

Expression of a Protein Tyrosine Phosphatase in Normal and *v-src*-Transformed Mouse 3T3 Fibroblasts

Terry A. Woodford-Thomas, Janette D. Rhodes, and Jack E. Dixon*

*Department of Biological Chemistry, The University of Michigan Medical School, Ann Arbor, Michigan 48109-0606; and the Walther Cancer Institute, Indianapolis, Indiana 46208

Abstract. A rat cDNA encoding a 51-kD protein tyrosine phosphatase (PTP1) was cloned into a mammalian expression vector and transfected into normal and *v-src*-transformed mouse NIH 3T3 fibroblasts. In the stable subclones isolated, PTP1 expression at the mRNA level was elevated twofold to 25-fold. The highest constitutive level of phosphotyrosine-specific dephosphorylating activity observed without cytotoxic effects or significant clonal instability was ~10-fold over the endogenous activity. The expressed PTP1 was found to be associated with the particulate fraction of the fibroblasts. Subcellular fractionation and immunofluorescent microscopic examination of PTP1-overexpressing cells has shown the phosphatase to be localized to the reticular network of the ER. PTP1 was readily solubilized by detergents, but not by high salt. Limited proteolysis of membrane-associated PTP1 resulted in the release of lower molecular mass (48 and 37 kD)

forms of the enzyme to the cytosol. Thermal phase partitioning of isolated membranes with Triton X-114 indicated that the full-length PTP1 was strongly integrated into the membrane in contrast to the proteolytically derived fragments of PTP1. Overexpression of PTP1 caused little apparent change in the rate of cell proliferation, but did induce changes in fibroblast morphology. A substantial increase in the proportion of bi- and multinucleate cells in PTP1-expressing cell populations was observed, and, in the case of the *v-src*-transformed cells, cell flattening and loss of refractility occurred. Although no apparent difference in the tyrosine phosphorylation of pp60^{*v-src*} was noted in *v-src*-transformed control and PTP1-overexpressing fibroblasts, the phosphotyrosine content of a 70-kD polypeptide was decreased in PTP1-overexpressing cells.

THE phosphorylation of specific tyrosine residues in cellular proteins is recognized as a critical event in the regulation of the cell cycle and neoplastic transformation. Protein tyrosine kinases generate part of the intracellular signal transduction pathway that mediates the biological response of cells to growth factors and oncogenic viruses. Protein tyrosine phosphatases (PTPase;¹ protein-tyrosine-phosphate phosphohydrolase, EC 3.1.3.48) modulate and even attenuate the activities of tyrosine kinases in addition to functioning in the dephosphorylation of other specific phosphotyrosine (PTYR)-containing cellular substrates. Thus, the equilibrium established between protein

tyrosine kinases and phosphatases is vital in determining both the cell growth properties and the differentiation state of the cell.

There is evidence that certain PTPases may act in a positive manner to regulate cell cycle progression (12), cell proliferation (16, 30), and differentiation (11, 18, 40). PTPases could also act in an opposing manner to inhibit cell growth, because tyrosine kinases have often been implicated as positive regulators of cell proliferation. Certain PTPases may therefore function as potential antioncogenes or suppressors of cell transformation. Protein tyrosine phosphatases are potently inhibited *in vitro* by micromolar concentrations of orthovanadate (17, 31, 38). Treatment of intact cells with noncytotoxic levels of vanadate can induce a reversible transformed phenotype and changes in the cytoskeletal architecture that are similar to that reported for Rous sarcoma virus (RSV)-transformed cells (19). Vanadate has been shown to mimic the mitogenic effects of certain growth factors such as EGF, FGF, and IL-3 on intact cells (19, 25, 37, 40). Despite the fact that vanadate has multiple biochemical effects in the cell, the observation that vanadate treatment results in an elevation in the tyrosine phosphorylation level underscores the important regulatory role that PTPases play.

Terry A. Woodford-Thomas' present address is Department of Pathology, Washington University School of Medicine, St. Louis, MO 63110.

Janette D. Rhodes' present address is Division of Infectious Diseases, VA Medical Center, USD, San Diego, CA 92161.

1. *Abbreviations used in this paper:* CMV, cytomegalovirus; DiOC6(3), 3,3'-dihexyloxycarbocyanine iodide; GST, glutathione-S-transferase; LAR, leukocyte antigen-related phosphatase; LCA, leukocyte common antigen; LRP, leukocyte-related phosphatase; PTPase, protein tyrosine phosphatase; PTYR, phosphotyrosine.

The elevated PTYR content of at least 20 different polypeptides identified by immunoblot analysis indicates that under such conditions the endogenous activities of at least a subset of tyrosine phosphatases are suppressed. This allows for specific PTYR-containing cellular substrates to be shifted to their phosphorylated forms. Unfortunately, the identity and role played by many of these proteins is still uncertain.

A rat brain cDNA encoding a 51-kD PTPase has been isolated and characterized in our laboratory (15). PTP1 is a vanadate- and molybdate-sensitive phosphatase that is widely distributed, is highly active, and shows an absolute specificity for PTYR-containing proteins. PTP1 contains a single catalytic domain in contrast to the integral membrane protein receptor-like enzymes such as leukocyte common antigen (LCA), leukocyte antigen-related (LAR) phosphatase, and LCA-leukocyte-related phosphatase (LRP) that contain duplicated PTPase domains (21, 24, 35, 36). Two other mammalian, nonreceptor-like tyrosine phosphatases have been cloned and sequenced: PTP1B, characterized from human placenta (3, 5, 6) and T cell PTP from human lymphocytes (8). PTP1 shares 97% amino acid sequence identity with human PTP1B and 72% identity with the core phosphatase domain of T cell PTP.

In this study, the overexpression of PTP1 transfected into both normal and *v-src*-transformed mouse 3T3 fibroblasts was investigated to determine its subcellular localization and effects on cell morphology, cell growth properties, and pattern of intracellular tyrosine phosphorylation. The transforming gene (*v-src*) of RSV encodes a highly active 60,000-kD protein tyrosine kinase (pp60^{*v-src*}), which is autoactivating and capable of phosphorylating a variety of cellular proteins (7). Because pp60^{*v-src*} has been implicated in playing a major role in the establishment and maintenance of the neoplastic phenotype (7, 22), overexpression of a PTPase in *v-src*-transformed fibroblasts provided an interesting model system to study the effects of tyrosine dephosphorylation induced by PTP1.

Materials and Methods

Cell Culture and Transfections

NIH 3T3 mouse fibroblasts were routinely grown at low density at 37°C in a water-saturated 10% CO₂/90% air atmosphere in DME (Flow Laboratories, Inc., McLean, VA) supplemented with 10% (vol/vol) calf serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). New cultures were started from frozen stocks every 4–6 wk. The highly transformed parental NIH 3T3 subline (3T3 JHV2B) was established by initial transfection with a plasmid containing the *v-src* gene derived from the Prague A strain of RSV (kindly provided by Elizabeth Taparowsky, Purdue University, West Lafayette, IN). 3T3 JHV2B was isolated as a single clone following focus formation in 0.5% calf serum. Cells were cotransfected with pKO neo, which encodes the aminoglycoside transferase gene, and PTP1 plasmid DNA using the calcium phosphate precipitation method (41). After 24 h, cells were replated into medium containing 400 µg/ml geneticin (G418; Gibco Laboratories, St. Lawrence, MA). Stable neomycin (geneticin)-resistant 3T3 subclones were isolated at 12–14 d posttransfection and maintained in selective medium.

Expression of PTP1 in *Escherichia coli*

PTP1 cDNA was originally isolated from a rat hypothalamic cDNA library using a mixed oligonucleotide probe based upon the amino acid sequence FWEMVWEQK derived from human placenta PTP1B (15). In addition to the full-length PTP1 used for transfection studies, a truncated form of PTP1 (PTP U323) was used for affinity purification of PTP1 specific antibody. This mutated PTP1 protein is ~37 kD compared to 51 kD for the wild type

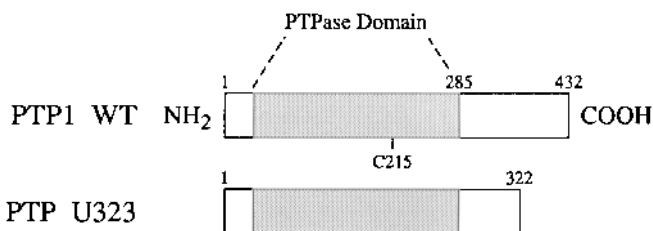


Figure 1. Schematic diagram of the recombinant wild type PTP1 and carboxyl-terminal truncated mutant designated PTPU323. The proposed active site in the single PTPase domain encompasses a region surrounding Cys 215 that has been shown to be essential for catalysis (14).

PTP1 and more resistant to proteolytic degradation (Fig. 1). A 1.8-kb EcoRI-SalI fragment encoding the truncated PTP1 was isolated from p7-PTP U323 and then ligated into EcoRI/SalI-digested pGEX-KG (13). This modified vector allowed the expression of PTP1 in frame with a 26-kDa fragment of glutathione-S-transferase (GST) for purification of the fusion protein from crude bacterial extracts in a single step by affinity chromatography on glutathione agarose. Purification of the GST-PTP U323 fusion protein was done according to Guan and Dixon (13) with modification. Bacterial cultures were incubated at 37°C until reaching an A₆₀₀ of 1.0. IPTG was added to 0.2 mM, and cells were harvested by centrifugation after 3 h of additional growth. Bacterial pellets were lysed by vigorous shaking with 0.1-mm glass beads in 50 mM imidazole, pH 7.5, 0.5 mM EDTA, 300 mM NaCl, and 0.1% β-mercaptoethanol using 25 ml of buffer/liter of culture. After centrifuging lysates at 10,000 g for 10 min at 4°C, the cleared supernatant was incubated with a 50% (vol/vol) slurry of glutathione agarose for 2 h at 4°C. The agarose beads were washed with 20 mM imidazole, pH 7.5, 25 mM NaCl, 0.05% Triton X-100, 10% glycerol, and 0.1% β-mercaptoethanol. The fusion protein was cleaved directly from the resin by incubation for 20 min at 24°C in thrombin cleavage buffer (20 mM imidazole, pH 7.5, 150 mM NaCl, 2.5 mM CaCl₂, and 0.1% β-mercaptoethanol) using ~4 µg of thrombin per 2 mg of fusion protein. After centrifuging the sample at 5,000 g for 5 min at 4°C, purified PTPase was recovered in the supernatant. 2 mg of active PTPase protein were routinely isolated from 100 ml of bacterial culture. After further purification by SDS-PAGE, the 37-kD PTP U323 protein was used for antibody production.

Production and Affinity Purification of PTP1 Antibody

Polyclonal antibody to purified PTP U323 protein was generated by subcutaneous injection of a New Zealand white rabbit according to standard protocol. Serum was mixed with 0.5 vol of saturated ammonium sulfate, and the suspension was centrifuged at 3,000 g for 30 min. The pellet was resuspended in PBS and dialyzed against the same buffer at 4°C. For antibody purification, the GST-PTP U323 fusion protein was bound to activated Affigel 10 in 0.1 M MOPS, pH 7.5, containing 0.2% β-mercaptoethanol. After covalent attachment of GST-PTP U323, the resin was reacted with 0.1 M ethanolamine, pH 8, and washed with phosphate buffer. Antibody was adsorbed to the affinity matrix in 100 mM sodium phosphate, pH 7.6, containing 0.1% Triton X-100, extensively washed, eluted with 0.2 M glycine-HCl, pH 2.3, containing 0.1 M NaCl and 0.1% Triton X-100, and immediately neutralized with concentrated Tris-HCl, pH 8. After the addition of RIA-grade BSA to 0.2 mg/ml, purified PTP1 antibody was concentrated in 0.1 M sodium phosphate, pH 7.2, and stored in 0.02% sodium azide at -20°C. Western blot analysis was used to verify antibody specificity. Only a single band migrating at 63 kD (GST-PTP U323 fusion protein) was detected after electrophoretic separation, electrotransfer, and immunoblot analysis of extracts from bacteria harboring the pGEX-PTP U323 construct.

