA ends were cloned into an EcoRI linearized pCMV vector. The inactive TC-C PTP was generated by coding codon 216 from TGT to AGT, coding for serine instead of cysteine. PCR was performed on TC cDNA with primer 1' (5'TGACCATGGGCTGATCTACAGTTGCAAG-3') containing the mutation and a cleavage site for Ncol and primer 2' (5'TGATACCTGGATCCTGCATCAG-3') containing a cleavage site for BamHI. The DNA fragment was isolated, cleaved with the restriction nucleases, and the fragment inserted into the pCMV expression vector.

The EK-R and HER1-2 expression plasmids have been described earlier (Herbst et al., 1991; Lee et al., 1989). The cDNA of the EGFR (Ullrich et al., 1984) was cloned into a XbaI/HpaI pre-cut pCMV as a XbaI/SalI 4658-bp fragment. The XbaI site was at the 5'-end of the cDNA, and derived from the cloning linker. Transfer of the 5'-end of the EGFR into the pCMV vector was done in several steps. The complete HER2 coding region was available in a pGEM vector (bp 1-3944 of Coussens et al. (1985)). From this plasmid a BarEI/HindIII fragment (bp 3412-HindIII site in pGEM polylinker) was taken and ligated into an XbaI/HindIII pre-cut pCMV vector together with a XbaI/BstEI (bp 1-3412, partial BarEI digest of HER-2 cDNA). The IR cDNA (Ullrich et al., 1985) was cloned as a 4336-bp ClaI/SphI fragment into the XbaI/HindIII pre-cut pCMV. The SphI site was filled in with Klenow polymerase to generate a blunt end. The Clal site was at the 5'-end of the cDNA and derived from the cloning linker. The IGF-1-R cDNA (Ullrich et al., 1986) was cloned into pCMV using the BamHI site at bp 4156 of the cDNA. The aPDGF-R cDNA (Clasen-Welsh et al., 1987) had been cloned as a 6.7-kilobase NotI cDNA fragment and was transferred as a 4527-bp NotI/HindIII fragment into the NotI/HindIII pre-cut pCMV vector. Similarly, from a 5.7-kilobase EcoRI cDNA encoding the \(\beta\)PDGF-R (Gronwald et al., 1989) a 3798-bp EcoRI/HindIII fragment was subcloned into the pCMV vector pre-cut with EcoRI and HindIII. Finally, the CSF-1-R cDNA (Coussens et al., 1986) was cloned as a 3584-bp BamHI fragment into a BamHI cut pCMV vector. The BamHI site at the 5'-end of the cDNA was derived from the cloning linker.

**Transient Expression Experiments**—CaCl\(_2\) gradient purified DNA was used for transfections. Human embryonic kidney fibroblasts 293 cells (ATCC CRL 1573) were grown, transfected, and analyzed as described (Lammers et al., 1990). Briefly, cells were grown in F-12/Dulbecco's modified Eagle's medium, 50:50, with 10% fetal calf serum, 0.5% \(\alpha\)-MEM, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 10 \(\mu\)g/ml aprotinin, 100 \(\mu\)g/ml NaF, 10 mM sodium pyrophosphate, and 1 mM sodium orthovanadate). The lysate was concentrated for 4 h (12,000 x \(g\)) of the supernatant taken, sample buffer added, proteins boiled for 0 min, and analyzed by SDS-PAGE and immunoblotting.

**Antibodies**—Antibodies used were the mouse monoclonal anti-phosphotyrosine antibody 5E.2 (Fendly et al., 1990), mouse monoclonal 9.4 against CD45, and rabbit antisera against phosphatases 1B, TC, and the carboxy-terminal peptides of p85 and the different receptors. For detection of phosphotyrosine and protein antigens on immunoblots, the enhanced chemiluminescence system (Amersham) was used in conjunction with goat anti-mouse and anti-rabbit antibodies (Bio-Rad). For reprobing, blots were stripped in 67 mM Tris-HCl, pH 8.8, 2% SDS, and 0.1% \(\beta\)-mercaptoethanol at 50 °C for 30 min.

