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Multiple Requirements for SHPTP2 in Epidermal Growth Factor-Mediated Cell Cycle Progression

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Using transient overexpression and microinjection approaches, we examined SHPTP2's function in growth factor signaling. Overexpression of catalytically inactive SHPTP2 (PTP2CS) but not catalytically inactive SHPTP1, inhibited mitogen-activated protein (MAP) kinase activation and Elk-1 transactivation following epidermal growth factor (EGF) stimulation of 293 cells. An SHPTP2 mutant with both C-terminal tyrosyl phosphorylation sites converted to phenylalanine (PTP2YF) was also without effect; moreover, PTP2YF rescued PTP2CS-induced inhibition of EGF-induced Elk-1 transactivation. PTP2CS did not inhibit transactivation by activated Ras, suggesting that SHPTP2 acts upstream of or parallel to Ras. Neither PTP2CS nor PTP2YF inhibited platelet-derived growth factor (PDGF)-induced Elk-1 transactivation. Thus, protein-tyrosine phosphatase activity, but not tyrosyl phosphorylation of SHPTP2, is required for the immediate-early responses to EGF but not to PDGF. To determine whether SHPTP2 is required later in the cell cycle, we assessed S-phase entry in NIH 3T3 cells microinjected with anti-SHPTP2 antibodies or with a glutathione *S*-transferase (GST) fusion protein encoding both SH2 domains (GST-SH2). Microinjection of anti-SHPTP2 antibodies prior to stimulation inhibited EGF- but not PDGF- or serum-induced S-phase entry. Anti-SHPTP2 antibodies or GST-SH2 fusion protein could inhibit EGF-induced S-phase entry for up to 8 h after EGF addition. Although MAP kinase activation was detected shortly after EGF stimulation, no MAP kinase activation was detected around the restriction point. Therefore, SHPTP2 is absolutely required for immediate-early and late events induced by some, but not all, growth factors, and the immediate-early and late signal transduction pathways regulated by SHPTP2 are distinguishable.

Growth factor receptors possessing intrinsic tyrosine kinase activity (receptor protein tyrosine kinases [RPTKs]) are critical regulatory enzymes for the control of cell growth and differentiation. Binding of a growth factor to its cognate RPTK results in receptor dimerization and "trans-phosphorylation" on multiple tyrosyl residues (8, 22, 73). These phosphotyrosyl residues serve as high-affinity binding sites for the recruitment of secondary signaling molecules containing *src* homology 2 (SH2) domains, most of which also are RPTK substrates (11, 27, 37, 40). Two general classes of SH2-containing proteins have been identified. Phospholipase C-γ1, SH2-containing protein-tyrosine phosphatase 2 (SHPTP2) (Syp, PTP-1D, and PTP2C), and rasGTPase-activating protein (rasGAP) are examples of SH2-containing proteins possessing intrinsic enzymatic activity (11, 27, 37, 40). Adapters, such as Grb2, Nck, Crk, Shc, and the p85 regulatory subunit of phosphatidylinositol 3′-kinase (PI-3K), lack intrinsic enzymatic activity and consist largely of SH2 and SH3 domains (11, 27, 37, 40). Adapters can couple non-SH2-containing catalytic subunits to activated RPTKs. For example, p85 binds to the PI-3K catalytic subunit p110, whereas Grb2 (6, 14, 19, 32, 59) and Nck (24) bind the guanine nucleotide exchange factor Sos. SH2-RPTK interactions can serve to relocate enzymes that are normally cytosolic to the vicinity of their substrates. Such interactions also can increase the enzymatic activity of SH2-containing proteins either directly, as for PI-3K (2, 9) and SHPTP2 (31, 65), or indirectly, by promoting receptor-directed tyrosyl phosphorylation, as is the case for phospholipase C-γ1 (48, 58).

These initial signaling complexes promote the activation of several downstream pathways, culminating in the transcriptional induction of specific genes. One important downstream pathway includes Ras, which is required for the initiation of a serine/threonine kinase cascade composed of Raf, MEK, and mitogen-activated protein kinase (MAPK) (Erk1/Erk2) (12, 38). A key MAPK target is Elk-1, which is a component of the ternary complex factor that binds to the serum response element within the promoter of c-*fos* and other "immediate-early" growth factor-induced genes. MAPK-induced phosphorylation within the Elk-1 C terminus promotes Elk-1-mediated transactivation of these genes, which occurs without a requirement for new protein synthesis (23). Growth factors also are required for later events in cell cycle progression; indeed, growth factors must be present throughout G_1 until the restriction point, which occurs approximately 2 h prior to S phase (50, 61). Much less is known about RPTK signal transduction during these later times.

The role that PTPs play in RPTK signal transduction also is unclear. The identification of SHPTPs suggested that these molecules might participate in RPTK signal transduction. SHPTP1 (also known as PTP-1C, HCP, and SHP), the first of the SHPTPs described (39, 54, 60, 80), is expressed predominantly in hematopoietic cells. SHPTP1 appears to function primarily as a negative regulator of multiple hematopoietic cytokine and growth factor signaling pathways, as vividly illustrated by the motheaten mouse phenotype, which results from mutations in the SHPTP1 gene (62, 71). The second mammalian SHPTP, SHPTP2 (17), also referred to as Syp (15), PTP-1D (76), PTP-2C (82), and SH-PTP3 (1), is ubiquitously

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expressed. Like SHPTP1, SHPTP2 contains two SH2 domains, a functional PTP domain, and a C-terminal hydrophilic domain containing tyrosyl phosphorylation sites (4). SHPTP2 has strong sequence similarity to the *Drosophila* gene *corkscrew* (*csw*) (52) and is its likely mammalian homolog (51a). *Xenopus* homologs of SHPTP1 and SHPTP2 have also been identified (68).

Several lines of evidence indicate that mammalian SHPTP2 and its *Xenopus* and *Drosophila* counterparts have a positive (i.e., signal-promoting) role in RPTK signaling. Genetic epistasis (52) and embryo microinjection (34) experiments strongly suggest that Csw is required to transduce signals downstream of *Torso*. SHPTP2 also is required for vertebrate development. Overexpression of catalytically inactive SHPTP2 prevents fibroblast growth factor-induced mesoderm induction, MAPK activation, and normal gastrulation in *Xenopus* embryos. These inhibitory effects are rescued by coexpression of *Xenopus* SHPTP2 with human SHPTP2 but not by *Xenopus* or human SHPTP1 (68). In mammalian systems, SHPTP2 participates in several signal transduction pathways, although its precise function(s) in these pathways is unknown. SHPTP2 has been implicated as a positive mediator of some, but not all, actions of insulin signaling (21, 41, 49, 78, 79). Upon insulin stimulation, SHPTP2 associates with insulin receptor substrate 1 via its SH2 domains (28). In vitro, this interaction is sufficient for PTP domain activation (65) and is likely to be a mechanism for PTP activation in vivo. Overexpression of catalytically inactive SHPTP2 inhibits insulin-induced GTP loading of Ras (49), MAPK activation (41, 49, 79), and transcriptional activation of a c-*fos* promoter element (79). These data suggest that SHPTP2 enzymatic activity is required for activation of the Ras-Raf-MEK-MAPK cascade in response to insulin.