Construction of the pCMV-PTP1 Mammalian Expression Vectors

PTP1 cDNA was subcloned into the mammalian expression vectors, pCMV4 and pCMV5 (kindly provided by David Russell, Southwestern Medical Center, Dallas, TX) from which constitutive expression is directed from the strong cytomegaloviral (CMV) promoter (Fig. 2). pCMV4 contained a translational enhancer sequence corresponding to the 5' untrans-

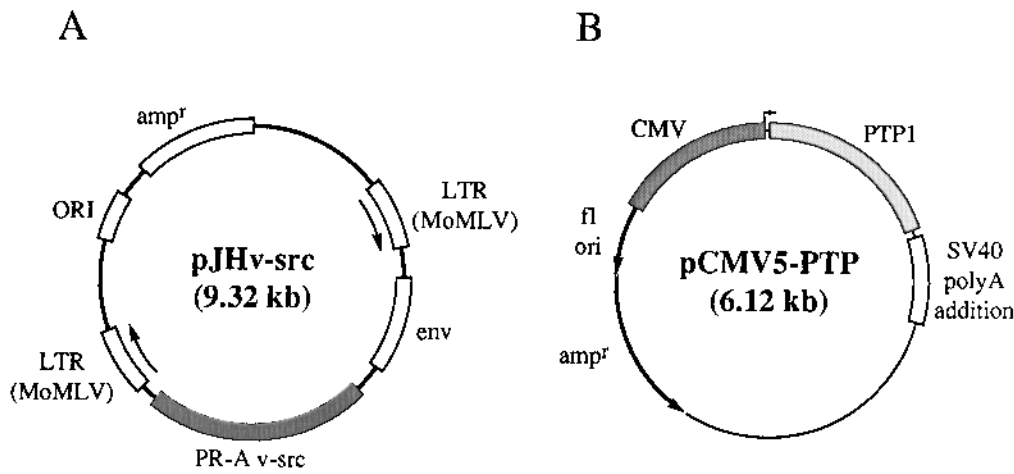


Figure 2. Mammalian expression vectors for the establishment of pp60^{v-src} and rat PTP1 expression in mouse 3T3 fibroblasts. Expression of pp60^{v-src} and PTP1 are under control of the Moloney murine leukemia virus LTR (A) and the cytomegalovirus promoter (B), respectively.

lated region of Alfalfa Mosaic Virus 4 RNA positioned so that the enhancer is located in the 5' untranslated region of the mRNA transcribed from the CMV promoter. This sequence functions by decreasing the initiation factor requirement for protein synthesis. A 1.4-kb HgaI fragment from pT7-PTP1 (15) encoding the entire PTP1 sequence was blunt-ended and ligated into HindIII-linearized, blunt-ended pCMV4/5, and the correct orientation was determined by KpnI digestion.

Analysis of PTP1 and pp60^{v-src} Expression

The levels of PTP1 mRNA as well as relative levels of PTP1 and pp60^{v-src} mRNAs in the transfected 3T3 subclones were determined by Northern blot analysis. Total RNA was prepared by extraction into guanidine isothiocyanate, followed by cesium chloride ultracentrifugation. RNA samples (10 µg/lane) were heat-denatured and electrophoresed on 1.5% formaldehyde agarose gels, transferred to nitrocellulose, and hybridized at 42°C in 50% formamide, 5 × SSPE, 5 × Denhardt's, 0.1% NaDodSO₄, and 100 µg/ml denatured salmon sperm DNA, and radiolabeled probe (1 × 10⁶ cpm/ml). The PTP1 hybridization probe was a 600-bp XhoI fragment from pT7-PTP1 labeled with [³²P]dCTP using random primers. The entire pJHv-src plasmid was labeled by nick translation for use as a hybridization probe. Both probes were radiolabeled to a specific activity of 2–5 × 10⁸ cpm/µg DNA. Blots were washed under high stringency (0.1 × SSC, 0.1% SDS) at 65°C and autoradiographed.

The expression of the PTP1 protein was demonstrated by immunoblot analysis. Trypsinized cells were washed, pelleted, and boiled in SDS sample buffer. Whole cell extracts were subjected to electrophoresis on 10% SDS-polyacrylamide gels and electrotransferred to nitrocellulose (BA85). Filters were incubated with 3% RIA-grade BSA in TBST (50 mM Tris, pH 7.2, 150 mM NaCl, 0.5% Tween 20) at room temperature for 4 h. For detection of PTP1, filters were incubated with affinity-purified PTP1 antibody (1:1,000) for 2 h at 4°C and then incubated with 0.1 µCi/ml [¹²⁵I]-labeled protein A, washed, and exposed to XAR film at -70°C. Alternatively, detection was done using an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody. The level of pp60^{v-src} protein expression was assessed similarly using mAb EC10 (kindly provided by Tom Parsons, University of Virginia, Charlottesville, VA), or Mab327 (donated by Joan Brugge, University of Pennsylvania, Philadelphia, PA) and [¹²⁵I]-sheep anti-mouse IgG.

PTPase Assay

PTYR-specific phosphatase activity was measured using [³²P]-Raytide (Oncogene Science, Inc., Manhasset, NY). The substrate was phosphorylated to a specific activity of 4–5 × 10⁵ cpm/nmol peptide (~0.2–0.3 mol phosphate/mol peptide) by p43^{v-abl} tyrosine kinase (Oncogene Science, Inc.) in the presence of [³²P]ATP (3,000 Ci/mmol). Tyrosine dephosphorylation was measured in a 50-µl reaction mixture containing 20 mM imidazole, pH 7.0, 5 mM EDTA, 20 mM sodium fluoride, 0.2% β-mercaptoethanol, and 0.2 µM [³²P]-tyrosine-phosphorylated substrate. After incubation for 10 min at 30°C, the reaction was terminated by addition of 750 µl of activated charcoal solution (0.9 M HCl, 90 mM sodium pyrophosphate, 2 mM NaH₂PO₄ 4% (vol/vol) Norit A (35)). The mixture was microfuged for 2 min, and radioactivity in the supernatant was deter-

mined. Protein concentrations were analyzed according to the Bradford method (2). 1 U of PTPase activity is defined as that amount of enzyme which releases 1 nmol of phosphate per minute. Linearity with respect to time and protein concentration was verified. Specific phosphatase activities were corrected for zero time determinations or for control reactions containing all constituents except whole cell or fractionated cell extracts. Because type 2 serine/threonine protein phosphatases, as well as acid phosphatases, can dephosphorylate PTYR-containing substrates under certain conditions, divalent cations were excluded and 5 mM EDTA and 20 mM NaF were included in the reaction mixture. In addition, contaminating tyrosine dephosphorylating activity of other phosphatases was assessed by conducting PTP assays in the presence and absence of 200 µM sodium vanadate.

Immunoprecipitations and Immunoblot Analyses with anti-pp60^{v-src} and Antiphosphotyrosine Antibodies

When metabolic labeling of cells was done, cultures were incubated in phosphate-free culture medium containing 1% dialyzed FCS and 1 mCi/ml [³²P]-orthophosphate for 12 h. Immunoprecipitation of pp60^{v-src} was done using mAb 327 or EC10 (kindly provided by J. Brugge, University of Pennsylvania, and T. Parsons, University of Virginia, respectively). Cells were lysed in RIPA buffer containing 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM sodium vanadate 15 µM phenylarsine oxide, 1 mM PMSF, 100 µM aprotinin, and 1 µM leupeptin and then passed three times through a 26-gauge needle. Protein concentrations of lysates were determined using the BCA protein assay kit (Pierce Chem. Co., Rockford, IL). Lysates were adjusted to 1 mg/ml protein and incubated with an excess of antibody for 2 h at 4°C. 50 µl of a 1:3 slurry of protein A-Sepharose CL-4B (Pharmacia Diagnostics Inc., Fairfield, NJ) previously incubated with rabbit anti-mouse IgG (Accurate Chem. & Sci. Corp., Westbury, NY) was added, and the lysate was rotated for 1 h at 4°C. Immunoprecipitates were washed three times with RIPA buffer containing 400 mM NaCl, three times with buffer containing 150 mM NaCl, and twice with 40 mM Tris-HCl, pH 7.2. All wash buffers contained 0.2 mM sodium vanadate.

Washed pp60^{v-src} immunoprecipitates were resuspended in 10 mM Tris-HCl, pH 7.2. For [³²P]-labeled samples, or when probing with an anti-PTYR antibody, extracts were analyzed by 10% SDS-PAGE and subjected to autoradiography or immunoblot analysis following electrotransfer to nitrocellulose, respectively.

The protein tyrosine kinase assay was performed by resuspending the immunoprecipitates in 30 µl of kinase buffer containing 20 mM Tris-HCl, pH 7.2, 5 mM MgCl₂, 5 µCi[γ-³²P]ATP (10 mCi/ml; Amersham Corp., Arlington Heights, IL) and 5 µg of acid-denatured enolase as an exogenous substrate. The reactions were allowed to proceed at room temperature for 2 min. Samples were electrophoresed as described above, and the gels dried and subjected to autoradiography.

For immunoblot analysis with anti-PTYR antibodies, cell extracts were prepared by adding lysis buffer (50 mM Heps, pH 7.6, containing 50 mM NaCl, 10% glycerol, 1% Triton X-100, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 200 µM sodium orthovanadate, 15 µM phenylarsine oxide, 5 mM EDTA, and protease inhibitors) directly to culture dishes, then incubating on ice for 20 min. Samples were microfuged at 4°C for 5 min.

The supernatants were normalized for protein concentration and then resolved by SDS-PAGE. After electrotransfer to nitrocellulose, filters were blocked with 3% BSA in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Tween 20), then incubated with a 1:1,000 dilution of anti-PTYR antibody in TBST (mAb Ig2bk, Upstate Biotechnology, Inc., Lake Placid, NY; or Mab 6G9, kindly provided by Tom Parsons, University of Virginia) followed by [¹²⁵I]-sheep anti-mouse IgG at 1 μ Ci/ml in TBST. Blots were washed and subjected to autoradiography.

Subcellular Fractionation

Exponentially growing cells were washed three times with PBS, scraped from culture dishes with a rubber policeman, and collected by centrifugation at 800 *g* for 5 min. Initially, cytosolic and total membrane particular fractions were prepared. Cells were allowed to swell and then lysed by Dounce homogenization in hypotonic buffer (5 mM Hepes, pH 7.2, 1 mM MgCl₂, 5 mM β -mercaptoethanol) containing 0.5 μ g/ml each of pepstatin, antipain, leupeptin, and chymostatin, 0.1 mM benzamide, 1 mM PMSF, and 15 μ g/ml L-1-chloro-3-[4-tosylamido]-7-amino-2-heptanone HCl. In the case where the membrane fraction was subsequently treated with trypsin, these protease inhibitors were omitted. Nuclei were removed by centrifugation at 2,000 *g*, and cell extracts were further centrifuged at 100,000 *g* for 1 h at 4°C to separate the cytosolic (S100) and total membrane (P100) fractions. Limited trypsin treatment of the membrane fraction resuspended in hypotonic buffer followed a time course of 20 min. Aliquots were taken at designated minute intervals, and the digestions were terminated by the addition of a fivefold excess of soybean trypsin inhibitor. Samples were recentrifuged at 100,000 *g* and separated into cytosolic and post trypsin-digested membrane fractions.

For further fractionation of cell extracts, cells were collected and lysed in hypotonic buffer containing protease inhibitors as described above. Nuclei were pelleted by centrifugation at 2000 *g*, and the supernatant was applied to a two-phase system and centrifuged at 100 *g* to partition the plasma membrane to the upper phase (26). The lower phase was then layered onto a 0.2 M sucrose cushion and centrifuged at 164 *g* to separate the ER (upper phase) and mitochondria (lower phase). Standardized amounts of protein for each fraction were subjected to Western immunoblot analysis as previously described. Subcellular fractions were analyzed for purity by EM (data not shown).