**RESULTS**

A transient expression system was employed to analyze the interaction of different PTPs with RTKs within the cell. As shown previously (Gorman et al., 1989; Lammers et al., 1990), cDNAs can be efficiently expressed in 293 cells upon transfection, and coexpression of different cDNAs is possible. Eight receptor tyrosine kinases (RTKs) from three different structural subclasses were used, including the epidermal growth factor receptor (EGF-R), the chimeric receptor HER1-2 (Lee et al., 1989), consisting of the extracellular domain of the EGF-R and the intracellular domain of the human EGF-R-like receptor (HER2 or c-erbB2), the insulin receptor (IR), the insulin-like growth factor-1 receptor (IGF-1-R), and the receptors for \(\alpha\)- and \(\beta\)-platelet-derived growth factor (PDGF-R), colony stimulating factor 1 (CSF-1-R), and stem cell factor (SCF-R/c-kit). For the latter, we also used a chimera, EK-R, which consists of the extracellular domain of the EGF-R and the transmembrane and intracellular domains of the protooncogene product \(p40^{c-abl}\) (Herbst et al., 1991).

For coexpression studies, three different: PTPs and two mutant derivatives were employed: the transmembrane phosphatase, CD45 (Charbonneau et al., 1988), PTP 1B, which is localized on the cytoplasmic face of the endoplasmic reticulum (Frangioni et al., 1992), and TC-PTP, which is found in the same cellular compartment (Cool et al., 1989). Introduction of a stop codon into the TC cDNA after codon 317 for arginine has been shown to yield a deregulated hyperactive enzyme, TC-M (also designated \(\Delta\)C11.PTP; Cook et al. (1990)), while mutation of cysteine 216 to serine inactivated the catalytic function of the phosphatase (TC-C).

The cDNAs for kinases and phosphatases were cloned into a cytomegalovirus promoter-based expression vector and transfected into human 293 embryonic fibroblasts. Expression was tested by either metabolic labeling of transfected cells followed by immunoprecipitation and analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), or by immunoblotting and probing with specific antibodies.

**Coexpression of RTKs and PTPs in 293 Cells**—To investigate the effects of PTPase expression on the phosphorylation state of coexpressed RTKs, equal amounts of each expression plasmid were transfected into human 293 fibroblasts. After stimulation with the appropriate ligand for 10 min, the cells were lysed and the phosphotyrosine content of the receptors was determined by gel electrophoresis and immunoblotting with anti-phosphotyrosine antibody. Fig. 1A shows the phosphotyrosine content analysis of four subclass I and II RTKs

\(^{1}\) C. Dadabay, L. Lorenzen, and E. Fischer, unpublished observations.
PTP Substrate Specificity

after transfection of receptor expression vector alone or together with one of the phosphatases. To verify that the observed changes in tyrosine phosphorylation signals were not due to varying expression levels, immunoblots were re-

probed with RTK- and PTP-specific antibodies. As a representative example, in aliquots of lysates used in Fig. 1, A and B, coexpressed PTP 1B levels were determined separately by immunoblot analysis with anti-PTP 1B antiserum (Fig. 1C).

Fig. 1. A and B, differential effects of PTPs on phosphorytrosine content of coexpressed activated RTKs. Receptors were expressed transiently either alone or together with PTP in 293 cells. After 24 h of serum starvation, ligand was added for 10 min, cells lysed, an aliquot analyzed by SDS-PAGE, transferred to nitrocellulose, and immunobotted using an antiphosphotyrosine antibody. C, as a representatve PTP expression control, an aliquot of cell lysates from all transfections coexpressing RTKs with PTP 1B was analyzed by immunoblotting with PTP 1B antiserum. R, receptor protein. P, receptor precursor. β, β-subunit of IR or IGF-1-R.