Biochemical studies implicate SHPTP2 in other RPTK signaling pathways. SHPTP2 associates, via its N-terminal SH2 domain, with the activated platelet-derived growth factor receptor β (PDGFR) and epidermal growth factor (EGF) receptor (15, 26, 30, 31, 76). Following stimulation of cells by either EGF or PDGF, SHPTP2 becomes rapidly tyrosine phosphorylated (4, 15, 26, 30, 76). In the case of PDGF stimulation, tyrosyl phosphorylation of SHPTP2, which occurs within its C terminus (4), creates a binding site for the Grb2-Sos complex. This finding led to the suggestion that SHPTP2 could activate Ras via an adapter mechanism (4, 33). Indeed, a PDGFR mutant lacking most tyrosyl phosphorylation sites but retaining its SHPTP2 binding site is able to activate Ras in response to PDGF (75). Thus, two potential signaling mechanisms might exist for SHPTP2 downstream of the PDGFR and the EGF receptor. One mechanism could involve recruitment of Grb2-Sos to tyrosyl-phosphorylated SHPTP2. The other, by analogy to insulin signal transduction, could involve the dephosphorylation of a substrate(s) required for Ras activation. Although these mechanisms need not be mutually exclusive, only the latter mechanism could operate in insulin signaling, since insulin stimulation does not induce tyrosyl phosphorylation of SHPTP2. However, no studies have established a functional requirement for tyrosyl phosphorylation of SHPTP2. Likewise, it has not been clear whether SHPTP2 is required only for immediate-early events in growth factor signal transduction or also later in the cell cycle.

In this study we have addressed these issues. Using a transient transfection approach with 293 cells, we have found that, as for insulin signaling, SHPTP2 is required for MAPK activation in response to EGF. An intact PTP domain, but not the C-terminal tyrosyl phosphorylation sites, of SHPTP2 is necessary for these functions. In addition to these immediate-early functions, timed microinjection of either anti-SHPTP2 antibodies or a glutathione *S*-transferase (GST) fusion protein encoding both SH2 domains of SHPTP2 into NIH 3T3 cells inhibited exit from G_1 into S phase in response to EGF. SHPTP2 was found to be required late within G_1, around the restriction point for EGF-mediated S-phase entry, and may be required throughout G_1. This later requirement for SHPTP2 occurs during a period when MAPK activation is not observed, suggesting that MAPK is not a target for SHPTP2 late in G_1. Finally, although SHPTP2 is absolutely required for cell cycle progression at various times following EGF stimulation, we have found no evidence that it is required for either immediate-early events or S-phase entry in response to saturating amounts of PDGF or serum. Our studies suggest that SHPTP2 is a highly specific modulator of RPTK signal transduction.

MATERIALS AND METHODS

Materials. Elkc-GAL4, encoding amino acids 307 to 428 of Elk-1 and the DNA binding domain of GAL4 (36), and GAL4-luciferase (36) were kindly provided by R. Treisman (Imperial Cancer Research Fund, London, England). pMT3Ras 61 (L), expressing a codon 61 mutation of c-*ras*^{H}, was a gift from L. Feig (Tufts University Medical School, Boston, Mass.), and pLN42K, expressing an activated version of c-*raf*, was a gift from T. Roberts (Dana Farber Cancer Institute, Boston, Mass.). Amino-terminal myc-tagged Erk1 (pJ3MErk1) (51) was provided by J. Chernoff (Fox Chase Cancer Center, Philadelphia, Pa.), and the 9E10 monoclonal anti-myc antibody was the generous gift of F. McKeon (Harvard Medical School, Boston, Mass.). Wild-type human PDGFR subcloned into pLNSX (pLNSPR) was provided by J. Cooper (Fred Hutchinson Cancer Center, Seattle, Wash.) (25). pSV-βGalactosidase was purchased from Promega. 3′-Bromo-5′-deoxyuridine (BrdUrd) and mouse monoclonal anti-BrdUrd (plus nuclease) antibodies were purchased from Amersham. Tetramethyl rhodamine isocyanate (TRITC)-coupled anti-rabbit and fluorescein isothiocyanate (FITC)-coupled anti-mouse antibodies were purchased from Tago Corporation and Jackson Laboratories, respectively. Anti-PTP-1D/SHPTP2 (P17420) antibodies were purchased from Transduction Laboratories for Western blotting (immunoblotting), and 4G10 antiphosphotyrosyl antibodies were a gift from T. Roberts. Activation-specific (phosphotyrosyl-specific) anti-MAPK antibodies (9101S) were purchased from New England Biolabs. Polyclonal anti-MAPK antiserum (10) was the generous gift of John Blenis (Harvard Medical School). Anti-Raf-1 antibodies (SC-227) and anti-Erk2 antibodies (SC-154) were purchased from Santa Cruz Biotechnology Co., Inc.

Cell culture. NIH 3T3 and 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) plus antibiotics (penicillin plus streptomycin). Recombinant mouse EGF and human PDGF BB were purchased from Upstate Biotechnology, Inc.

Construction of SHPTP2 mutants. The cloning of wild-type human SHPTP2 (PTP2WT) and the generation of tyrosine-to-phenylalanine mutations at amino acids 542 and 580 (PTPYF) and an internal deletion within the PTP domain (PTP2ΔP) have been described previously (4, 17, 68). To generate a cysteine-to-serine mutation at amino acid 459 (the essential catalytic cysteine) of SHPTP2, overlapping primer sets (5′-TCACTATAGGGCGAATTGGGTACC-3′ with 5′-TCCAGCACTCGAGTGCACCACGAC-3′ and 5′-GTCGTGGTGCACTCGAGTGCTGGA-3′ with 5′-ACGCCAAGCTCGAAATTAACCCTC-3′) were used for PCR overlap extension, as described previously (68). The resulting PCR products were subcloned individually into pBSKS+ (Stratagene) and excised with *Eco*RI and *Xho*I, and a three-way ligation was performed into the *Eco*RI site of pBSKS+ to yield PTP2CS. The sequence of PTP2CS was confirmed by automated DNA sequencing (Applied Biotechnology). For the experiments described, these mutants were subcloned into the *Eco*RI site of the mammalian expression vector pJ3Ω (44). All SHPTP2 mutants were confirmed for their expression by transient transfection into COS-1 cells, followed by indirect immunofluorescence with anti-SHPTP2 antibodies (3). PTP1WT and the mutant containing a cysteine-to-serine point mutation in the conserved catalytic domain of SHPTP1 (PTP1CS) have been described previously (54).

SHPTP2 antibodies and GST fusion proteins. Rabbit polyclonal anti-SHPTP2 antibodies were generated against a full-length SHPTP2-GST fusion protein (30). Preimmune immunoglobulin G (IgG) and immune IgG were purified by passing antisera over a protein G-Sepharose column. The final IgG concentration was 10 mg/ml. GST and a GST fusion protein encoding the N- and C-terminal SH2 domains (GST-SH2) of SHPTP2 (amino acids 1 to 251) were prepared as described previously (21).

Immunoprecipitation and immunoblotting. Total cell lysates were prepared by using Nonidet P-40 (NP40) lysis buffer as described previously (21). SHPTP2 was immunoprecipitated from NP40 lysates by using anti-SHPTP2 antibodies (50 μg of antibody 075) for 2 h at 4°C. Immune complexes were collected on protein A-Sepharose, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto Immobilon (Millipore). Immunoblots were probed with either monoclonal antiphosphotyrosine antibody 4G10 at

0.5 μg/ml or a 1:1,000 dilution of monoclonal anti-PTP-1D/SHPTP2 antibody. Sheep anti-mouse IgG peroxidase-linked secondary antibodies (Amersham) were used at a 1:5,000 dilution, and blots were developed by using Enhanced Chemiluminescence (Amersham).