Triton X-114 Partitioning

Triton X-114 extractions were performed according to the method described by Bordier (1). Membranes (2 mg/ml protein) were lysed in 200 μ l of 10 mM Tris-HCl, pH 7.4, 1% Triton X-114, 1 mM EDTA, 5 mM 2-mercaptoethanol, and protease inhibitors at 4°C. The mixture was then layered onto a 300- μ l cushion of 6% (wt/vol) sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.06% Triton X-114 in a 1.5-ml microfuge tube (Eppendorf Inc., Fremont, CA) and warmed to 30°C with gentle agitation during which clouding of the solution occurred. The phases were separated at room temperature by centrifugation for 2 min at 300 *g*. The upper aqueous phase was reextracted with buffer containing 0.5% Triton X-114 and separated on the same sucrose cushion. The Triton X-114 detergent pellet was washed once with buffer in the absence of detergent. Both phases were normalized for protein and detergent concentrations and subjected to immunoblot analysis.

Immunofluorescence Studies and Microscopy

Mouse 3T3 fibroblasts were grown on 12 \times 12 mm coverslips. Normal fibroblasts were synchronized by arresting cells in 0.5% calf serum for 48 h followed by their release from quiescence by addition of media containing 10% serum. Cell cycle progression was monitored by measuring [³H]thymidine incorporation into DNA and by determination of the mitotic index as cells entered the G₂/M phase. Cells were fixed and stained as described below. All steps were carried out at room temperature. Different cell fixation procedures were necessary for use of the various antibodies and conjugated lectins.

For staining with affinity-purified PTP1 antibody, cells were fixed for 20 min with 3.7% formaldehyde in PBS (25 mM), and then permeabilized for 3 min with 0.3% Triton X-100 in PBS. Coverslips were then rinsed and incubated with PTP1 antibody for 45 min. After rinsing, coverslips were incubated with FITC-labeled goat anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA), rinsed again, and mounted in 4% *n*-propyl gallate in 10% PBS/90% glycerol. FITC-conjugated ConA and FITC or TRITC-conjugated

wheat germ agglutinin (Vector Laboratories, Inc.) were used as markers for the RER and Golgi apparatus, respectively (39). Cells were fixed and permeabilized as described above, and incubated with the conjugated lectins for 30 min, rinsed, and mounted. The ER was also visualized by staining with the lipophilic dye 3,3'-dihexyloxycarbocyanine iodide [DiOC₆(3)] (Molecular Probes, Inc., Eugene, OR). A stock solution of DiOC₆(3) was made at 0.5 mg/ml in ethanol and stored protected from light. A working solution of 2.5 μ g/ml was prepared in Dulbecco's phosphate-buffered saline just before use. Cells were fixed for 10 min in 0.25% glutaraldehyde in Dulbecco's phosphate-buffered saline and stained with either DiOC₆(3) for 5 min or with PTP1 antibody as described above. Coverslips were rinsed, mounted, and examined immediately.

As a control, bacterially expressed PTP1 was used to adsorb affinity-purified PTP1 antibody. PTP1 antibody was incubated with a 10-fold excess of PTP U323 protein for 30 min at 37°C and then stored at 4°C until used. Stained cells were photographed with a microscope (model Axioskop; Carl Zeiss, Inc., Thornwood, NY) using a plan-neofluar 100X/1.30 oil objective. AxioLine exciter-barrier filters were used for visualizing FITC and TRITC fluorescence. Photographic prints were made from slides taken with color film (Ektachrome 400; Kodak, Rochester, NY).

Results

Expression of the 51-kD Rat PTP1 in Mouse 3T3 Fibroblasts

The full-length rat PTP1 cDNA encodes a PTYR-specific phosphatase that is 432 amino acids in length (Fig. 1). The single PTPase catalytic domain possesses the signature sequence VHCSAGIGRSG characteristic of the enzyme-active site. Within this sequence lies Cys 215, which is proposed to be essential in catalysis by participating in the formation of a phosphoenzyme intermediate (14). The PTP1 cDNA was transfected into both normal 3T3 and *v-src*-transformed mouse 3T3 fibroblasts. The potential effects of PTP1 overexpression on cell morphology and cell growth properties were studied. In addition, the cellular distribution of PTP1 was examined for future studies aimed at identifying biological substrates for PTP1-catalyzed dephosphorylation. Transformation of the parental NIH 3T3 line was achieved by initial transfection of cells with a plasmid containing the *v-src* gene from the Prague A strain of RSV from which expression of the oncogene product was under control of the Moloney murine leukemia virus LTR (Fig. 2 A). Subcloning of the PTP1 cDNA behind the strong CMV promoter in the pCMV4 or pCMV5 vectors (Fig. 2 B) allowed for the establishment of normal and *v-src*-transformed 3T3 fibroblast subclones that constitutively expressed the 51-kD protein tyrosine phosphatase. Several hundred neomycin (geneticin)-resistant subclones were generated, of which 30 were selected for analysis of PTP1 expression at both the mRNA and protein levels. The levels of PTP1 mRNA as well as the relative levels of PTP1 and pp60^{*v-src*} mRNA were determined in transfected subclones by Northern blot analysis of total cellular RNA. The levels of PTP1 mRNA expression in normal 3T3 fibroblast subclones were increased fivefold to 10-fold over the endogenous level by introduction of the CMV promoter-driven PTP1 vector (Fig. 3 A). Expression levels of the ~2.0-kb rat PTP1 mRNA in *v-src*-transformed 3T3 fibroblast subclones were in a range from twofold to 25-fold higher than levels obtained in PTP1-transfected normal 3T3 subclones (Fig. 3, A and B). The translational-enhancer sequence in pCMV4-PTP1 proved to be useful in establishing stable expression of rat PTP1 in normal 3T3 fibroblasts. It was generally the case that normal 3T3 subclones transfected

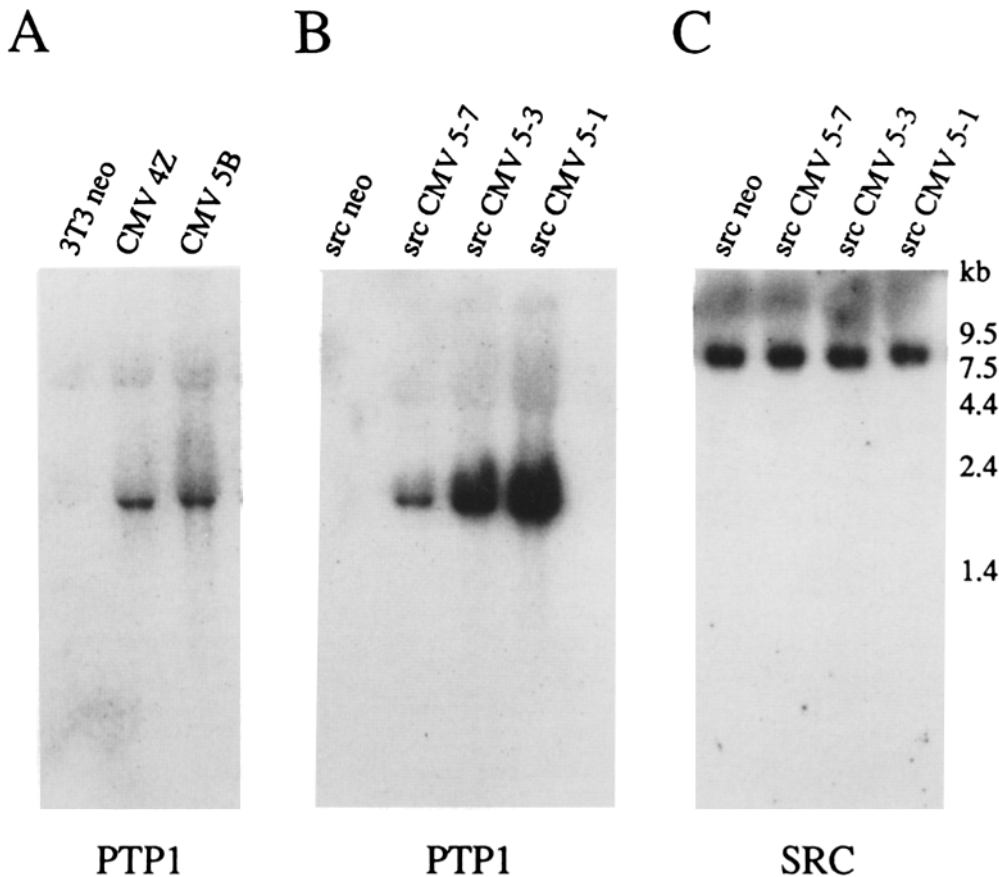


Figure 3. Northern blot analysis showing PTP1 expression levels in a selected series of normal and *v-src*-transformed, pCMV PTP1-transfected mouse 3T3 subclones. Total RNA (10 μ g/lane) isolated from individual subclones was electrophoresed on a 1.5% formaldehyde gel, transferred to nitrocellulose and probed with either radiolabeled 600-bp XhoI fragment derived from pT7-PTP1 or radiolabeled pJHv-*src*. RNA size markers are indicated below blots. (A) Normal 3T3 neo^R control subclone and two representative PTP1-transfected subclones. (B and C) *v-src*-transformed, neo^R control subclone and three *v-src*-transformed, PTP1-transfected subclones.

with pCMV5-PTP1 demonstrated an initial high level of rat PTP1 expression that was subsequently diminished with serial passage of the cells as observed by immunofluorescent microscopy. The normal 3T3 subclone, CMV4-Z, which showed stable PTP1 expression was selected for further studies. A series of stable *v-src*-transformed 3T3 subclones showing stepwise increases in the level of PTP1 mRNA expression were also selected for further characterization (Fig. 3 B). As indicated in Fig. 3 C, these subclones expressed PTP1 mRNA upon a similar background level of pp60^{*v-src*} mRNA.

Western blot analysis using affinity-purified PTP1 antibody was used to determine the level of PTP1 protein expression in whole cell extracts. Endogenous levels of PTP1 (or a similar tyrosine phosphatase that may have shared epitopes) were barely detectable in neomycin-resistant normal or transformed control subclones at low passage number. In PTP1-transfected subclones, the relative levels of the 51-kD tyrosine phosphatase protein reflected the PTP1 mRNA levels. PTP1 and pp60^{*v-src*} protein expression levels in the transformed control subclone (*src* neo) and *src* CMV5-1 PTP, a transformed PTP1 overexpressing subclone, are shown in Fig. 4. Vanadate-sensitive PTPase activities were measured in cell extracts using [³²P-Tyr]-Raytide as the substrate. In the case of *src* CMV5-7 PTP and *src* CMV5-1 PTP, PTYR-specific phosphatase activities were increased threefold and 10-fold over the endogenous activity, respectively (Table I). Interestingly, the endogenous level of tyrosine phosphatase activity increased by ~30–50% as a consequence of *v-src*-induced cell transformation alone. In the PTP1-transfected

normal 3T3 subclones examined, PTPase activities were elevated approximately twofold to threefold over the endogenous level.

The growth rate of PTP1-expressing subclones only showed minor alteration in that the doubling time was increased by 1–2 h. However, certain changes in the morphological appearance of the mouse fibroblasts were observed. An increased number of multinucleate cells were noted in the populations of both normal and transformed, PTP1 expressing subclones. In these subclones, 2–3% of the cells were bi- or multinucleate. Cells of the *src* neo control subclone were rounded, were refractile, showed no indication of growth saturation at high cell density, and, as such, were highly transformed (Fig. 5 C). Cells of the *src* CMV5-1 PTP expressing subclone, which synthesized the highest achievable levels of the 51-kD PTP1 protein, were nonrefractile and resumed a more flattened morphology that was different from the fusiform cell morphology of the normal cells (Fig. 5, A and D). Morphological changes in the *src* CMV5-7 PTP subclone were intermediate (Fig. 5 B). The ability of the *src* CMV5-1 PTP subclone to grow in low serum (0.2%) was unaltered by PTP1 overexpression. However, preliminary studies indicated that this subclone had a 75% reduction in its ability to form colonies when plated in soft agar, although the normal plating efficiency of the subclone was comparable to the parental transformed clone (>98%).