In comparison to cells overexpressing the EGF-R alone, coexpression of EGF-R and either of the PTPs reduced receptor autophosphorylation by less than 50%, as determined by densitometric scanning, after stimulation with EGF. In contrast, the unstimulated, yet nevertheless phosphorytosine-containing, receptor was relatively more efficiently dephosphorylated by coexpressed PTP 1B and TC-PTP, and completely dephosphorylated by the plasma membrane-bound CD45 phosphatase. Similarly, the TC-M mutant reduced phosphorytosine content to a barely detectable level for both ligand-stimulated and unstimulated EGF receptor. This complete dephosphorylation was a further control to demonstrate that residual receptor phosphorytosine was not due to insufficient transfection efficiency resulting in partially segregated expression of PTP and RTK in different cell populations. Since the in vitro activity of TC and TC-M against p-nitropheny phosphate were roughly equal (not shown), their differential activities in intact 293 cells provides further evidence for the proper localization of TC in the cell, in contrast to the truncation mutant TC-M. As an additional control, cotransfection of receptor with the inactive TC showed a similar degree of phosphorytosine as that of receptor alone. In contrast to the closely related EGF-R, the ligand-independent basal phosphorytosine content of HER1-2 was insensitive to coexpressed CD45. Interestingly, however, a band migrating just below the major activated HER1-2 chimera protein, which presumably represents an incompletely processed precursor form of the receptor, was resistant to CD45 but not to 1B and TC dephosphorylation.

A similar result was obtained with IR and IGF-1-R. These subclass II RTKs are synthesized as precursor polypeptides which are proteolytically cleaved into ligand-binding α and tyrosine kinase domain-containing β subunits during their transport to the cell surface. Overexpressing cells revealed the presence of constitutively tyrosine-phosphorylated IR and IGF-1-R precursor bands with an apparent molecular mass of 190 kDa, in addition to 95-kDa β subunits whose phosphorytosine content increased significantly upon ligand stimulation. Again, there was a weak dephosphorylation of all bands when CD45 was coexpressed in 293 cells, with the β subunits of both IR and IGF-1-R showing the most pronounced effect in the absence of ligand. Co-overexpression of PTP 1B or TC, however, resulted in almost complete dephosphorylation of both IR and IGF-1-R precursors and the unstimulated β subunits, and reduced the phosphorytosine content of ligand-activated β subunits to less than 50% of the TC-C control.

For subclass III RTKs α and β PDGF-R, CSF-1-R, and c-kit (represented by the ER chimera), the PTP dephosphorylation assay yielded some similar as well as diverging results. The phosphorytosine residues of all forms of the four structurally related RTKs were sensitive to the catalytic action of the deregulated PTP TC-M mutant, with only the CSF-1-R showing some residual signal. CD45 coexpression had some activity on α and β PDGF-R bands from both "unstimulated" and ligand-exposed cells, resulting in reduction of the phosphorytosine signal of the respective bands to approximately 30 and 50%. The lower precursor band in the doublet and the upper, presumably fully processed receptor were about equally affected by CD45 action, whereas in PTP 1B- and TC-overexpressing cells the incompletely processed precursor of both
PDGF-Rs was completely dephosphorylated and the mature receptor was more resistant. Interestingly, phosphotyrosine residues in the EK-R chimera were good substrates for all three PTPs. Only the mature form of the ligand-activated EK-R retained approximately 5% of the phosphotyrosine signal when CD45, PTP 1B, or TC were coexpressed in 293 cells. In contrast, the fully processed main band of the CSF-1-R was highly resistant to all of the PTPs used. However, consistent with the effects on EK-R, unstimulated receptors and precursor forms migrating just below the main band of the mature CSF-1-R were highly sensitive to CD45, PTP 1B, and TC. This was especially evident upon overexposure of the blot (not shown).

All of the better characterized RTKs, such as EGF-R, IR, and βPDGF-R, contain several phosphotyrosylated tyrosine residues in the activated state. We therefore tested whether the observed dephosphorylation activity of different PTPs was directed preferentially at specific phosphotyrosine residues, or whether a targeted RTK molecule was always completely dephosphorylated. Since the signals obtained with the antiphosphotyrosine immunoblot analysis (Fig. 1B) represent average phosphotyrosine content values, we used metabolic labeling of transfected cells with [35S]methionine and immunoprecipitation with the antiphosphotyrosine antibody for further analysis. Assuming that all phosphotyrosine residues are equally well recognized by the antiphosphotyrosine monoclonal antibody, we expected a less pronounced decrease in the immunoprecipitation signals from PTP-coexpressing cells if only selected phosphotyrosines were targeted, because a larger number of partially dephosphorylated receptor molecules would be immunoprecipitated. As shown in Fig. 2, using this approach with the βPDGF-R, we obtained a result with approximately the same relative receptor band intensities as detected in the experiment shown in Fig. 1B. A polyclonal rabbit antiphosphotyrosine antiserum yielded the same result (not shown). This strongly suggested that dephosphorylation by either of the PTPs tested was complete for each of the targeted receptor molecules.