For time course experiments assessing total cellular tyrosyl phosphorylation, MAPK tyrosyl phosphorylation, and Raf-1 mobility shifts, quiescent NIH 3T3 cells were either left unstimulated or stimulated with EGF (100 ng/ml) and lysed in 2× sample buffer at various times following stimulation. Equal amounts of protein were resolved by SDS–8 or 10% PAGE as indicated, and transferred onto Immobilon (Millipore). Phosphotyrosine-containing proteins were monitored by immunoblotting with antiphosphotyrosine antibody 4G10 as described above. MAPK tyrosyl phosphorylation was assessed using phosphospecific rabbit polyclonal antibodies (1:1,000) that recognize tyrosyl-phosphorylated p44Erk1 and p42Erk2. Levels of MAPK were monitored with an anti-p44Erk1/p42Erk2 rabbit polyclonal antiserum (10) used at a 1:5,000 dilution. Anti-Raf-1 antibodies were used at a 1:1,000 dilution. Phospho-MAPK, MAPK, and Raf-1 immuno blots were processed by using donkey anti-rabbit IgG peroxidase-linked secondary antibodies (Amersham) at a dilution of 1:5,000 and developed as described above.

p44Erk1 and p42Erk2 activity assays. Quiescent NIH 3T3 cells were either left unstimulated or stimulated with EGF (100 ng/ml) for various times as indicated. Cells were washed twice with ice-cold phosphate-buffered saline (PBS). Lysates from each time point were aliquoted into two equal fractions. The first fraction was subjected to immunoprecipitation with anti-p44Erk1 antiserum (5 μl). This antiserum recognizes only p44Erk1 under nondenaturing conditions; under denaturing conditions, both p44Erk1 and p42Erk2 are recognized by this antiserum (10; data not shown). The second fraction was subjected to immunoprecipitation with anti-p42Erk2 antibodies (0.5 μg). Following incubation overnight at 4°C, anti-p44Erk1 and anti-p42Erk2 immune complexes were collected on protein A-Sepharose and washed four times in 1% NP-40 buffer plus 1 mM sodium orthovanadate and then once in myelin basic protein (MBP) kinase buffer (20 mM HEPES [*N*-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.4], 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetracetic acid], 1 mM dithiothreitol, and 10 mM $MgCl_2$) without MBP. Washed immune complexes were incubated in MBP kinase buffer plus MBP (0.25 mg/ml) for 5 min at 30°C, prior to the initiation of the kinase reaction with 50 μM ATP containing 5 μCi of [γ-^{32}P]ATP for a further 15 min at 30°C. Immune-complex kinase reactions were stopped by the addition of 0.5 M EDTA, and equal aliquots of supernatant were spotted onto P81 phosphocellulose paper. These filters were washed extensively in 0.5% phosphoric acid, dried, and Cerenkov counted.

In vitro GST-SH2/anti-SHPTP2 antibody binding assay. GST (10 μg) or GST-SH2 (10 μg) bound to glutathione agarose beads was preincubated with 500 μg of preimmune or immune anti-SHPTP2 IgG (986) for 2 h at 4°C. Following antibody incubation, GST and GST-SH2 beads were washed extensively to remove unbound antibody. The GST- and GST-SH2/anti-SHPTP2 antibody complexes were incubated for a further 2 h at 4°C with equal amounts of cell lysates (equivalent to 2×10^6 cells) prepared from quiescent NIH 3T3 cells that either had been left unstimulated or had been stimulated for 5 min with PDGF (50 ng/ml). Bound protein complexes were collected, washed four times in 1% NP-40 lysis buffer containing 1 mM sodium orthovanadate, resolved by SDS–8% PAGE, and transferred to Immobilon for immunoblotting with 4G10 antiphosphotyrosine antibody.

Microinjection and mitogenesis assays. NIH 3T3 cells were seeded onto glass coverslips, grown to approximately 80% confluence, and rendered quiescent by serum deprivation in DMEM plus 0.1% FCS for 24 to 36 h. Cytoplasmic microinjection was performed as described previously (21), by using an Eppendorf injector (model 5242) and a Narishige hydraulic manual micromanipulator. Immune or matched preimmune antibodies were injected at a concentration of 10 mg/ml 1 to 2 h prior to stimulation of cells with either 10% FCS, EGF (100 ng/ml), or PDGF (50 ng/ml) in the presence of BrdUrd (1:1,000 dilution) for 18 h. Alternatively, antibodies or GST-SH2 (0.5 mg/ml), prepared as previously described (21), were microinjected into NIH 3T3 cells at the indicated times following growth factor treatment in the presence of BrdUrd and incubated for a total of 18 h. For detection of BrdUrd-labeled and microinjected cells, coverslips were washed three times with PBS, fixed with 95% methanol for 10 min, and rehydrated with PBS twice for 5 min. Cells were permeabilized in 0.1% NP-40 in PBS for 4 min and incubated with primary monoclonal antibody to BrdUrd (plus nuclease) for 1 h at room temperature. Following incubation with primary antibody, cells were washed three times for 1 min in 0.1% NP-40 in PBS, and secondary antibodies were applied for 30 min at room temperature. Antibody-injected cells were identified by staining for the presence of rabbit IgG using a TRITC-coupled anti-rabbit secondary antibody, and GST-SH2-injected cells were detected by coinjection with FITC-dextran (1 mg/ml). For antibody injections BrdUrd-labeled cells were detected by using a FITC-coupled anti-mouse secondary antibody, and for GST-SH2 injections BrdUrd-labeled cells were detected by using a TRITC-coupled anti-mouse secondary antibody. The coverslips were washed three times in 0.1% NP-40 in PBS, incubated for 2 min with DAPI (4′,6-diamidino-2-phenylindole) (0.1 μg/ml) to stain nuclei, washed three times with PBS, and mounted in Mowiol. Cells were viewed with an Olympus fluorescence microscope, and BrdUrd-positive and microinjected cells were quantitated. Results are expressed as the percentage of cells (either injected or uninjected) entering S phase, as indicated by BrdUrd staining. All results were analyzed for statistical significance by a one-tailed Student's *t* test.

Transfection assays. (i) Elk-GAL4 luciferase assays. 293 cells were seeded at 2.5×10^5/60-mm plate 24 h prior to transfection. Three to four hours before transfection, fresh DMEM plus 10% FCS was added to the cells. 293 cells were transfected by the calcium phosphate precipitation technique using 0.5 μg of GAL4-luciferase, 1 μg of Elk-GAL4, 1 μg of pSV-βGal, and various concentrations of pJ3SHPTP2, pLCSHPTP1, pMT3Ras61(L), pLN42K, and/or pLNSPR, to a total of 8.5 μg of DNA. After 12 to 16 h, DNA precipitates were removed, and the cells were washed twice in Hanks balanced salt solution and allowed to recover for 2 to 3 h in 10% FCS plus DMEM. Transfected cells were rendered quiescent by being washed twice with Hanks balanced salt solution and replacing the medium with DMEM plus 0.1% FCS for 24 h. The cells were either left unstimulated or stimulated with either EGF (100 ng/ml), PDGF (50 ng/ml), or 10% serum for 5 h. For luciferase and β-galactosidase activity assays, cells were washed twice in PBS and lysed in 0.3 ml of 1% Triton X-100–15 mM $MgSO_4$–4 mM EGTA–5 mM dithiothreitol. After centrifugation in an Eppendorf Microfuge at 10,000 rpm for 5 min at 4°C, the resulting supernatants were used for luciferase and β-galactosidase activity assays. Luciferase activity was determined by incubating cell lysates (30 to 60 μl) with 0.3 ml of luciferase reaction buffer (25 mM Gly-Gly, 15 mM $MgSO_4$, 4 mM EGTA, 15 mM KH_2PO_4, 5 mM dithiothreitol, 2 mM ATP, and 0.2% Triton X-100), and luciferase activity was measured by automated injection of 0.1 ml of luciferin (1 mM) using a Monolight 2010 Luminometer. β-Galactosidase activity was determined by incubating 0.15 ml of cell lysate with 0.15 ml of β-galactosidase reaction buffer (120 mM Na_2HPO_4, 80 mM NaH_2PO_4, 2 mM $MgCl_2$, 100 mM β-mercaptoethanol, and 2 mg of *o*-nitrophenyl-β-galactosidase) at 37°C until a faint yellow color appeared. Reactions were stopped by the addition of 1.0 M sodium carbonate, and spectrophotometric readings were taken at 420 nm. Results are expressed as luciferase activity normalized for transfection efficiency by using β-galactosidase. Unless otherwise stated, results shown are presented as means ± standard errors of the means (SEM) from duplicate samples taken from an experiment representative of at least three such experiments. Data were analyzed for statistical significance by a one-tailed Student's *t* test.