Examination of Potential Cellular Substrates for PTP1

After oncogenic transformation of 3T3 fibroblasts by *v-src*, the level of PTYR in cellular proteins is elevated severalfold

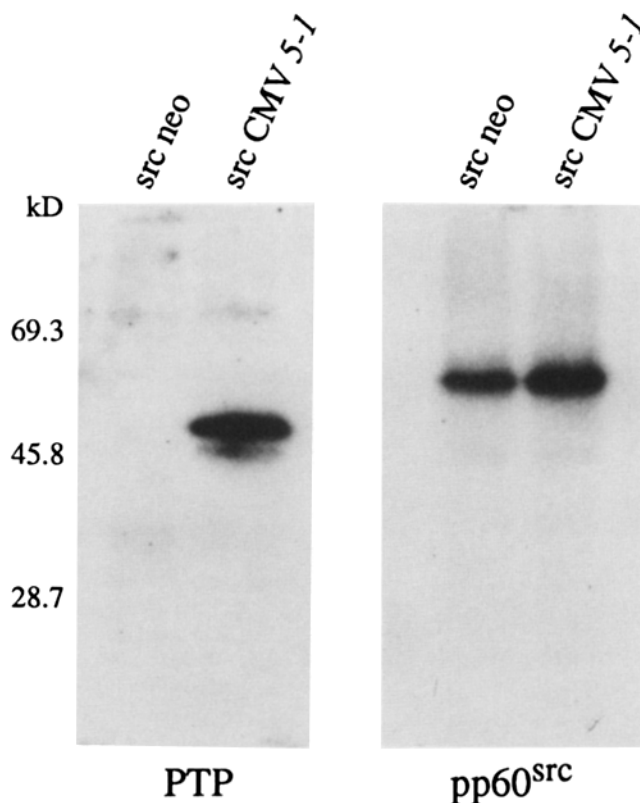


Figure 4. Western blot analysis of PTP1 and pp60^{v-src} expression in the *src neo* control and *src CMV5-1* PTP1 subclones. Total cell extracts prepared as described in Materials and Methods were resolved by 10% SDS-PAGE, electrotransferred to nitrocellulose, and probed with affinity-purified rat PTP1 antibody or antibody to pp60^{v-src} (mAb EC10), followed by [²⁵I]-protein A or [¹²⁵I]-sheep anti-mouse IgG, respectively. Protein molecular weight markers are indicated.

over that found in normal proliferating cells. Therefore, it is advantageous to use these cells for the examination of *in vivo* substrates for PTP1-catalyzed dephosphorylation. One tyrosine phosphorylated protein initially analyzed was pp60^{v-src} itself. *In vitro*, PTP1 had the ability to dephosphorylate a synthetic *v-src* peptide (RR-*src*, RRLIEDAEY AARG, Peninsula labs) which corresponds to the sequence surrounding Tyr 416, the major autophosphorylation site in pp60^{v-src} (not shown). In addition, purified recombinant PTP1 protein could dephosphorylate native pp60^{v-src} when added to [³²P] autophosphorylated kinase immunoprecipitates. However, no evidence has been obtained *in vivo* to suggest that the transfected rat PTP1 can alter the tyrosine phosphorylation state of pp60^{v-src}.

When pp60^{v-src} was immunoprecipitated from lysates from the *src neo* and *src CMV5-1* PTP subclones and immunoblot analysis performed using anti-PTYR antibody, no difference was noted (Fig. 6, *A* and *B*). Additionally, metabolic labeling of cells with [³²P]orthophosphate, followed by SDS-PAGE of pp60^{v-src} immunoprecipitates and direct autoradiography revealed no phosphorylation differences (Fig. 6 *C*). Finally, pp60^{v-src} immune complex kinase assays were performed. The levels of pp60^{v-src} autophosphorylation and the phosphorylation of an exogenous substrate, enolase, were similar with both subclones (not shown).

Table I. Characteristics of Control and PTP1 Overexpressing Cells

Cell line/ subclone	Cell morphology/ growth properties*	PTPase activity [†]
(U)		
Parental		
NIH 3T3	Normal line, cells fusiform, contact inhibited, no growth in soft agar	105
JHV2B	<i>v-src</i> transformed line, cells rounded, refractile, not contact inhibited, good growth in soft agar	155
Transfected		
3T3 CMV4Z	Cells fusiform, contact inhibited, 2-3% binucleate, no growth in soft agar	350
<i>src neo</i>	Cells rounded, refractile, not contact inhibited, good growth in soft agar	170
<i>src CMV5-1</i>	80% cells flattened, nonrefractile, 2-3% bi- or multinucleate, growth in soft agar reduced by 75%	1,660
<i>src CMV5-7</i>	50% cells flattened, nonrefractile, often bi- or multinucleate, growth in soft agar comparable to <i>src neo</i>	485

* For soft agar growth experiment, 1 × 10⁴ cells/60-mm dish were plated in 2.5% low melt agarose (Bio-Rad Laboratories) in DME/10% CS overlaid on 5% low melt agarose (FMC) in DME/10% CS. Colonies were allowed to grow for 2 wk. Plates were stained with p-iodonitrotetrazolium violet (1 mg/ml) and then scored.

[†] PTPase activity was measured in total cell lysates using [³²P]-Raytide as described in Materials and Methods. 1 unit of PTPase hydrolyzes 1 nmol of [³²P]-Tyr (P) per min.

The observation that pp60^{v-src} is similarly tyrosine phosphorylated in control and PTP1-overexpressing cells implies that this oncoprotein is not a physiological substrate for rat PTP1. Therefore, this tyrosine kinase is highly active in both the control and PTP1-transfected 3T3 subclones. Thus, the repertoire of PTYR-containing proteins induced concomitant with cellular transformation represents a further set of candidate substrates for PTP1. A comparison of the PTYR protein pattern was made using the *src neo* and *src CMV5-1* PTP subclones.

Cell lysates prepared in the presence of phosphatase inhibitors were resolved by SDS-PAGE and electroblotted. After probing with an anti-PTYR antibody, one notable difference was observed. In the PTP1 overexpressing subclone, the level of tyrosine phosphorylation was significantly decreased in a polypeptide migrating at ~70 kD (Fig. 7). The identity of this potential substrate of PTP1 is currently being sought.

Subcellular Localization of PTP1

To examine the subcellular distribution of the expressed 51-kD PTP1, as well as endogenous immunoreactive PTPase, cell lysates were prepared from control and PTP1-expressing cells. Lysates were subjected to high-speed centrifugation to separate the cytosolic (S100) and membrane (P100) fractions. More than 90% of the expressed 51-kD PTP1 protein sedimented with the P100 fraction from the *src CMV5-1* subclone (Fig. 8 *A*, lane 1 and Fig. 8 *B*, lane 1). Similar results were obtained using the normal CMV4Z 3T3 subclone (not shown). In addition, low levels of an endogenous 51-kD immunoreactive protein could be detected in the P100 mem-

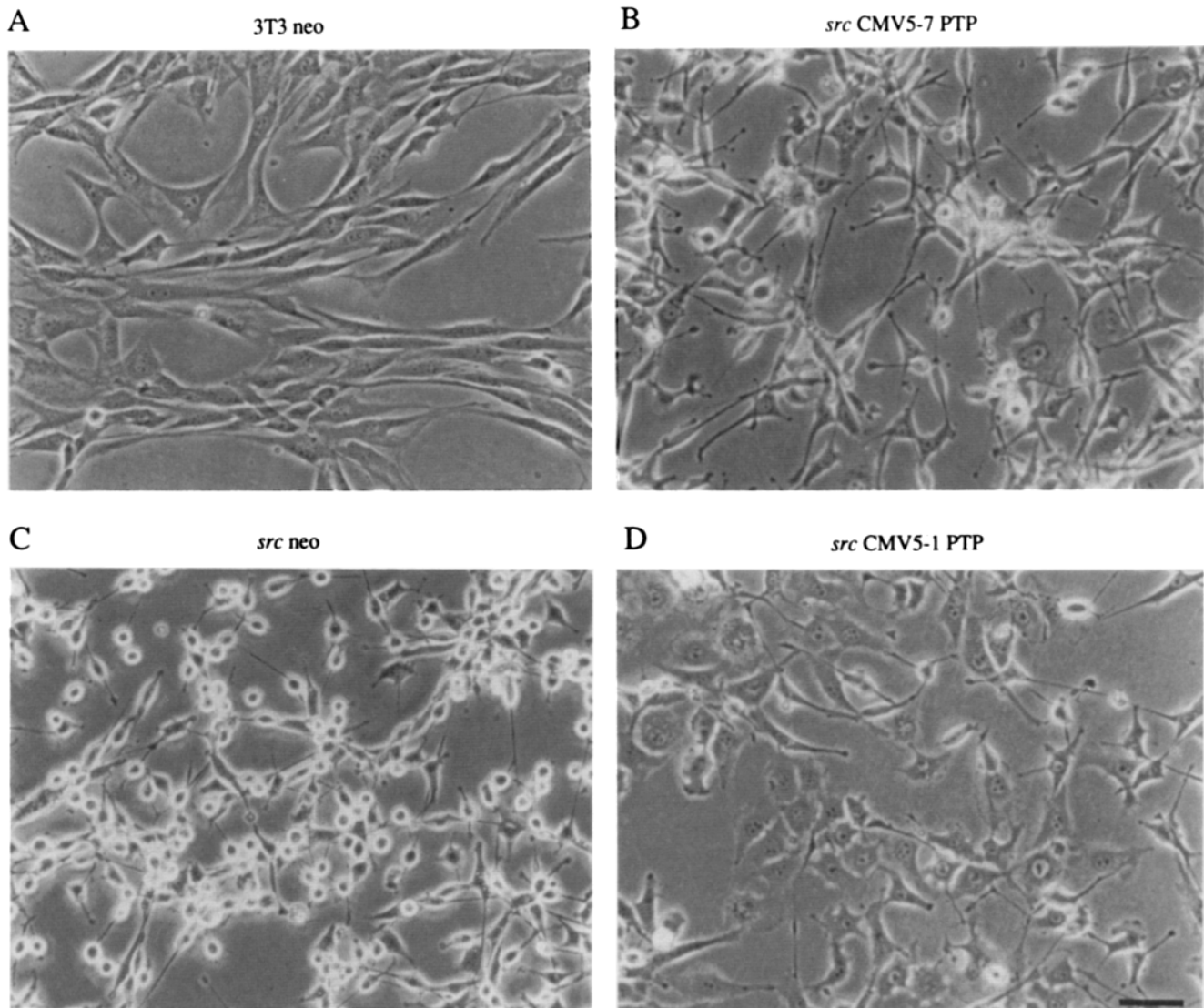


Figure 5. Morphological appearance of transfected 3T3 fibroblast subclones. (A) 3T3 neo control. (B and D) Two different PTP1 overexpressing *v-src*-transformed 3T3 subclones, *src* CMV5-7 PTP1 and I CMV5-7 PTP1. (C) *src* neo control. The photographs were taken under phase-contrast. Bar, $\sim 100 \mu\text{m}$.

brane fraction isolated from the *src* neo control cells upon prolonged autoradiographic exposure of immunoblots after using [^{125}I]-protein A for detection. The 51-kD PTP1 could be effectively released from the P100 fraction by treatment with 0.5% Triton X-100/1% CHAPS (Fig. 8 B, lane 4), but not by salt extraction with 0.6 M KCl. In the case of the *src* CMV5-1 PTP subclone, measurement of PTYR-specific phosphatase activity using ^{32}P -labeled Raytide as the substrate indicated that at least 85% of the enzyme was membrane associated. Incubation of the P100 fraction at 4°C for 2 h in the absence of added protease inhibitors resulted in the proteolytic breakdown of the full-length 51-kD PTP1 first to a 48-kD form and then to a 37-kD form (Fig. 8 B, lane 2). This latter molecular weight form comigrated with bacterially expressed 37-kD PTP U323, which represents the mutated PTP1 truncated at its COOH-terminal region. The degradation of PTP1 by a “membrane-associated” protease that resulted in the formation of the 48- and 37-kD protein fragments could be mimicked by limited trypsin cleavage of membrane-associated PTP1. Limited trypsin digestion of the

P100 fraction over a time course of 10 min at 30°C resulted in the generation of similar forms of the phosphatase, of which the lower molecular weight forms (<51 kD) were primarily released into the cytosol (Fig. 9).

The cleavage products of PTP1 resulting from endogenous membrane-associated proteolysis or from trypsin digestion were also examined using thermal phase partitioning. When either whole cells or the P100 membrane pellet were extracted with Triton X-114 and the phases partitioned at 30°C, >90% of the expressed 51-kD PTP1 was recovered in the detergent phase (Fig. 10). When the P100 pellet was allowed to undergo proteolytic breakdown at 4°C or subjected to limited trypsin proteolysis, the lower molecular weight forms of PTP1 that were generated partitioned almost exclusively with the aqueous phase.