Because of the excellent quantitative responsiveness of the transient 293 cell overexpression system to varying amounts of transfected expression plasmid DNA, we were able to examine the influence of changed relative RTK and PTP expression levels and the dephosphorylation process. While we had used equal amounts of RTK and PTP expression plasmids in the experiments shown in Fig. 1 and therefore analyzed the PTP/RTK interaction under roughly equimolar conditions, we next used increasing amounts of βPDGF-R vector in conjunction with a parallel decrease of the different PTP expression plasmids (Fig. 3). After stimulation with ligand for 10 min, cells were treated and analyzed by anti-phosphotyrosine antibody immunoblotting as described above. While the incompletely processed precursor band migrating just below the mature βPDGF-R was sensitive to PTP 1B, TC, and TC-M action under all conditions, it remained mostly resistant to CD45 up to a 3-fold overexpression of the phosphatase. At a PTP/RTK ratio of 7, all PTPs completely dephosphorylated all forms of the βPDGF-R, whereas at the equivalent inverse ratio even TC-M was unable to dephosphorylate the receptor. Thus, quantitative parameters play an important role in the regulation of the tyrosine phosphorylation state of RTKs by PTPs, an aspect which may become significant in situations of abnormal RTK expression in some forms of human cancer.

Coexpression of p85 Prevents EK-R Dephosphorylation—Receptors with tyrosine kinase activity associate with and phosphorylate several identified SH2 domain-containing proteins in vitro and in vivo. As demonstrated previously in vitro, addition of PLC-γ to lysates of EGF-R-overexpressing cells inhibits receptor dephosphorylation (Rotin et al., 1992). To investigate whether the additional expression of an SH2 domain-containing protein interacting with the EK-R would influence dephosphorylation by a specific PTP, we employed EK-R, PTP TC-M, and the noncatalytic subunit of PI 3'-kinase, p85. The EK-R was chosen because it had proved to be most susceptible to dephosphorylation by all of the phosphatases. Furthermore, the deregulated phosphatase mutant TC-M exhibited a highly potent dephosphorylating activity on all forms of the eight receptors tested. The recently characterized PI 3'-kinase subunit, p85 (Skolnik et al., 1991; Otsu et al., 1991; Escobedo et al., 1991) contains an SH2 domain and can be expressed at high levels in 293 cells upon transfection with an expression plasmid. p85 associates with and is directly phosphorylated by the kit tyrosine kinase in 293 cells (Herbst et al., 1991).

The result of this three-protein overexpression experiment is shown in Fig. 4. Overexpression of EK-R alone resulted in ligand-induced tyrosine phosphorylation of the receptor, which was abolished upon coexpression of PTP TC-M, as

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**FIG. 2. Immunoprecipitation of [35S]methionine-labeled βPDGF-R from cotransfected 293 cells.** Cells transfected with βPDGF-R alone or with receptor and phosphatase were labeled overnight with [35S]methionine in methionine-free minimal essential medium containing 0.5% fetal calf serum. After cell lysis, phosphotyrosine-containing proteins were immunoprecipitated with anti-phosphotyrosine antibody and analyzed by SDS-PAGE. A fluorography is shown. The molecular mass in kDa (×10^-2) is indicated.

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**FIG. 3. Effect of βPDGF-R and PTP expression levels on receptor phosphotyrosine content.** 293 cells were cotransfected with expression plasmids coding for βPDGF-R and different PTPs and analyzed as described in the legend to Fig. 1. The amount of expression plasmid (μg) used for each transfection is indicated. Immunoblotting analysis with antiphosphotyrosine antibody (A) and a control for receptor expression with anti-βPDGF-R antiserum for the cotransfection with PTP 1B (B) are shown.

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**FIG. 4. Overexpression of EK-R alone resulted in ligand-induced tyrosine phosphorylation of the receptor, which was abolished upon coexpression of PTP TC-M, as**
amount of proteins are indicated. After serum starvation for 10 min, solubilized, and an aliquot of the cell lysate analyzed by SDS-PAGE. Transfer to nitrocellulose was followed by immunoblotting with antiphosphotyrosine antibody. The positions of EK-R and p85 proteins are indicated.