(ii) p44Erk1 kinase assays. 293 cells were seeded at 2.5×10^5/60-mm plate prior to transfection by calcium phosphate precipitation with 5 μg of pJ3SHPTP2 and 0.5 μg of pJ3MErk1. Following transfection, cells were rendered quiescent and either left unstimulated or stimulated with EGF for 5 min. After stimulation, cells were washed with PBS and lysed in 1% NP-40 buffer plus protease and phosphatase inhibitors, as described previously (21). Anti-Myc (9E10) antibodies (1:100 dilution) were added to the lysate overnight at 4°C, and p44Erk1 immune complex kinase assays were performed as described above.

RESULTS

SHPTP2 is required for immediate-early events in EGF signaling. In order to address the role of SHPTP2 in growth factor signaling, we first sought to determine whether SHPTP2 is required for immediate-early events mediated by the Ras-Raf-MEK-MAPK cascade in EGF and PDGF pathways, and, if so, which domain(s) of SHPTP2 mediates the signal. Our initial approach was to transiently overexpress wild-type and mutant versions of SHPTP2 (Fig. 1) into 293 cells. These cells express sufficient levels of the EGF receptor endogenously to permit studies of EGF signaling. Since 293 cells lack detectable PDGFRβ (PDGFR) expression, for experiments in which PDGFR signaling was assessed an expression vector encoding human PDGFRβ (see Materials and Methods) was cotransfected. When phosphorylated in vitro by the PDGFR or when overexpressed in cells stimulated with either PDGF or EGF, SHPTP2 becomes phosphorylated on both Y-542 and Y-580 (3, 4). Both of these tyrosyl phosphorylation sites conform to the consensus binding site for the SH2 domains of Grb2 (63), and Grb2 can bind to both of these sites in vitro and in vivo (3). A mutant in which both Y-542 and Y-580 have been converted to phenylalanine (PTP2YF) should be able to bind via its SH2 domains to appropriate upstream RPTKs but would be incapable of transmitting a signal by binding Grb2. If the Grb2/Sos adapter model were operative, expression of sufficiently high levels of PTP2YF could compete with binding of endogenous wild-type SHPTP2 to the RPTK and might interfere with growth factor-induced signaling; thus, PTP2YF could act as a "dominant-negative" mutant. To assess the potential role of the PTP domain, we transiently overexpressed two distinct

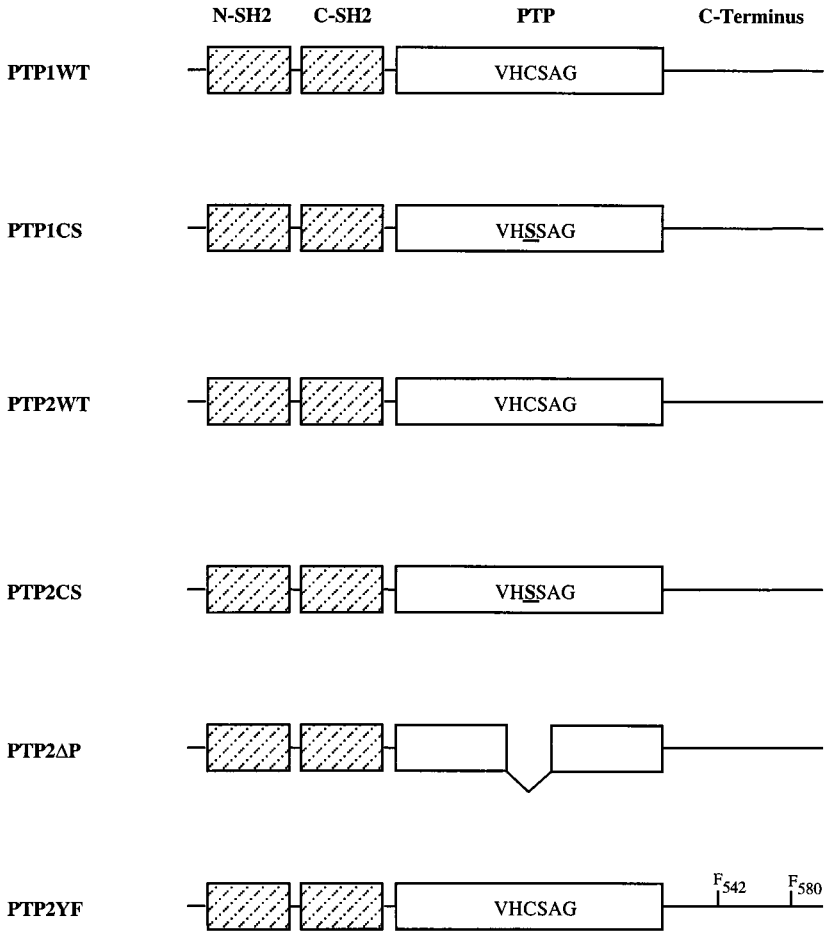

FIG. 1. Schematic representation of wild-type and mutant SHPTP2 and SHPTP1 clones. SHPTP1 (PTP1WT) and SHPTP2 (PTP2WT) have been described previously (17, 54). N-SH2 and C-SH2, N- and C-terminal SH2 domains respectively; PTP1CS and PTP2CS, cysteine-to-serine point mutations at amino acids 453 (SHPTP1) and 459 (SHPTP2) within the conserved catalytic motif (VHCSAG); PTP2ΔP, internal 31-amino-acid deletion encompassing the conserved catalytic motif within the PTP domain of SHPTP2 (68); PTP2YF, tyrosine-to-phenylalanine mutations at amino acids 542 and 580 within the C terminus of SHPTP2 (4, 68).

PTP domain mutants: a 31-amino-acid deletion within the PTP domain (PTP2ΔP) and a cysteine→serine mutation (PTP2CS) of the essential catalytic cysteine (C-459) found in all PTP family members (64). PTP2ΔP and PTP2CS both lack tyrosine phosphatase activity. If PTP activity is required for signaling by a given RPTK, PTP2ΔP or PTP2CS might be expected to compete with endogenous SHPTP2 for binding to its target and be unable to transmit the appropriate signal. Unlike PTP2ΔP, in which a large part of the PTP domain catalytic pocket has been deleted, PTP2CS retains, in principle, the ability to bind substrates. For many PTPs, the rate-limiting step in catalysis is substrate release (20). Accordingly, cysteine →serine mutants of some PTPs (5, 42, 66) have been shown to tightly bind and "trap" substrates. Thus, PTP2ΔP and PTPCS might be expected to have different actions and/or potencies, depending on the details of the particular signaling pathway regulated by SHPTP2.