PTP1 Localization Pattern in PTP1-overexpressing 3T3 Fibroblasts

Staining of PTP1-transfected 3T3 fibroblasts with affinity-

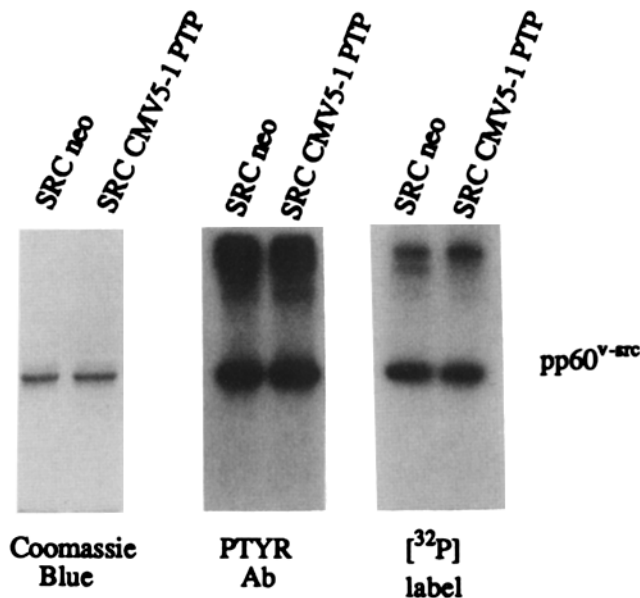


Figure 6. Analysis of the tyrosine phosphorylation state of pp60^{v-src} in the *src neo* and *src CMV5-1 PTP* subclones. (A) Coomassie blue staining of pp60^{v-src} immunoprecipitates prepared using mAb 327. (B) Autoradiograph showing the level of phosphotyrosine in pp60^{v-src} immunoprecipitates after probing with an anti-PTyr antibody. (C) Autoradiograph of pp60^{v-src} immunoprecipitates from ³²P-labeled cells.

purified PTP1 antibody indicated that the tyrosine phosphatase was associated with a reticular network resembling the ER. A similar staining pattern was observed for PTP1 in both normal and transformed fibroblasts (Fig. 11, A–C). This localization on the ER was supported by use of FITC-labeled Con A, which recognizes specific mannose-rich carbohydrate cores in glycoproteins of the RER (Fig. 11 D). In addition, PTP1 staining was similar to the image produced after staining cells with the cationic dye, DiOC₆ [DiOC6(3)] (not shown). Fixation of fibroblasts with 0.25% glutaraldehyde resulted in the best preservation of the ER polygonal network (Fig. 11 B). The staining pattern observed after treatment of fixed permeabilized cells with TRITC-WGA to visualize the Golgi apparatus is shown for comparison to the localization pattern of PTP1 (Fig. 11 E). Lack of fluorescent staining by PTP1 antibody was observed in non-transfected fibroblasts or in PTP1-transfected cells stained with PTP1 antibody that had been previously adsorbed with purified PTP U323 protein (Fig. 11 F).

Additional Evidence for Association of PTP1 with the ER

Cellular membranes from the *src neo* and *src CMV5-1 PTP* subclones were further fractionated into plasma membrane, ER, and mitochondria-enriched components. Both the endogenous immunoreactive mouse PTPase from the *src neo* subclone and the expressed 51-kD rat PTP1 from the PTP1-transfected cells partitioned primarily with the ER-enriched fraction as determined by immunoblot analysis (Fig. 12). A lower level of signal was detected in the mitochondria-enriched fraction, which, by EM examination, was ~30% contaminated with ER membrane. These results support the

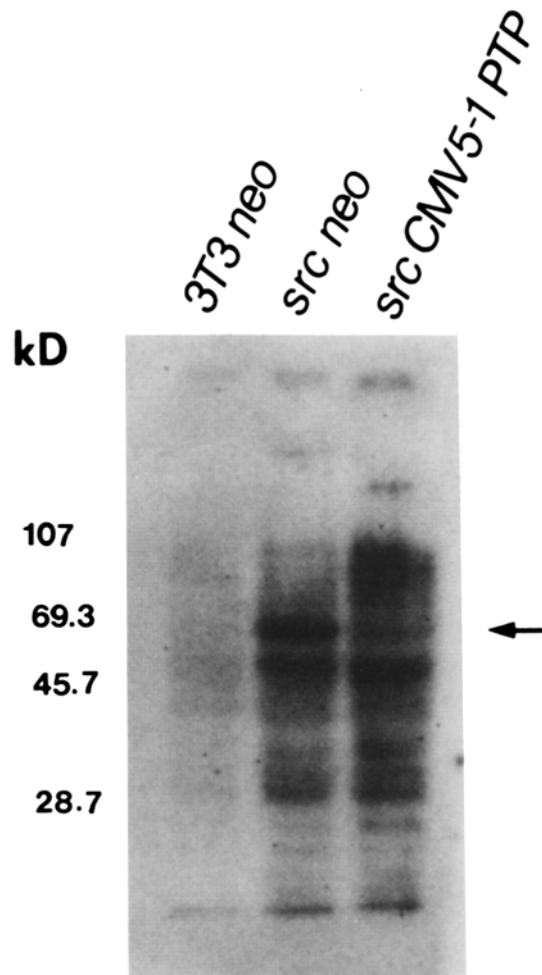


Figure 7. Immunoblot analysis of phosphotyrosine-containing proteins in lysates from the *src neo* control and *src CMV5-1 PTP* subclones. Cellular proteins were resolved by 10% SDS-PAGE. After electrotransfer, the blot was probed with an anti-PTYR antibody, then [¹²⁵I]-sheep anti-mouse IgG, and autoradiography performed. Molecular mass markers are indicated.

immunofluorescent localization of PTP1 using permeabilized, fixed PTP1-transfected 3T3 fibroblasts, which indicated that the tyrosine phosphatase was associated primarily with the ER.

Discussion

The constitutive overexpression of rat PTP1, which shows an absolute specificity for the dephosphorylation of PTYR-containing substrates, has been demonstrated in both normal and *v-src*-transformed 3T3 fibroblasts. The levels of rat PTP1 mRNA and protein expression achieved in transformed cells was on average 10-fold higher than in normal cells. This observation suggested that overexpression of a highly active tyrosine kinase, pp60^{v-src}, may have allowed for increased tolerance for rat PTP1 overexpression at levels that might otherwise be cytotoxic to the cells. During the course of establishing and maintaining stable PTP1-expressing subclones, the cytotoxic effects of PTP1 overexpression in certain cell subclones became obvious. This emphasized the fact that un-

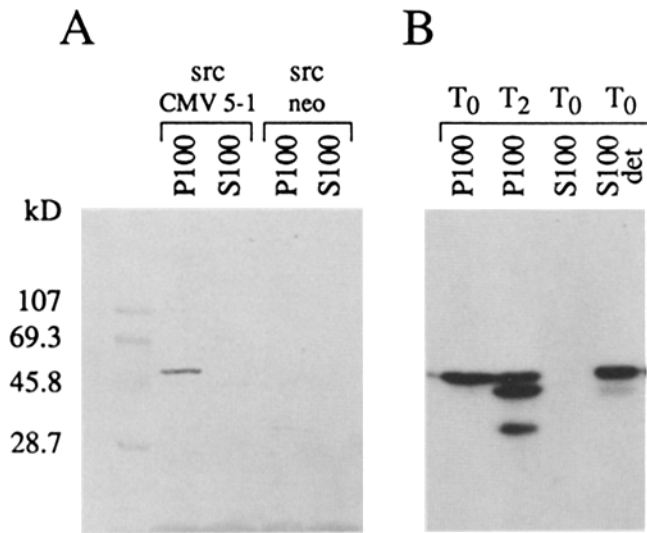


Figure 8. Membrane localization of the 51-kD rat PTP1. (A) Immunoblot analysis of the particulate (P100) and cytosolic (S100) fractions from the *src* CMV5-1 PTP1 and *src* neo subclones. Aliquots of each fraction (20 μ g protein/lane) were resolved by 10% SDS-PAGE, electrotransferred to nitrocellulose, and probed with PTP1 antibody, followed by alkaline phosphatase-conjugated goat anti-rabbit IgG. (B) Immunoblot analysis of the P100 and S100 fractions from *src* CMV5-1 PTP using PTP1 antibody, followed by [125 I]-protein A. Each lane represents the loading of 10 μ g protein: lane 1, 51 kD PTP1 associated with the P100 fraction; lane 2, endogenous proteolysis of the 51-kD PTP1 after 2 h at 4°C; lane 3, S100 fraction; lane 4, S100 fraction following pretreatment of the P100 fraction with 0.5% Triton X-100/1% CHAPS.

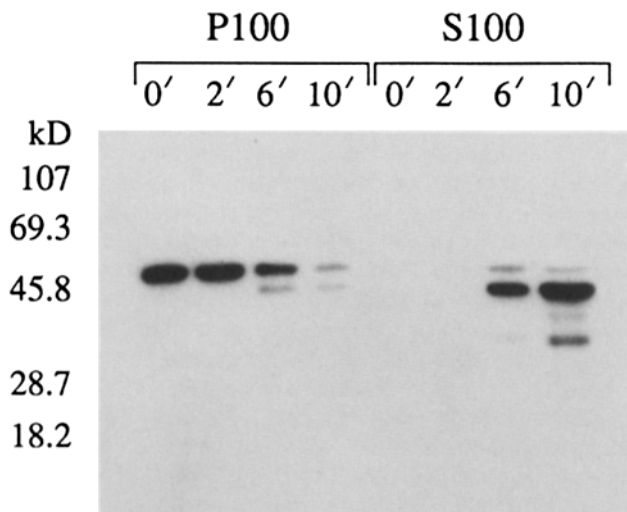


Figure 9. Limited trypsin treatment of the P100 fraction isolated from *src* CMV5-1. Membranes derived from 2×10^6 cells were resuspended in 200 μ l of digestion buffer and treated with trypsin (0.5 μ g/ml) for 0, 2, 6, and 10 min at 30°C. Proteolytic reactions were terminated addition of excess soybean trypsin inhibition and extracts recentrifuged at 100,000 g to yield the S100 fragment. Immunoblot analysis was performed as described. Protein molecular weight markers are indicated.

der normal conditions, cellular tyrosine phosphatase activity is necessarily tightly regulated.

The highest level of constitutive PTP1 expression was observed in the *src* CMV5-1 PTP1 subclone in which the Va^{2+} -sensitive phosphatase activity level was found to be 10-fold higher than the endogenous activity in the *src* neo control subclone. Initial examination of the *src* CMV5-1 PTP subclone by immunofluorescence microscopy using affinity-purified PTP1 antibody indicated that >90% of the cells expressed detectable levels of PTP1. This high degree of expression did not appear to be stable in this subclone, however, as serial passage of this subclone resulted in a reestablishment of the phosphatase equilibrium such that only ~30% of the cell population demonstrated detectable immunofluorescent PTP1 staining under the cell growth conditions described. This expression level was then maintained as a characteristic feature of this particular subclone. In contrast, 60–70% of the cells from the *src* CMV5-7 PTP1 subclone consistently demonstrated PTP1 specific immunofluorescent staining. This particular subclone showed a lower level of PTP1 expression and tyrosine phosphatase activity that was threefold higher than the endogenous activity.

Interestingly, the endogenous level of Va^{2+} -sensitive PTPase activity was found to increase 30–50% in the control cells as a consequence of a *v-src* transformation alone. This has also been reported to occur in chicken embryo fibroblasts following RSV transformation (27). In both cases, induction of an endogenous PTPase might have occurred to allow reestablishment of a tyrosine kinase-phosphatase equilibrium. The endogenous PTPase level in the *src* neo control line also increased as cells were carried to a higher passage number (>15 passages). The cell culture conditions were such that by growing in medium containing 10% serum, selection for a highly transformed phenotype was not maintained. At a higher passage number, the *src* neo subclone characteristically had a slower growth rate and appeared less transformed morphologically, i.e., less rounded, less refractile. It is, therefore, of interest to determine if a direct correlation exists between the rise in endogenous PTPase activity and the manifestation of a phenotype which appeared to be less transformed. Whether this increase in PTPase occurred at the level of mRNA or protein expression and/or stabilization, or whether this increase was the result of a gene amplification event is currently being studied.