![Figure 4](image)

**FIG. 4.** Co-overexpression of p85 prevents dephosphorylation of EK-R by TC-M. 293 cells were transfected with the indicated amount (in μg) of expression plasmids coding for EK-R alone or with a mixture containing plasmids coding for EK-R, TC-M, and p85. After serum starvation for 24 h, cells were stimulated with EGF for 10 min, solubilized, and an aliquot of the cell lysate analyzed by SDS-PAGE. Transfer to nitrocellulose was followed by immunoblotting with antiphosphotyrosine antibody. The positions of EK-R and p85 proteins are indicated.

![Figure 5](image)

**FIG. 5.** Inhibition of v-erbB and v-fms transformation of NIH 3T3 cells by PTP overexpression. 10^6 cells were infected with virus as indicated (colony forming units). After 36 h cells were trypsinized and plated in medium with 4% serum. Medium was changed every other day until foci appeared, and cells were then stained with crystal violet.

already shown in Fig. 1B. Transfection of two times more p85 than TC-M expression plasmid led to a weak recovery of receptor phosphorylation, which improved greatly at a ratio of 3:1. Reprobing of the blot with anti-receptor and anti-p85 antibody demonstrated equal expression of EK-R in all lanes and increasing expression of p85 (not shown). In an analogous experiment, substitution of p85 with PLCγ and EK-R with EGF-R expression plasmids yielded similar results (not shown).

**Inhibition of NIH 3T3 Cell Transformation by PTP Overexpression**—To test the physiological relevance of our 293 cell experiments, we examined the effect of PTP overexpression on v-erbB and v-fms oncogene-mediated transformation of NIH 3T3 cells. Recombinant retroviruses containing DNA sequences coding for PTP 1B, TC, and TC-M were generated and used for coinfection experiments. Replication-defective retroviruses encoding the tyrosine kinase oncogenes v-erb-B and v-fms were used to infect NIH 3T3 cells either alone or together with PTP retroviruses (Fig. 5). The multiplicity of infection (m.o.i.) of 0.1 (10^4 virus particles/10^6 cells) for the transforming retrovirus was chosen to allow a suitable number of foci to form. In 293 transient expression experiments (Fig. 1), neither PTP 1B nor TC had significant dephosphorylation activity on the mature forms of EGF-R or CSF-1-R. Therefore an m.o.i. of 10 was chosen to achieve high overexpression levels for these PTPs. As shown in Fig. 5, the number of foci formed in cell monolayers infected with v-erbB or v-fms viruses was not significantly reduced upon superinfection with PTP 1B virus. While the transformation inhibition was somewhat more pronounced for PTP TC, it was much weaker than that achieved with a 10-fold lower m.o.i. (m.o.i of 1) of the TC-M virus (Fig. 5). Apparently, the expression resulting from infection by a single virus particle yielded enough PTP activity to abolish transformation. At an m.o.i of 1, one cannot expect every cell to be infected, which is likely to account for the residual number of foci.

**DISCUSSION**

Since the first discovery of protein tyrosine phosphatases, their role as negative regulators of tyrosine kinase-generated biological signals has been postulated. However, to date, no evidence for such a function in natural systems has been presented. In fact, where an involvement for PTPs is best understood, dephosphorylation of target phosphotyrosine residues leads to signal activation or removal of a regulatory block. For example, CD45 activation regulates T-cell receptor-mediated signalling (reviewed in Trowbridge (1991)), the corkscrew gene product PTP is a positive regulator of the torso signal (Perkins et al., 1992), and cdc25 participates in cell cycle regulation by dephosphorylating and thus activating the histone H1 kinase p34^cd25 (Russell and Nurse, 1996; Dunphy and Kumagai, 1991; Gautier et al., 1991). Yet, the large number of identified intracellular and receptor-like transmembrane PTPs suggests that in addition to their role as positive regulators, PTPs may also be negative regulators of specific tyrosine kinase signals. In addition to the problem of RTK/PTP specificity, many other questions remain unanswered, including those related to target recognition, catalytic activity regulation, the possible existence of specific ligands for receptor-like PTPs, the role of PTPs in dephosphorylation of specific PTK substrates, and the significance of cellular localization signals, such as that identified in PTP 1B (Franzoni et al., 1992).