We used two assays to determine the consequences of overexpressing the SHPTP2 mutants for immediate-early signal transduction. MAPK activity was monitored indirectly by adopting a previously described technique that measures the ability of a cotransfected GAL4 DNA binding domain/Elk-1 C-terminus chimera (Elk-GAL4) to activate a GAL4-luciferase reporter construct (36). This assay reflects the ability of MAPK to phosphorylate Elk-1 (Elk) within its C terminus, which enhances its ability to transactivate (see the introduction). MAPK activation also was assessed directly by cotransfecting a myc epitope-tagged Erk-1 expression construct and measuring the ability of anti-myc immune complexes to phosphorylate MBP.

Wild-type or mutant SHPTP2 constructs were transiently transfected into 293 cells, which then were rendered quiescent by serum deprivation and either left unstimulated or stimulated with EGF. Compared with transfection of the parental expression vector alone, transfection of PTP2WT slightly potentiated Elk-GAL4-directed transactivation in response to EGF (Fig. 2A). This effect, although small (nine- versus sevenfold stimulation), was reproducible and statistically significant ($P < 0.05$) and suggests that SHPTP2 function may be at least partly limiting for EGF signaling in 293 cells. In marked contrast, expression of either PTP2ΔP or PTP2CS significantly inhibited EGF-induced transactivation (Fig. 2A). Although the tyrosyl phosphorylation status of PTP2CS could not be assessed (owing to comigration with endogenous SHPTP2), PTP2ΔP was efficiently tyrosyl phosphorylated upon EGF stimulation (Fig. 3A), indicating that the ability of PTP2ΔP to inhibit EGF-mediated Elk-GAL4 activation was not due to inability to become tyrosyl phosphorylated. Notably, PTP2ΔP reproducibly inhibited EGF-induced transactivation less well than PTP2CS; the latter completely abolished transactivation,

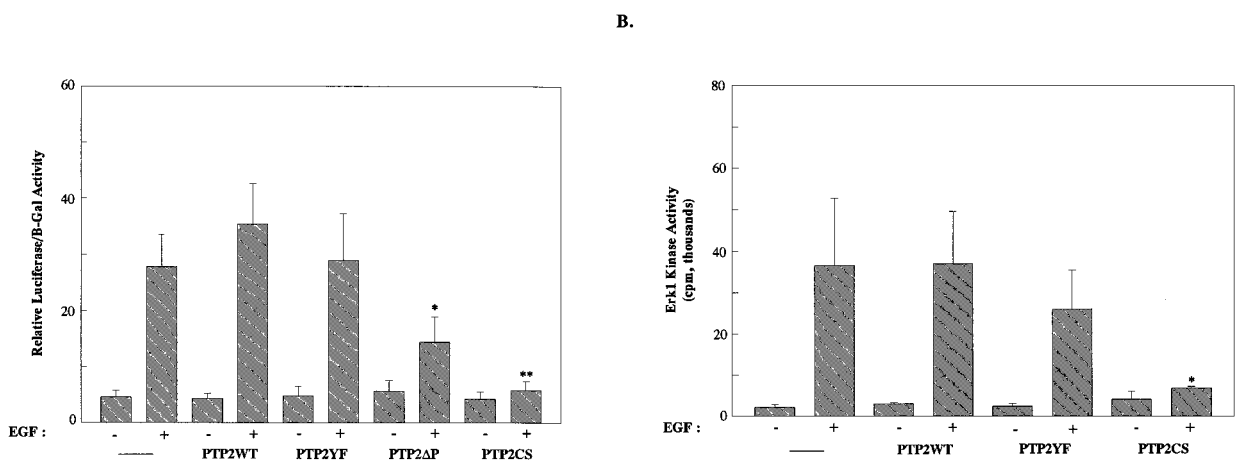

FIG. 2. SHPTP2's phosphatase domain is required for EGF-induced MAPK activation and Elk-1 transactivation. (A) 293 cells were cotransfected with either 4 μg of vector alone (—) or 4 μg of the indicated SHPTP2 mutant plus Elk-GAL4, GAL4-luciferase, and pSVβGal, as described in Materials and Methods. Serum-starved cells were either left unstimulated (−) or were stimulated (+) with EGF for 5 h, and GAL4-luciferase activity was measured. Results are expressed as relative luciferase activity (normalized to β-galactosidase activity) and are representative of the means and SEM of four to six separate experiments using three different preparations of SHPTP2 expression constructs. *, $P < 0.05$; **, $P < 0.001$. (B) 293 cells were cotransfected with 5 μg of vector alone (—) or 5 μg of the indicated SHPTP2 mutant plus 0.5 μg of Myc-Erk1. Cells were serum starved and either left unstimulated (−) or stimulated for 5 min with EGF (+). Myc-Erk1 was immunoprecipitated with monoclonal antibody 9E10, and immune-complex kinase assays were performed with MBP as substrate. Results are representative of the means ± SEM of three separate experiments. *, $P < 0.05$.

whereas the former inhibited it approximately twofold. This difference in inhibitory potency was highly significant ($P < 0.001$) (see Discussion). Consistent with the observation that Elk-1 and MAPK activation correlate in vivo (23), overexpression of PTP2CS also significantly inhibited Erk1 kinase activity ($P < 0.05$) (Fig. 2B). The finding that two distinct catalytically inactive mutants of SHPTP2 function as dominant-negative inhibitors of MAPK-induced transactivation of Elk-1 in response to EGF strongly suggests that SHPTP2 must dephosphorylate some key substrate(s) in the EGF signaling pathway for MAPK induction to occur.

Expression of PTP2YF had no effect on EGF-stimulated transactivation compared with the vector alone control (Fig. 2A). Likewise, overexpression of PTP2YF was without significant effect on EGF-induced MAPK activity (Fig. 2B). EGF-induced transactivation in cells expressing PTP2YF was reproducibly lower than that in cells expressing PTP2WT. These results argue that C-terminal tyrosyl phosphorylation is not essential for SHPTP2 function, but phosphorylation at one or both of these sites may slightly enhance the ability of PTP2WT to potentiate EGF signaling (see Discussion). If this interpretation is correct, then PTP2CS-induced inhibition of Elk-GAL4 activity should be rescued by cotransfection of PTP2YF. To test this hypothesis, PTP2CS and PTP2YF were overexpressed either individually or together at different ratios in 293 cells, and Elk-GAL4 activity was assayed following EGF stimulation. When PTP2CS was coexpressed with an equal ratio of PTP2YF, Elk-GAL4 activity was inhibited to a magnitude similar to that for PTP2CS expressed alone. However, coexpression of PTP2CS and PTP2YF at a 1:4 ratio resulted in EGF-stimulated transactivation at levels comparable to that of vector alone, PTP2WT or PTP2YF (Fig. 3B). In addition to arguing that tyrosyl phosphorylation of SHPTP2 is not essential for its positive signaling role, these data strongly support the notion that the PTP-inactive mutants of SHPTP2 function as bona fide dominant-negative mutants in the EGF signaling pathway.