That constitutive PTP1 overexpression was capable of inducing alterations in cell morphology was best exemplified by the appearance of the *src* CMV5-1 PTP1 subclone. A flattened cell morphology was observed with the cells spreading well on a plastic surface in contrast to the less adherent, more rounded, refractile morphology of the *src* neo control subclone. Despite this difference, cytoskeletal staining of both subclones using FITC-phalloidin demonstrated a similar disorganization of actin cables characteristic of the transformed phenotype (not shown). Also, no differences in the serum growth factor requirement of PTP1-expressing and control transformed subclones was observed (not shown). However, the *src* CMV5-1 PTP subclone showed 75% decreased ability to form colonies and proliferate in soft agar. Another characteristic noted in all PTP1-expressing cell populations was a higher percentage of bi- and multinucleate cells.

The 51-kD PTP1 protein in PTP1-expressing 3T3 fibro-

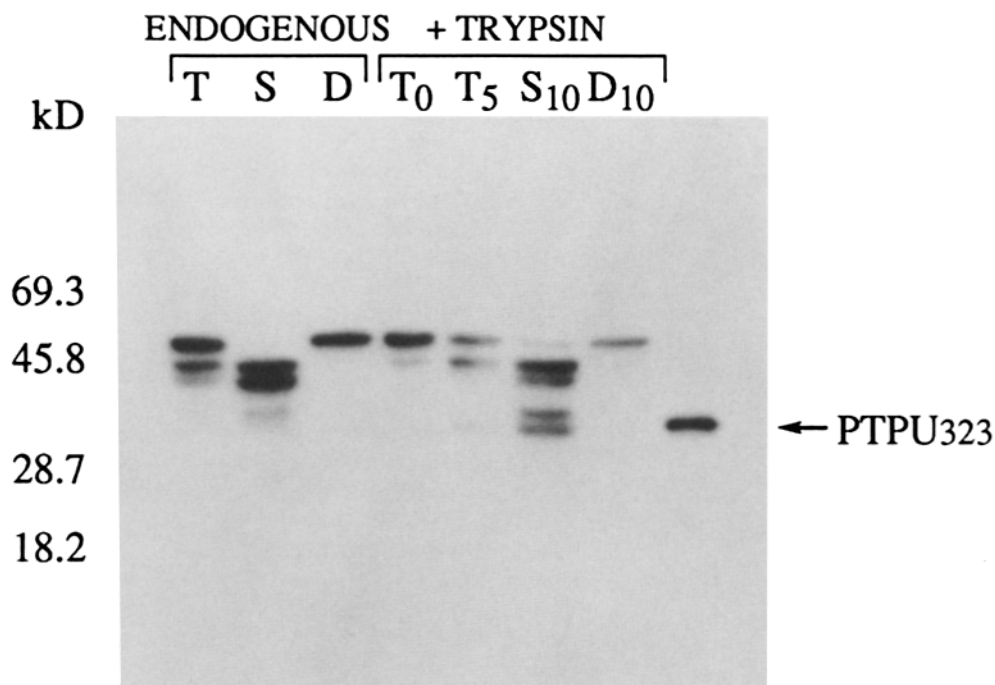


Figure 10. Triton X-114 phase partitioning of the P100 fraction from *src* CMV5-1. Membranes were allowed to undergo proteolysis by endogenous proteases (2 h, 4°C): T, total membranes; S, supernatant (aqueous); D, detergent phase; or with added trypsin (0.5 μg/ml, 10 min, 24°C): T₀, total membrane fraction, T₅, total membrane fraction, 5-min trypsin digestion; S₁₀, supernatant, 10-min trypsin digestion; D₁₀, detergent phase, 10-min trypsin digestion. Immunoblot analysis was performed as described. Protein molecular weight markers are indicated. Bacterially expressed 37-kD PTPU323 is shown as an additional marker.

blasts was found to be almost entirely membrane-associated (>85%) as demonstrated by immunoblot analysis in combination with PTPase activity measurements. Localization of PTP1 to the membrane network forming the ER was shown by immunofluorescent microscopy and was further supported by subcellular fractionation studies in which the 51-kD PTP1 partitioned primarily with the ER-enriched subfraction. In addition, immunolocalization of PTP1 to the ER was verified by EM (not shown). When cells were permeabilized by brief treatment with 0.3% Triton X-100 before formaldehyde fixation, localization of PTP1 to the ER was abolished, most likely because of its solubilization from the membranes. If cells were then stained with FITC-Con A (for ER imaging) or antitubulin antibodies followed by fluorescein-conjugated second antibody, only the microtubular cytoskeleton was observed.

It was of concern that the association of overexpressed rat PTP1 with the ER may not have reflected the natural site for PTP1 localization within the cell, but rather was an abnormal reflection of the level of overexpression itself. This has been reported to occur during the expression of T cell receptor components (23), as well as expression to high levels of a chimeric growth hormone-influenza hemagglutinin protein (32), and mutant LDL receptors (29). These proteins were found to accumulate in an abnormally expanded component of smooth-surfaced membrane that was thought to represent a hypertrophied transitional zone between the ER and the Golgi complex or else confined to the RER itself and irregular extensions of the RER. These localizations most likely reflected the site of blockage in the movement of either artificial or naturally occurring mutant proteins or unused components of membrane receptor complexes. The rat PTP1 shows 97% amino acid sequence identity with the ubiquitous PTP1B and is a naturally occurring mammalian tyrosine phosphatase. It would therefore not likely be recognized as a foreign protein in mouse cells. Two pieces of evidence sup-

port the contention that PTP1 and a similar endogenous immunoreactive mouse PTPase are naturally associated with the ER. First, immunofluorescence microscopic examination of the normal 3T3 CMV4Z subclone and the transformed *src* CMV5-7 PTP and *src* CMV5-1 PTP subclones gave similar staining patterns. Therefore, the localization of PTP1 was not altered by the level of PTP1 expression. The distribution pattern of PTP1 was also similar in normal and *v-src*-transformed PTP1-expressing subclones even though their cytoskeletal architecture was quite different. Second, a similar subcellular distribution was observed by immunoblot analysis of fractionated membranes from the *src* neo and *src* CMV5-1 PTP1 subclones indicating that rat PTP1 was colocalized to the same ER-enriched membrane subfraction as a similar endogenous, immunoreactive mouse PTPase.

The 51-kD PTP1 could be detergent solubilized from isolated cell membranes, but could not be extracted with salt concentrations up to 600 mM KCl. This biochemical data suggests that the PTPase may be integrated into the membrane. Triton X-114 extraction of isolated membranes followed by thermal partitioning also demonstrated that >90% of the 51-kD PTP1 could be recovered in the detergent pellet, suggesting that it is an integral membrane protein. This result agrees well with results from the standard membrane preparation in that 85–90% of the enzyme activity was associated with the P100 fraction. Natural degradation of the particulate 51-kD PTP1 occurred using washed, isolated cell membranes. It is significant that limited trypsinization of these membranes generated protein fragments of a size similar to that occurring under endogenous conditions. These fragments are believed to be generated by proteolysis at the COOH terminus, because these forms of PTP1 show high phosphatase activity and it is known the deletion of as few as 36 amino acids from the NH₂ terminus renders the enzyme catalytically inactive (K. Guan and J. Dixon, unpublished observations). Examination of the primary sequence

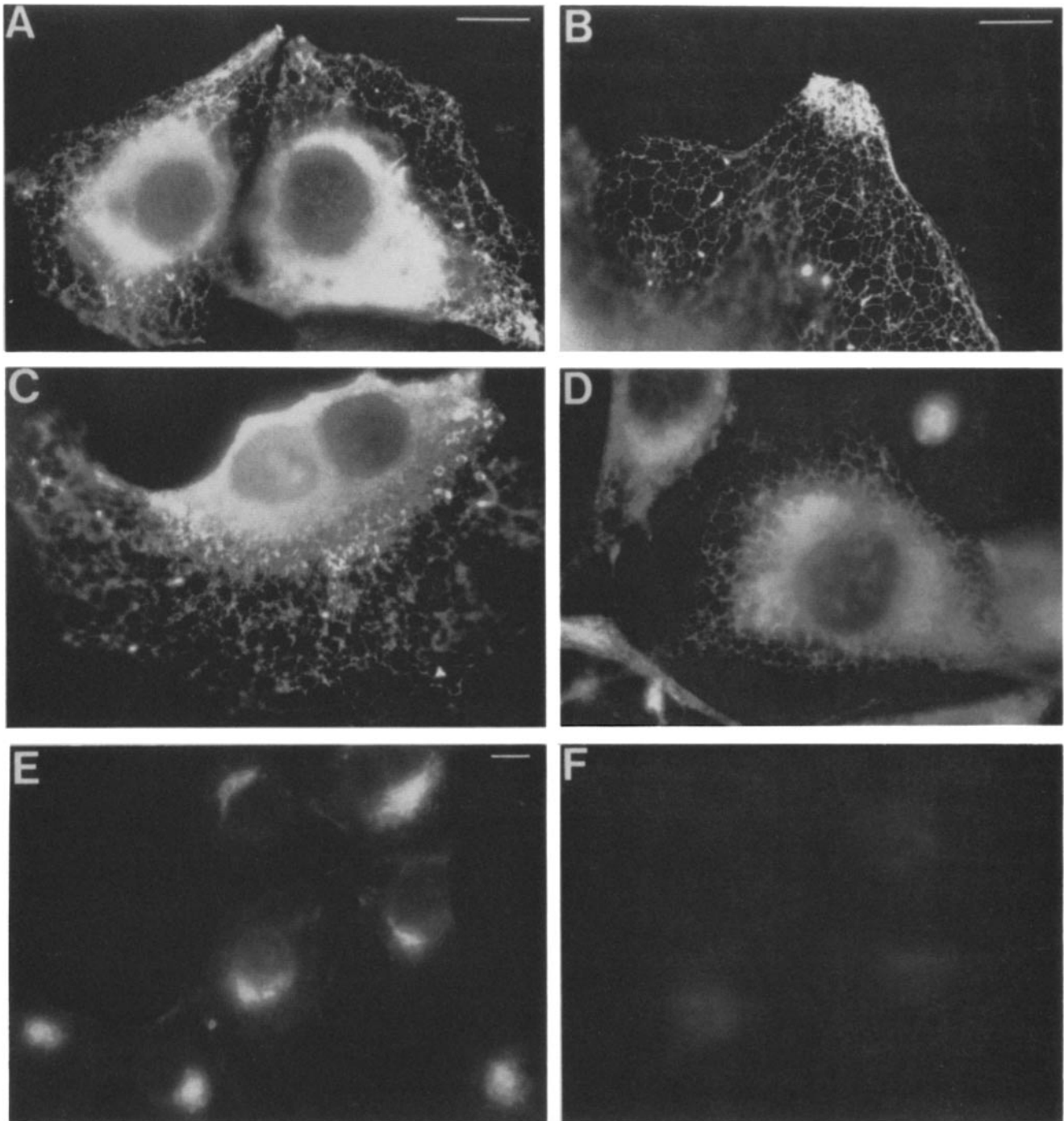


Figure 11. Fluorescent staining of NIH 3T3 fibroblasts for PTP1 localization. Cells were stained for PTP1 as described. (A and B) *src* CMV5-7 PTP subclone, PTP1 antibody. (C) 3T3 CMV4Z PTP subclone, PTP1 antibody. (D) *src* CMV5-7 PTP subclone, FITC-ConA. (E) *src* CMV5-7 PTP1 subclone, FITC-WGA. (F) *src* CMV5-7 PTP subclone stained with PTP1 antibody that was preadsorbed with PTPU323 37-kD protein. Bars: (A, and C-F) 10 μ m; (B) 5 μ m.

reveals a region in which at least one cleavage site is suspected to be located that would generate the 37-kD form. Within this region occurs a proline-rich stretch (residues 101-113) followed by at least four basic residues that may provide the structural basis for accessibility and susceptibility to proteases. The 48- and 37-kD forms of the enzyme did not appear to be intimately associated with the membrane and were released into the S100 fraction with a hypotonic buffer wash. The lower molecular mass 37-kD form comi-

grated with an authentic truncated, recombinant form of PTP1 in which the carboxyl terminal 110 amino acids have been eliminated by introduction of a stop codon at amino acid residue 323 in the PTP1 coding sequence. After thermal partitioning of Triton X-114 membrane extracts, the lower molecular mass 48- and 37-kD forms of PTP1 were also recovered in the aqueous phase and would therefore be biochemically more hydrophilic than the 51-kD PTP1.