PTP function has been studied largely in vitro using para-nitrophenyl phosphate or synthetic phosphopeptides as substrates. In the present study we used a transient expression assay system to investigate several aspects of PTP activity regulation in intact cells. By pairwise co-overexpression of three PTPs and eight RTKs, we addressed the question of target specificity, the significance of cellular localization, and the influence of relative concentrations of PTPs, RTKs, and RTK-binding proteins on RTK dephosphorylation.

Our results indicate strongly that PTP specificity is defined by a combination of cellular localization and target recognition. As shown in Fig. 1 and in previously published reports (Lammers et al., 1990; Herbst et al., 1991), the 293 cell overexpression system generates high levels of expression and, for most RTKs, high basal levels of tyrosine phosphorylation. Our data indicate that this ligand-independent, i.e. unstimulated, phosphorylation signal may represent the result of spontaneous auto- or transphosphorylation of receptor molecules that reside in different intracellular membrane compartments while being transported to the cell surface. This interpretation was suggested by the higher sensitivity of the unstimulated phosphotyrosine-containing RTK bands, as seen in the case of EGF-R, α- and βPDGF-R, and CSF-1-R, to the catalytic activity of intracellular PTPs 1B and TC. The same was true for incompletely glycosylated receptor forms of lower apparent molecular weights in the cases of HER1-2, α- and βPDGF-R, and CSF-1-R. Moreover, PTP 1B and TC
sensitivity was most pronounced for the uncleaved IR and the IGF-1-R receptors, which are likely to be localized in the endoplasmic reticulum. For these receptor types, the partial sensitivity of the β subunit phosphorylation signals suggested that PTP 1B and TC may also be found in membrane-bound compartments containing already cleaved and processed IR and IGF-1-R precursor molecules. On the other hand, weak PTP 1B and TC-resistant phosphotyrosine signals at 190 kDa suggested that a fraction of uncleaved precursors were present on the surface of transfected cells, where they were tyrosine-phosphorylated upon ligand binding. Further support for this interpretation of our data was provided by experiments in which a COOH-terminally truncated PTP TC mutant TC-M was employed. In analogy with PTP 1B, the deleted sequence of 98 amino acids apparently contains a localization signal for intracellular membrane compartments which, when removed, "deregulates" the phosphatase, resulting in unspecific dephosphorylation of all forms of the RTKs tested.

Taken together, the results of our coexpression experiments with PTP 1B and TC suggest a role in the cellular system that controls the kinase function of nascent PTKs during biosynthesis and transport to the cell surface. This would require that such PTPs exhibit a broad spectrum of substrate specificity, and PTP 1B and TC may simply reflect either redundant or cell-type-specific systems with similar functions, since both PTPs exhibited similar activities on all RTKs tested, with the exception of HER1-2 and βPDGF-R, which appeared more sensitive to TC than 1B. Failure of this control system either due to abnormal RTK overexpression, as in the case of the HER2/neu RTK, or subnormal expression of the PTPs, could then lead or at least contribute to malignant transformation of the cell. The possibility of cell transformation by a deregulated, overexpressed RTK that is localized in the ER was recently demonstrated with a transmembrane domain deletion mutant of HER2/neu (Hudziak and Ullrich, 1991).

The significance of PTP localization is further emphasized by an experiment in which recombinant retroviruses encoding PTP 1B, TC, and TC-M were used to investigate the effect of phosphatase overexpression on v-erbB- or v-fms-mediated NIH 3T3 cell transformation (Fig. 5). Strong suppression of focus formation in cell monolayers without apparent inhibition of cell growth was only obtained with the truncated nonlocalized TC-M mutant. Even though under our experimental conditions the intact TC-PTP exhibited 1.5–2 times higher specific activity in an in vitro phosphatase assay system with 3-nitrophenyl phosphate as substrate than the mutant (not shown), a significantly lower inhibitory effect was observed with 10 times the colony-forming units of the corresponding wild type PTP virus. Whether this inhibition of v-erbB and v-fms transforming activity, which was similar to that obtained with the PTP 1B virus for both oncogenes, reflects the dephosphorylation of ER- or Golgi-associated activated oncoproteins by coexpressed PTP 1B and TC is not certain, but may be possible in view of our previous findings (Hudziak and Ullrich, 1991) and our observations summarized above.