To further address the specificity of SHPTP2's role in the EGF pathway, we investigated the effects of overexpressing

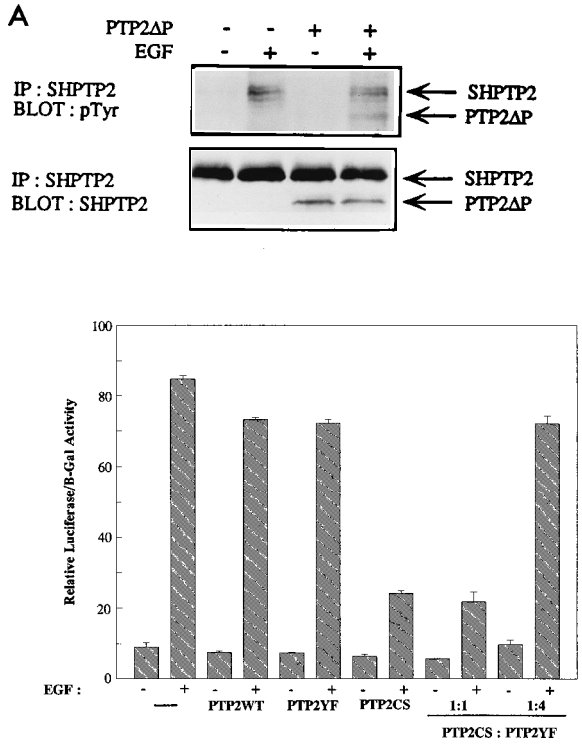

FIG. 3. SHPTP2 C-terminal tyrosyl phosphorylation sites are dispensable for EGF signaling. (A) 293 cells were transfected with either 10 μg of vector alone or 10 μg of PTP2ΔP. Cells were then rendered quiescent by serum deprivation. SHPTP2 was immunoprecipitated (IP) from vector control (−) and PTP2ΔP-transfected (+) cells that were either left unstimulated (−) or stimulated with EGF (+) for 5 min. Immune complexes were resolved by SDS–10% PAGE, transferred to Immobilon and immunoblotted with an antiphosphotyrosine (pTyr) antibody (upper panel). The antiphosphotyrosine immunoblot was reprobed with an anti-SHPTP2/PTP-1D antibody (lower panel). The positions of endogenous SHPTP2 and transfected PTP2ΔP are indicated. (B) 293 cells were transfected with either 1 μg of vector alone (−) or 1 μg of the indicated SHPTP2 mutants. For coexpression studies, PTP2CS and PTP2YF were transfected at a 1:1 or a 1:4 (microgram) ratio, respectively. Serum-starved cells were either left unstimulated (−) or stimulated with EGF (+), and GAL4-luciferase activity was assessed. Results are means and SEM for duplicate samples and are representative of three separate experiments.

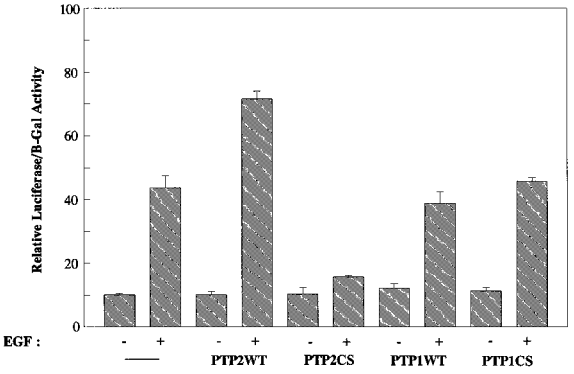

FIG. 4. SHPTP1 does not affect EGF-induced Elk-1 activation. 293 cells transfected with the indicated plasmids were rendered quiescent and then were left unstimulated (−) or were stimulated with EGF (+), and GAL4-luciferase activity was measured. Results are means ± SEM for duplicate samples and are representative of three separate experiments.

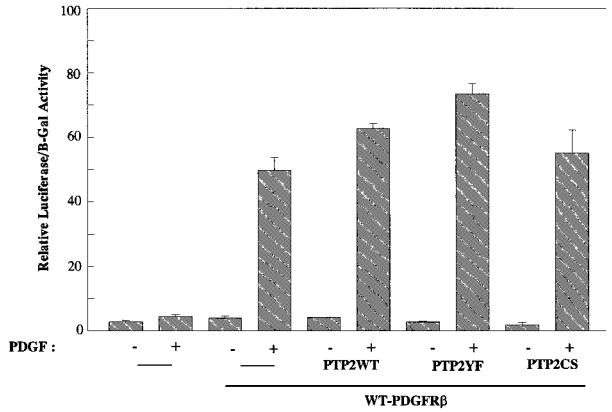

FIG. 6. SHPTP2 is not required for PDGF-induced Elk-1 activation. 293 cells were transfected with or without the PDGFR (2 μg), as indicated, plus 4 μg of the indicated mutant of SHPTP2. Cells were rendered quiescent by serum deprivation and were either left unstimulated (−) or stimulated with PDGF (50 ng/ml) for 5 h (+), and GAL4-luciferase activity was measured. Results are means and SEM for duplicate samples and are representative of four separate experiments.

the closely related SHPTP, SHPTP1. Overexpression of either wild-type (PTP1WT) or catalytically inactive (PTP1CS) SHPTP1 in 293 cells had no significant effect on EGF-induced transactivation when PTP2CS was completely inhibitory (Fig. 4). The lack of effect of PTP1WT or PTP1CS was not due to the failure to express either of these cDNAs in 293 cells, as indicated by immunoblot analysis of transiently transfected cells (3).

PTP2CS does not inhibit activated Ras transactivation of Elk-1. Our data provide evidence that SHPTP2 is an upstream mediator of MAPK activation in response to EGF. To further define the position of SHPTP2 relative to other signaling components of this pathway, PTP2CS was cotransfected into 293 cells with vectors expressing either activated c-*ras* or c-*raf* genes. At a dose of *ras* expression vector capable of promoting an amount of Elk-GAL4 activity equivalent to that of EGF-stimulated, untransfected controls, coexpression of PTP2CS had no effect on transactivation (Fig. 5). Thus, SHPTP2 is not necessary for activated Ras to promote transactivation via Elk. Treatment of the Ras-transfected cells with EGF results in a further increase in transactivation. Whether this increase reflects further GTP loading of activated Ras, activation of endogenous c-Ras in activated Ras-expressing cells, or activation of a parallel pathway is unclear (see Discussion). Nevertheless, cotransfection of PTP2CS blocks the EGF-stimulated potentiation of activated Ras activity (Fig. 5), indicating that SHPTP2 is required for this pathway. In agreement with these findings, PTP2CS also does not inhibit transactivation by cotransfected activated *raf* (3). Since SHPTP2 failed to inhibit either activated Ras or Raf, these data suggest that SHPTP2 lies upstream of, or parallel to, Ras in EGF-mediated signaling; similar conclusions have been reached previously by other workers studying insulin signaling (49, 79). Since both MAPK and subsequent Elk-1-mediated transactivation are dependent on the activation of Ras and Raf, which occurs within minutes following EGF stimulation (7), these results further support the conclusion that SHPTP2 is a required immediate-early component of EGF-mediated signaling.

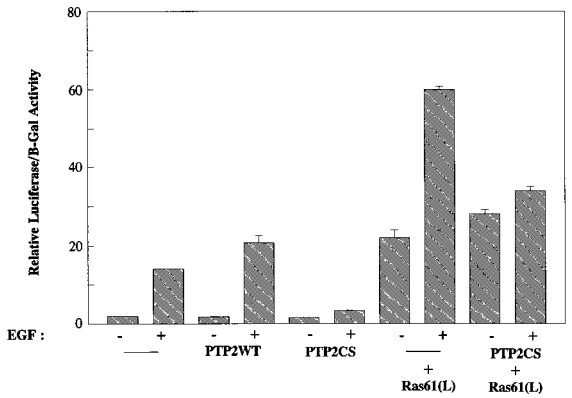

FIG. 5. PTP2CS does not inhibit Ras61(L) activation of Elk-1. Ras61(L) (5 ng) or Ras61(L) plus PTP2CS (4 μg) was cotransfected into 293 cells. Quiescent cells were either left unstimulated (−) or stimulated with EGF (+) for 5 h, and GAL4-luciferase activity was measured. Results are means and SEM for duplicate samples and are representative of four separate experiments.