The purpose of docking of PTPase activity to the ER net-

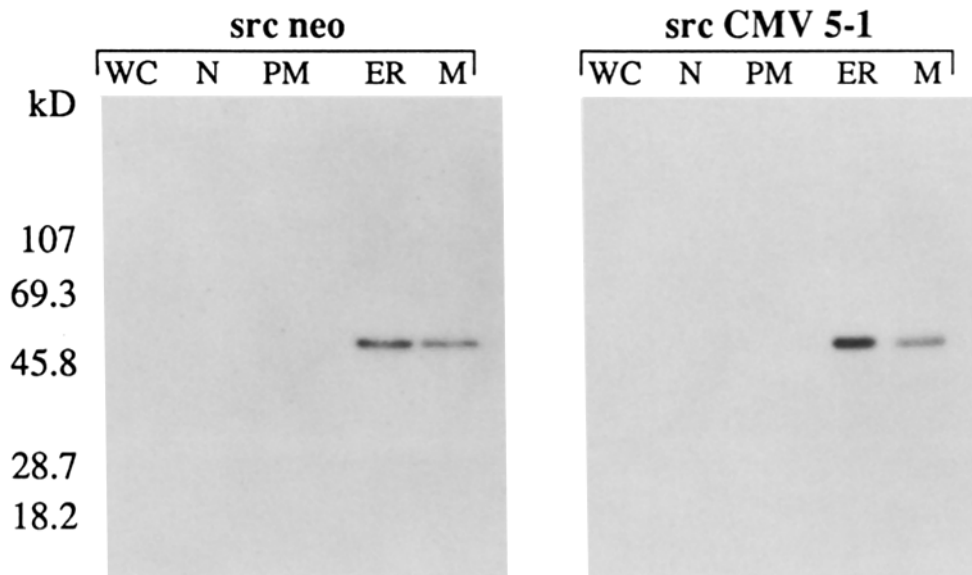


Figure 12. Fractionation of cell extracts prepared from the *src neo* and *src DMV5-7* PTP subclones. Subcellular separation of membrane fractions was done in the presence of protease inhibitors as described in Materials and Methods. 5 mg of protein/lane and 2 μ g of protein/lane were resolved for fractions derived from the *src neo* and *src CMV5-7* PTP subclones, respectively. WC, whole cell lysate; N, nuclear-enriched; ER, ER-enriched; M, mitochondria-enriched. Immunoblot analysis and detection of PTP1 was performed as described.

work is unknown. Association of PTP1 with the ER may limit the substrates available to the PTPase and thus would impose a form of regulation on the active phosphatase. This ER subcellular localization of PTP1 is in contrast to the transmembrane PTPases that are interpreted to be associated with the plasma membrane. These two locations suggest that PTP1 and the "receptor-like" PTPases are likely to recognize a different set of endogenous substrates and perhaps show different substrate specificity *in vivo*. The possibility also exists that proteolytic cleavage of PTP1 from the ER membrane occurs *in vivo* and this may represent an important mechanism for enzyme regulation. Expression of the full-length 51-kD and truncated 37-kD PTP1 proteins in *E. coli* renders PTPases that are both catalytically active towards exogenous [32 P-Tyr]-containing artificial substrates such as Raytide, *src* peptide erythrocyte band 3, angiotensin, or lysozyme peptide. The 37-kD form of PTP1 appears to be resistant to further proteolysis both in *E. coli* or in mammalian cells.

The results described herein with constitutive PTP1 overexpression in NIH 3T3 fibroblasts are in contrast to those reported for the inducible overexpression of the T cell PTP in BHK cells (9). The 48-kD T cell PTP existed as a component of a high molecular mass (>650 kD) complex that sedimented primarily with a 5,000 g pellet fraction. This PTPase was inactive *in vitro* towards exogenous PTYR-containing substrates unless first pretreated with trypsin, which generated a 37-kD form of the enzyme. A 37-kD carboxyl terminal-truncated form of the T cell PTP was also expressed in BHK cells resulting in a redistribution of PTPase activity to both particulate as well as supernatant fractions in which the bound enzyme could be released under less stringent conditions, as well as the generation of an enzyme which was fully active without trypsin treatment. A 50% reduction in the cell growth rate and morphological changes such as cell multinucleation were observed. Although the full-length and truncated forms of the T cell PTP apparently displayed considerable activity differences *in vitro* using reduced and carbomethylated (RCM) lysozyme as a substrate, in intact cells analysis of the PTYR-containing cellular protein pattern showed little difference.

It is thought that PTP1 most likely associates with cellular membranes through its carboxyl terminal tail. The carboxyl tail of PTP1 contains a sequence of amino acids free of charged residues (residues 408–427) that may participate in the association of the enzyme with the ER membrane. In addition, this region of PTP1 contains a sequence of amino acids that could be modified by polyisoprenylation or palmitoylation (15). Studies are currently underway using chimeric PTP1 C-tail constructs to evaluate the targeting function of this region of the molecule. The targeting function for PTP1 appears to be contained within the final 25 amino acids of the COOH terminus (L. Mauro and J. Dixon, manuscript in preparation). Alignment of the sequences of PTP1 with two other mammalian PTPases that contain a single catalytic domain indicates that these enzymes diverge primarily in their COOH-terminal sequence (Fig. 13). The specific subcellular distribution of PTP1B and T cell PTP have not been reported, but if subcellular targeting is directed by the C-tail region it may be speculated that at least PTP1 and the T cell PTP could differ in their cellular localization. The function of the C-tail could therefore be specific to each PTPase isoform examined. These functions could encompass the following: (a) to direct PTPase localization; (b) to direct PTPase translocation upon cell stimulation; (c) to regulate accessibility to cellular substrates; and (d) to modulate enzyme activity. In the latter instance, regulation of enzyme activity could occur through the shielding of the catalytic center. Another means by which PTP1 activity could be modulated is by phosphorylation. The carboxyl-terminal region of the rat PTP1 contains serine residues at positions 335 and 338 and threonine residues at positions 387 and 394 within sequence motifs that form specificity determinants for casein kinase II-catalyzed phosphorylation (15). PTP1 can be phosphorylated *in vitro* on tyrosine(s) by p43^{v-abl} tyrosine kinase, although the phosphorylation site(s) have yet to be identified. Purified PTP1 can be shown to undergo auto- or trans-dephosphorylation *in vitro* (T. A. Woodford-Thomas and J. E. Dixon, unpublished results). The effects of these phosphorylations on enzyme activity are under study.

The ER network is associated with a microtubular network that forms part of the cytoskeleton. Association of PTP1 with

```

RATPTP ( 1) MEM--EKEFEQIDKAGNWAAIYQDIRHEASDFPCRIAKLPKNKRRNRVYRDVSPFDH
HUMPTP ( 1) MEM--EKEFEQIDKSGSWAAIYQDIRHEASDFPCRVAKLPKNKRRNRVYRDVSPFDH
T-C PTP ( 1) MPTTIEREFEEELDTQRRWQPLYLEIRNESHDYVPHRVAKFPENRNRNRVYRDVSPYDH

RATPTP ( 55) SRIKLNQEDNDYINASLIKMEEAQRSYILTQGPLPNTCGHFWEMVWEQN SRGVVML
HUMPTP ( 55) SRIKLNQEDNDYINASLIKMEEAQRSYILTQGPLPNTCGHFWEMVWEQKSRGVVML
T-C PTP ( 57) SRVVKLQNAENDYINASLVDTIEEAQRSYILTQGPLPNTCCHFWMVMWQQKTKAVVML

RATPTP (111) NRIMEKGSCLKCAQYWPQKEEKEMVFD DTLNKLTLISEDVKSYYTVRQLELENLATQ
HUMPTP (111) NRVIMEKGSCLKCAQYWPQKEEKEMIFEDTLNKLTLISEDIKSYYTVRQLELENLTTQ
T-C PTP (113) NRIVMEKESVVKCAQYWP-TDDQEMLFKETGFSVKLLSEDVKSYYTVHLLQLLENINSG

RATPTP (167) EAAREILHFHYTTWPDFGVPESPASFLNFLFKVRESGSLSPHEGPIVVHCSAGIGRS
HUMPTP (167) ETREILHFHYTTWPDFGVPESPASFLNFLFKVRESGSLSPHEGPIVVHCSAGIGRS
T-C PTP (168) ETRTITSHFHYTTWPDFGVPESPASFLNFLFKVRESGSLNPDHGPAVITHCSAGIGRS

RATPTP (223) GTFCLADTCLLLMDKRRKDPSSVDIKKVLLEMRFRMGLIQTADQLRFSYLAVIEGA
HUMPTP (223) GTFCLADTCLLLMDKRRKDPSSVDIKKVLLEMRKFRMGLIQTADQLRFSYLAVIEGA
T-C PTP (224) GTFSLVDTCLVLMKGGD---INIKQVLLNMRKYRMGLIQTADQLRFSYMAIIEGA

RATPTP (279) KFIMGDSSVQDQWKELSHEDLEPPPEHV PPPPRPPKRTLEPHNGKCKELFSNHQWV
HUMPTP (279) KFIMGDSSVQDQWKELSHEDLEPPPEHI PPPPRPPKRILEPHNGKGRFFPNHQWV
T-C PTP (277) KCIKGDSSIQKRWKELSKEDLSPAFD-----SPNKIMTEKYNGNRIGL-----

RATPTP (335) SEESCDEEDILAREESRAPSIAVHSMSSMSQDTEVRKRMVGGGLQSAQAASVPTEE
HUMPTP (335) KEETQEDKDCPIKEEKGSPLNAAPYGIESMSQDTEVRSRVVGGSLRGAQAASAPAKG
T-C PTP (321) -----EEKLTGDRCTGLS-SKMQDT-----MEENSESALRKRIRFD

RATPTP (390) ELSPT-----EEEQAHRPVHVKPFLVNVCMATAATGAYLCYRVC--
HUMPTP (391) EPS-L-----PEKDEDHALS YWKPFLVNMGVATVLTAGAYLCYRFLFNS
T-C PTP (357) RKATMAQKVQMKQRLNENERKRWLYWQPIITKMGFMSVILVGA FVGWTLFEEQQ

RATPTP (432) --H
HUMPTP (434) M-T
T-C PTP (413) MAL

```

Figure 13. Alignment of the amino acid sequences of rat brain PTP1 (15), human human placental PTP1B (6), and the T cell PTP (8). Gaps (hyphens) have been introduced to optimize alignments. The single amino acid code is used. The carboxyl-terminal (C-tail) region is shown (gray).

the ER raises the possibility that PTPase activity could be differentially regulated during the cell cycle or when changes to the cytoskeleton are provoked. During mitosis, when nuclear membrane breakdown occurs, the perinuclear arrangement of the ER and the transcending reticular ER network become disrupted. At this phase of the cell cycle, the ER-associated PTP1 may be redistributed and/or enzymatically modulated. Immunofluorescent microscopic studies using synchronized 3T3 fibroblasts suggest a more diffuse pattern of PTP1 staining during the G2/M phase of the cell cycle (not shown).

Since the subcellular localization of overexpressed rat PTP1 in 3T3 fibroblasts is now known, it should be possible to more thoroughly study the phosphotyrosine protein patterns in transfected cells. Cell transformation induced by pp60^{v-src} is mediated by the tyrosine kinase activity that is intrinsic to this oncoprotein. pp60^{v-src} is located primarily at the cytoplasmic face of the plasma membrane in transformed cells (20, 28, 42). However, a subpopulation of pp60^{v-src} demonstrates perinuclear and cytoskeletal localization. In this study, no detectable differences in the tyrosine phosphorylation state or kinase activity of pp60^{v-src} could be seen in the *src* neo control and one PTP1-overexpressing *src* 3T3 subclone. Therefore, the changes in cellular morphology seen in these transformed subclones cannot be directly accounted for by the changes in the activity level of the *src* oncoprotein.