RTK coexpression with the transmembrane PTP CD45 revealed a more pronounced degree of target selectivity. While this PTP had no effect on the unstimulated HER2/neu RTK, it efficiently dephosphorylated the equivalent, presumably intracellular form of the closely related EGF-R. Neither IR nor IGF-1-R precursors and β subunits were significant substrates. Moreover, while all α- and βPDGF-R forms were partially (50–60%) dephosphorylated, the CD45 phosphatase was very effective in reducing the phosphotyrosine content of the kit cytoplasmic domain of ligand-activated ER-K, but not that of the CSF-1-R. Presumably, these differential effects of CD45 reflect constitutive catalytic activation of this enzyme during its transport to the cell surface in vesicles that also contain spontaneously activated and autophosphorylated forms of co-overexpressed RTKs. An apparent lack of dephosphorylating activity, such as in the case of the precursors for IR, IGF-1-R, and HER1–2, may therefore be caused by segregation of these polypeptides in the ER, while the PTP is exported on to the cell surface.

The most surprising result was obtained with the ER-chimaera (Fig. 1) and subsequently confirmed with wild type p145ER (not shown). The phosphotyrosine residues of the activated kit kinase domain were equally good substrates for all PTPs tested, irrespective of their predominant localization in the cell. Only a small population of molecules appeared to be resistant to either of the PTPs. One may postulate that ER-K and the kit RTK, which are predominantly on the surface of transfected 293 cells, as evidenced by the strong ligand-dependent kinase activation, accumulate in specific areas that are accessible to PTPs 1B and TC but exclude other, even closely related RTKs, such as PDGF-R and CSF-1-R. Since there is no precedent for such segregation on the cell surface, one could alternatively interpret the resistance of surface-localized phosphotyrosine-RTKs to be caused by differences in their microenvironments, which may influence PTP-RTK interactions. Since kit is normally not expressed in 293 fibroblasts, the molecules that may protect the activated receptor kinase from dephosphorylation may not be present in this cell type. Because this interpretation does not explain the resistance of CSF-1-R, which normally is not expressed in 293 cells, further experiments will be necessary for a better understanding of these differential effects.

The possibility that cellular RTK binding factors are involved in the regulation of signal transduction by receptor dephosphorylation is supported by the results of our competition experiment, in which overexpressed SH2-containing p85-PI3K protects ER-K from dephosphorylation by the deregulated TC-M phosphatase (Fig. 4). Together with the RTK versus PTP titration results shown in Fig. 3, these data indicate that both the relative cellular concentrations of PTP and RTK, and also the amounts of additional cellular proteins exhibiting affinity for the activated, tyrosine-phosphorylated receptor, are factors that define the kinetic parameters of RTK dephosphorylation and subsequent inactivation. From this it is reasonable to propose that high level overexpression of a cellular polypeptide with high affinity for RTK-phosphotyrosines could lead to cellular transformation and oncogenesis. Evidence for this possibility has been provided in vitro (Rotin et al., 1992) and by the identification of the oncogene v-crk (Matsuda et al., 1991), which encodes an SH2-containing protein with high affinity for phosphotyrosine residues. Similar high affinity binding factors, such as src, GRB-2/sem-5, and p85 may therefore act not only as docking proteins for catalytic subunits, as in the case of PI 3-kinase (reviewed in Schlessinger and Ullrich, 1992), but could be involved in regulating access of PTPs to activated RTKs and thereby modulate the duration of the receptor-generated signal.

In summary, we have shown that RTK and PTP localization plays a key role in the regulation of phosphotyrosine-mediated signal transduction. Moreover, as yet unidentified structural determinants and relative expression levels of the reaction partners seem to play a role in the definition of specificity and activity regulation. Our findings suggest that target selection may be broadly defined for certain cytoplasmic PTPs such as TC and 1B, which appear to fulfill
more general functions, while cell surface-associated PTPs will likely display more stringent substrate specificity. Finally, RTK-binding proteins may serve as signal enhancers or modulators by influencing PTP access to phosphotyrosine substrate sites. Further experiments will be necessary to elucidate the details of RTK/PTP interaction and to establish the role of specific PTPs in normal and oncogenic signal transduction.

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