SHPTP2 is not required for Elk-1 activation in response to PDGF. We used the same SHPTP2 mutants to ask whether SHPTP2 plays a similar role in PDGF-induced MAPK activation. For these experiments, 293 cells were cotransfected with the expression vectors for the PDGFR, and either PTP2WT-, PTP2YF-, or PTP2CS- and Elk-GAL4-mediated transactivation in response to PDGF was assessed. Under these conditions, neither PTP2CS nor PTP2YF inhibited PDGF-induced activation of Elk-GAL4 (Fig. 6). We also investigated whether overexpression of PTP2YF or PTP2ΔP could affect downstream signals from the PDGFR in a cell line that expresses endogenous PDGFR. When overexpressed in COS-1 cells, which contain endogenous PDGFR, neither PTP2YF nor PTP2ΔP inhibited MAPK activation in response to PDGF; under these conditions PTP2ΔP also was tyrosyl phosphorylated in response to PDGF (3). These data strongly suggest that neither the PTP domain nor the C-terminal tyrosyl phosphorylation sites of SHPTP2 are absolutely required to propagate the immediate-early signals required for MAPK activation in response to saturating concentrations of PDGF. Furthermore, they suggest that, if the Grb2/adapter model (see the introduction) is physiologically important, it must function in a pathway other than MAPK activation (at least in 293 and COS-1 cells). Similar results were also obtained for serum-induced Elk-1 transactivation. When 293 cells were transiently transfected with PTP2CS and serum stimulated, Elk-GAL4-mediated transactivation was not affected (3). Previous work

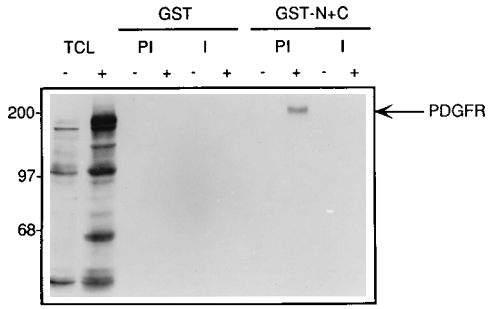

FIG. 7. SHPTP2 (986) antibodies inhibit binding by SHPTP2's SH2 domains. GST and GST-SH2 bound to glutathione-agarose beads were preincubated with either preimmune (PI) or immune (I) anti-SHPTP2 antibodies. GST and GST-SH2/anti-SHPTP2 antibody complexes were then incubated with cell lysates prepared from NIH 3T3 cells that were either left unstimulated (−) or stimulated with PDGF (+). Protein complexes were resolved by SDS–8% PAGE and immunoblotted with antiphosphotyrosine antibody 4G10. The migration positions of molecular weight markers (Gibco BRL) and the position of the PDGFR are indicated.

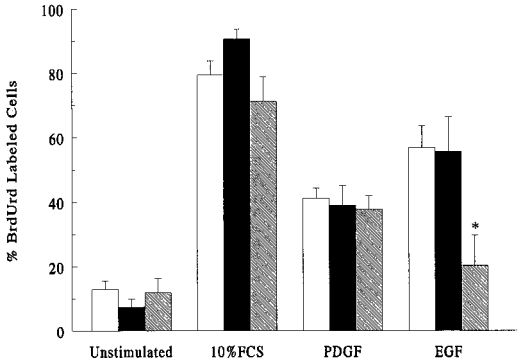

FIG. 8. SHPTP2 is required for EGF- but not PDGF- or serum-induced S-phase entry. Anti-SHPTP2 antibodies were microinjected into the cytoplasm of quiescent NIH 3T3 cells and were either left unstimulated or treated for 18 h with 10% serum, PDGF (50 ng/ml), or EGF (100 ng/ml) in the presence of BrdUrd. BrdUrd-positive uninjected or injected cells were detected by using a monoclonal antibody to BrdUrd and an anti-mouse–FITC secondary antibody. Injected NIH 3T3 cells were detected by staining for the presence of rabbit IgG using an anti-rabbit-TRITC secondary antibody. Results are means with SEM (*, $P < 0.05$) for several experiments ($n > 3$) and represent the accumulation of approximately 250 to 350 preimmune (closed bars) and immune anti-SHPTP2-injected cells (shaded bars). The percentage of BrdUrd-positive uninjected cells (open bars) was averaged over at least three separate fields per coverslip.

has suggested that lysophosphatidic acid is the major mitogen in serum (43). Thus, SHPTP2 does not appear to be required for MAPK induction in response to this G-protein-coupled receptor pathway. Our results are also consistent with previous microinjection studies in which SHPTP2 was reported to be dispensable for serum-induced mitogenesis (21, 78).

SHPTP2 SH2 domain interactions are inhibited by anti-SHPTP2 antibodies. To confirm the transient transfection studies, as well as to determine whether SHPTP2 is required at later times in EGF- and PDGF-mediated mitogenesis, we adopted a microinjection approach. We sought to perturb SHPTP2 function by microinjecting into NIH 3T3 cells anti-SHPTP2 antibodies that could neutralize the function of SHPTP2. One way such antibodies could act is by binding to the SH2 domains of SHPTP2 and preventing association of SHPTP2 with upstream phosphotyrosyl targets. We asked whether any of our polyclonal anti-SHPTP2 antibodies inhibited the association of SHPTP2 with the PDGFR, a known target for the SH2 domains of SHPTP2 (15, 26, 30, 31, 76). GST preincubated with either preimmune or anti-SHPTP2 antibodies did not associate with any phosphotyrosyl proteins in lysates prepared from PDGF-treated cells. However, upon PDGF stimulation, a GST fusion protein encoding the N- and C-terminal SH2 domains of SHPTP2 (GST-SH2) preincubated with preimmune antibodies bound a 180-kDa phosphotyrosyl protein, which we and others have previously identified as the PDGFR (15, 26, 30, 31, 76). In contrast, GST-SH2 preincubated with anti-SHPTP2 antibodies (986), failed to associate with the PDGFR when incubated with lysates prepared from PDGF-treated cells (Fig. 7). Similar results were obtained with some, but not all other anti-SHPTP2 polyclonal antibodies available in our laboratory (16). These in vitro studies suggested that 986 antibodies might neutralize SHPTP2 function in vivo.

SHPTP2 is required for EGF- but not PDGF-induced S-phase entry. Our transient transfection studies suggest that SHPTP2 is required for MAPK activation in response to EGF but not PDGF or serum. Since recent work has implicated MAPK activation in immediate-early signaling events as well as in G_1/S progression (35, 67), our results would predict that SHPTP2 is required for EGF-induced S-phase entry. By acting at a later stage of the cell cycle, SHPTP2 could also be required for PDGF and/or serum-induced S-phase entry. In order to determine whether SHPTP2 functions during later events in growth factor-induced mitogenesis, we microinjected 986 antibodies into NIH 3T3 cells and assayed the ability of these cells to enter S phase in response to EGF, PDGF, or serum. Since SHPTP2 associates with both the activated EGF receptor and PDGFR (15, 26, 30, 31, 76), we reasoned that microinjection of antibodies that block these associations could interfere with several features that may be required for SHPTP2 function. For example, such antibodies could inhibit the tyrosyl phosphorylation of SHPTP2 by the receptor and/or could prevent relocalization of SHPTP2 to its substrate(s).