Many cellular proteins have been found to contain increased levels of PTYR in *v-src*-transformed cells reflecting the interaction of this tyrosine kinase with both cytoplasmic and membrane components of the cell (4, 10, 33, 34). In addition to its own autophosphorylating ability, other potential substrates for pp60^{v-src} include p36 (calpactin I), 50- and 120-kD proteins, vinculin, ezrin, talin, GTPase activating protein (GAP), and the fibronectin receptor. The overexpression of PTP1 in *v-src*-transformed 3T3 fibroblasts in which the protein phosphotyrosine content is significantly elevated has revealed at least one candidate substrate for specific tyrosine dephosphorylation by PTP1. Activation of pp60^{v-src} in 3T3 fibroblasts has been shown to stimulate phosphatidylinositol turnover accompanied by an elevation in cellular diacylglycerol and downregulation of protein kinase C (43). It should prove interesting to study the effects of PTP1 overexpression on this integrated signal transduction network in *v-src*-transformed 3T3 fibroblasts.

We are indebted to D. James Morre, from the Purdue Cancer Center for his advice on subcellular fractionation procedures and expert assistance in EM, as well as Dorothy Werderitsch for her technical skills. The assistance of Elizabeth Taparowsky in helping to establish the parental *v-src*-transformed 3T3 fibroblast line was greatly appreciated. Our gratitude is given to Joan Brugge and Tom Parsons for their provision of antibodies used in this study. In addition, we acknowledge the contributions made by KunLiang Guan throughout the course of this study. We thank Ourania Andrisani and David Pot for their scientific advice.

This work was supported by grant 18849 from the National Institutes of Health (J. E. Dixon) and by the Walther Cancer Institute (Indianapolis, IN).

Received for publication 9 July 1991 and in revised form 14 January 1992.

References

1. Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* 256:1604-1607.
2. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
3. Brown-Shimer, S., K. A. Johnson, J. B. Lawrence, C. Johnson, A. Bruskin, N. R. Green, and D. E. Hill. 1990. Molecular cloning and chromosome mapping of the human gene encoding protein phosphotyrosyl phosphatase 1B. *Proc. Natl. Acad. Sci. USA.* 87:5148-5152.
4. Burr, J. G., G. Dreyfuss, S. Penman, and J. Buchanan. 1980. Association of the *src* gene product of Rous sarcoma virus with cytoskeletal structures of chicken embryo fibroblasts. *Proc. Natl. Acad. Sci. USA.* 77:3484-3488.
5. Charbonneau, H., N. K. Tonks, S. Kumar, C. D. Diltz, M. Harrylock, D. E. Cool, E. G. Krebs, E. H. Fischer, and K. A. Walsh. 1989. Human placenta protein-tyrosine-phosphatase amino acid sequence and relationship to a family of receptor-like proteins. *Proc. Natl. Acad. Sci. USA.* 86:5252-5256.
6. Chernoff, J., A. R. Schievella, C. A. Jost, R. L. Erikson, and B. G. Neel. 1990. Cloning of a cDNA for a major protein-tyrosine-phosphatase. *Proc. Natl. Acad. Sci. USA.* 87:2735-2739.
7. Collett, M. S., and R. L. Erikson. 1978. Protein kinase activity associated with the avian sarcoma virus *src* gene product. *Proc. Natl. Acad. Sci. USA.* 75:2021-2024.
8. Cool, D. E., N. K. Tonks, H. Charbonneau, K. A. Walsh, E. H. Fischer, and E. G. Krebs. 1989. cDNA isolated from a human T-cell library encodes a member of the protein-tyrosine-phosphatase family. *Proc. Natl. Acad. Sci. USA.* 86:5257-5261.
9. Cool, D. E., N. K. Tonks, H. Charbonneau, E. H. Fischer, and E. G. Krebs. 1990. Expression of a human T-cell protein-tyrosine-phosphatase in baby hamster kidney cells. *Proc. Natl. Acad. Sci. USA.* 87:7280-7284.
10. David-Pfeuty, T., and Y. Nouvian-Dooche. 1990. Immunolocalization of the cellular *src* protein in interphase and mitotic NIH *c-src* overexpresser cells. *J. Cell Biol.* 111:3097-3116.
11. Frank, D. A., and A. C. Sartorelli. 1986. Regulation of protein phosphotyrosine content by changes in tyrosine kinase and protein phosphotyrosine phosphatase activities during induced granulocytic and monocytic differentiation of HL-60 leukemia cells. *Biochem. Biophys. Res. Commun.* 140:440-447.
12. Gould, K. L., S. Moreno, N. K. Tonks, and P. Nurse. 1990. Complementa-tion of the mitotic activator, p^{50cdk2} by a human protein-tyrosine phosphatase. *Science (Wash. DC).* 250:1573-1576.
13. Guan, K., and J. E. Dixon. 1991. Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and protein purification procedure of fusion proteins with glutathione S-transferase. *Anal. Biochem.* 192:262-267.
14. Guan, K., and J. E. Dixon. 1991. Protein tyrosine phosphatase catalysis proceeds via a cysteine-phosphate intermediate. *J. Biol. Chem.* 266:17026-17030.
15. Guan, K., R. S. Haun, S. J. Watson, R. L. Geahlen, and J. E. Dixon. 1990. Cloning and expression of a protein-tyrosine-phosphatase. *Proc. Natl. Acad. Sci. USA.* 87:1501-1505.
16. Iivanainen, A. V., C. Lindquist, T. Mustelin, and L. C. Andersson. 1990. Phosphotyrosine phosphatases are involved in reversion of T lymphoplastic proliferation. *Eur. J. Immunol.* 20:2509-2512.
17. Ingebritsen, T. S., S. K. Lewis, V. M. Ingebritsen, B. P. Jena, K. T. Hiryan, S. W. Jones, and R. L. Erikson. 1989. Properties and regulation of protein tyrosine phosphatases. *Adv. Prot. Phosphatases.* 5:121-147.
18. Kato, Y., M. Iwamoto, T. Koike, and F. Suzuki. 1987. Effect of vanadate on cartilage-matrix proteoglycan synthesis rabbit costal chondrocyte cultures. *J. Cell Biol.* 104:311-319.
19. Klarlund, J. 1985. Transformation of cells by an inhibitor of phosphatases acting on phosphotyrosine in proteins. *Cell.* 41:707-717.
20. Krueger, J. G., E. Wang, E. A. Garber, and A. R. Goldberg. 1980. Difference in intracellular location of pp60^{src} in rat and chicken cells trans-formed by Rous sarcoma virus. *Proc. Natl. Acad. Sci. USA.* 77:4142-4146.
21. Krueger, N. X., M. Steuli, and H. Saito. 1990. Structural diversity and evolution of human receptor-like protein tyrosine phosphatases. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:3241-3252.
22. Levinson, A. D., H. Oppermann, L. Levintow, H. E. Varmus, and J. M. Bishop. 1978. Evidence that the transforming gene of avian sarcoma virus encodes a protein kinase associated with a phosphoprotein. *Cell.* 15:561-572.
23. Lippincott-Schwartz, J., J. S. Bonifacino, L. C. Yuan, and R. D. Klausner. 1988. Degradation from the endoplasmic reticulum: disposing of newly synthesized proteins. *Cell.* 54:209-220.
24. Matthews, R. J., E. D. Cahir, and M. L. Thomas. 1990. Identification of an additional member of the protein-tyrosine-phosphatase family: evidence for alternative splicing in the tyrosine phosphatase domain. *Proc. Natl. Acad. Sci. USA.* 87:4444-4448.
25. Montesano, R., M. S. Pepper, D. Belin, J. D. Vassalli, and L. Orci. 1988. Induction of angiogenesis *in vitro* by vanadate, an inhibitor of phosphotyrosine phosphatases. *J. Cell. Physiol.* 134:460-466.
26. Morré, D. J., and D. M. Morré. 1989. Preparation of mammalian plasma membranes by aqueous two-phase partition. *BioTechniques.* 7:946-958.
27. Nelson, R. L., and P. E. Branton. 1984. Identification, purification, and characterization of phosphotyrosine specific protein phosphatases from cultured chicken embryo fibroblasts. *Mol. Cell. Biol.* 4:1003-1012.
28. Nigg, E. A., B. M. Sefton, T. Hunter, G. Walther, and S. J. Singer. 1982. Immunofluorescence localization of the transforming protein of Rous sarcoma virus with antibodies against a synthetic *src* peptide. *Proc. Natl. Acad. Sci. USA.* 79:5322-5326.
29. Pathak, R. K., R. K. Merkle, R. D. Cummings, J. L. Goldstein, M. S. Brown, and R. G. W. Anderson. 1988. Immunocytochemical localization of mutant low density lipoprotein receptors that fail to reach the golgi complex. *J. Cell Biol.* 106:1831-1841.
30. Pingel, J. T., and M. L. Thomas. 1989. Evidence that the leukocyte-common antigen is required for antigen-induced T lymphocyte proliferation. *Cell.* 58:1055-1065.
31. Pot, D. A., T. A. Woodford, E. Remboutsika, R. S. Haun, and J. E. Dixon. 1991. Cloning, bacterial expression, purification and characterization of the cytoplasmic domain of rat LAR, a receptor-like protein tyrosine phosphatase. *J. Biol. Chem.* 266:19688-19696.
32. Rizzolo, L. J., J. Finidori, A. Gonzalez, M. Arpin, I. E. Ivanor, M. Adesnik, and D. D. Sabatini. 1985. Biosynthesis and intracellular sorting of growth hormone-viral envelope glycoprotein hybrids. *J. Cell Biol.* 101:1351-1362.
33. Rohrschneider, L. R. 1979. Immunofluorescence on avian sarcoma virus-transformed cells: localization of the *src* gene product. *Cell.* 16:11-24.
34. Rohrschneider, L. R. 1980. Adhesion plaques of Rous sarcoma virus-transformed cells contain the *src* gene product. *Proc. Natl. Acad. Sci. USA.* 77:3514-3518.
35. Streuli, M., N. X. Krueger, A. Y. Tsai, and H. Saito. 1989. A family of receptor-linked protein tyrosine phosphatases in humans and *Drosophila*. *Proc. Natl. Acad. Sci. USA.* 86:8698-8702.
36. Thomas, M. L., P. J. Reynolds, A. Chaun, Y. Ben-Neriah, and I. S. Trowbridge. 1987. B-cell variant of mouse T200 (Ly-5): evidence for alternative mRNA splicing. *Proc. Natl. Acad. Sci. USA.* 84:5360-5363.
37. Tojo, A., M. Kasuga, A. Urabe, and F. Takaku. 1987. Vanadate can replace interleukin 3 for transient growth of factor-dependent cells. *Exp. Cell Res.* 171:16-23.
38. Tonks, N. K., C. D. Diltz, and E. H. Fischer. 1988. Characterization of the major protein-tyrosine-phosphatase of human placenta. *J. Biol. Chem.* 263:6731-6737.
39. Virtanen, I., P. Ekblom, and P. Laurila. 1980. Subcellular compartmentalization of saccharide moieties in cultured normal and malignant cells. *J. Cell Biol.* 85:429-434.
40. Wice, B., J. Wildbrandt, and L. Glaser. 1987. Control of muscle differentiation in BC₃H1 cells by fibroblast growth factor and vanadate. *J. Biol. Chem.* 262:1810-1817.
41. Wigler, M., R. Sweet, G. K. Sim, B. Wold, A. Pellicer, E. Lacy, T. Maniatis, S. Silverstein, and R. Axel. 1979. Transformation of mammalian cells with genes from prokaryotes and eukaryotes. *Cell.* 16:777-785.
42. Willingham, M. C., G. Jay, and I. Pastan. 1979. Localization of avian sarcoma virus *src* gene product to the plasma membrane of transformed cells by electron microscopic immunocytochemistry. *Cell.* 18:125-134.
43. Wolfman, A., T. G. Wingrove, P. J. Blackshear, and I. G. Macara. 1987. Down-regulation of protein kinase C and of an endogenous 80 kDa substrate in transformed fibroblasts. *J. Biol. Chem.* 262:16546-16552.