Quiescent NIH 3T3 cells were microinjected with either preimmune or anti-SHPTP2 (986) antibodies and, after an approximately 1-h recovery, were either left unstimulated or stimulated for 18 h with serum, PDGF, or EGF in the presence of BrdUrd. Cells were scored for successful microinjection (as indicated by anti-IgG staining; see Materials and Methods), as well as for nuclear BrdUrd staining. Microinjection of preimmune antibodies did not affect either the basal (approximately 15%) or EGF-induced (approximately 55%) level of BrdUrd labeling. However, microinjection of 986 antibodies prior to EGF treatment significantly reduced the number of cells entering S phase (to approximately 20%; $P < 0.05$) (Fig. 8). Coinjection of anti-SHPTP2 antibodies with purified full-length SHPTP2 protein (at equimolar concentrations) rescued inhibition of S-phase entry induced by 986 antibodies (3), in agreement with the notion that the anti-SHPTP2 antibodies acted by interfering with the function of SHPTP2.

Microinjection of the same antibodies had no effect on the percentage of NIH 3T3 cells entering S phase in response to either serum or PDGF (Fig. 8). We attempted to determine whether 986 antibodies inhibited S-phase entry induced by subsaturating concentrations of PDGF; i.e., whether SHPTP2 might be required to potentiate the PDGF signal when PDGF is limiting. Although we did not observe any difference in the percentages of uninjected and injected cells that incorporate BrdUrd under these conditions, this was difficult to assess definitively, because at subsaturating concentrations of PDGF (5 ng/ml) only 20 to 25% of the cells entered S phase compared with approximately 10% of unstimulated controls (3). Therefore, we cannot exclude a subtle requirement for SHPTP2 to

promote S-phase entry of NIH 3T3 cells in response to PDGF. However, our data are most consistent with the notion that SHPTP2 is required for the entry of NIH 3T3 cells into S phase in response to EGF but not serum or PDGF.

SHPTP2 is required late in G_1 of the cell cycle in response to EGF. We next asked how long SHPTP2 is required following EGF stimulation of quiescent NIH 3T3 cells. First, we determined the length of time required for these cells to progress from G_0 through G_1 and enter S phase. Quiescent NIH 3T3 cells were stimulated with EGF in the presence of BrdUrd, and the percentage of BrdUrd-positive cells was quantitated at various times after the addition of EGF. As shown in Fig. 9A, these NIH 3T3 cells began to enter S phase 11 to 12 h following EGF addition. To enter S phase, quiescent cells require growth factors throughout G_1 until the restriction point; after passage through the restriction point, which takes place about 2 h prior to S phase, cells no longer require growth factors to enter and complete S phase (50, 61). To determine the restriction point in our NIH 3T3 cells, quiescent cells seeded on coverslips were stimulated for various times, after which the cells were washed free of growth factors and then incubated for an additional 12 h in the presence of BrdUrd to monitor progression into S phase. As shown in Fig. 9A, in these NIH 3T3 cells, the restriction point occurs approximately 10 h following stimulation of quiescent cells with EGF. On the basis of these data, anti-SHPTP2 antibodies were microinjected into cells either 1 h prior to EGF addition or at various times after the addition of EGF, representing mid- to late G_1 and S phase. All microinjected cells were incubated for 18 h in the presence of EGF plus BrdUrd, and the percentages of uninjected and injected BrdUrd-positive cells entering S phase were quantitated. Microinjection of anti-SHPTP2 antibodies 1 h prior to, and at 4 and 8 h following, EGF stimulation significantly inhibited ($P < 0.05$) the entry of these cells into S phase (Fig. 9B and 10A to C). However, cells microinjected at either 12 h or 16 h showed unimpaired BrdUrd labeling following EGF stimulation (Fig. 9B and 10D). Microinjection of matched preimmune antibodies 8 h following EGF stimulation had no effect on S-phase entry (3), indicating that microinjection alone did not perturb cell cycle progression. To define further the role of SHPTP2 in late G_1 progression following EGF stimulation, GST-SH2 was microinjected into NIH 3T3 cells. Microinjection of GST-SH2 1 h prior to or 8 h following EGF stimulation significantly (approximately twofold) inhibited EGF-induced S-phase entry (Table 1). No effect on EGF-induced S-phase entry was observed upon microinjection of GST alone 1 h prior to or 8 h following EGF stimulation (3). We conclude that SHPTP2 function is required until a point close to the restriction point but is dispensable for the process of S phase itself.

SHPTP2 regulates distinct pathways during EGF-mediated G_1 progression. Our findings indicate that SHPTP2 function is required for the immediate-early activation of MAPK and also for late G_1 events in the EGF signaling pathway. These observations led us to investigate whether SHPTP2 also participates in MAPK activation during the later cell cycle stages. Antiphosphotyrosine immunoblots of total cell lysates from NIH 3T3 cells at various times following EGF stimulation revealed a rapid increase in the tyrosyl phosphorylation of several proteins (Fig. 11A). Two bands, migrating with apparent molecular masses of 42 and 44 kDa (Fig. 11A) were particularly prominent; work in many laboratories has established that these correspond to MAPK isoforms (p44Erk1 and p42Erk2). In contrast, there was no tyrosyl phosphorylation of proteins migrating in the 42- to 44-kDa range during the later G_1 period (6 to 10 h). Work in many laboratories has established that the MAPK phosphorylation state correlates with its activation in vivo (12, 38). These results suggested that neither p44Erk1 nor p42Erk2 is active around the restriction point. We therefore monitored MAPK phosphorylation state directly by using a phosphospecific antibody that recognizes activated forms of both p44Erk1 and p42Erk2. To increase our ability to detect transient MAPK activation at later cell cycle stages, we examined more closely spaced time points at these times. Phospho-p44Erk1 and phospho-p42Erk2 were readily detected within

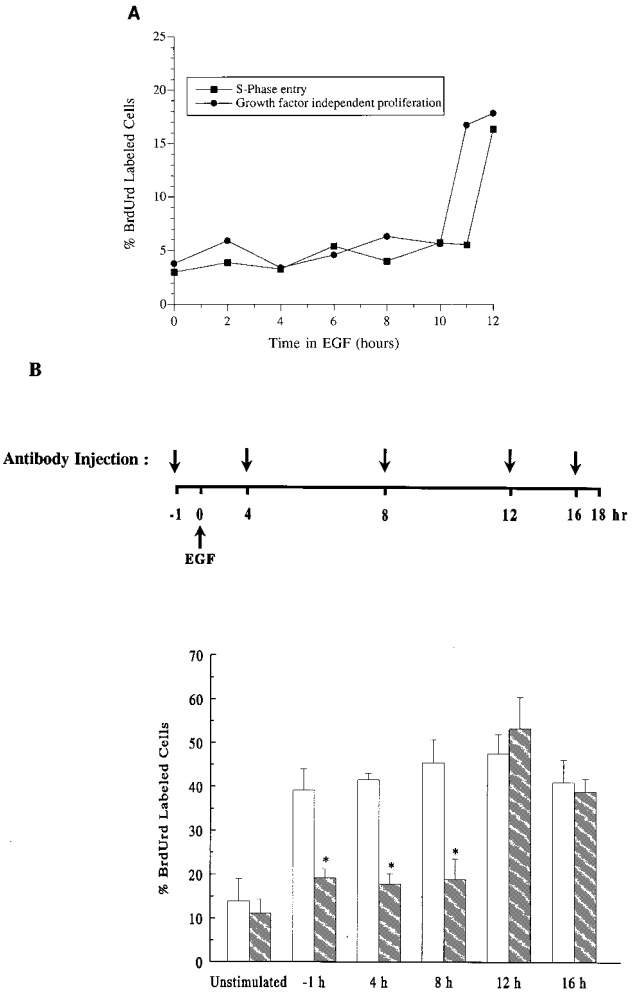

FIG. 9. SHPTP2 is required during late G_1 for EGF-mediated mitogenesis. (A) Determination of the restriction point and S-phase entry time in NIH 3T3 cells. For analysis of the restriction point (growth factor-independent proliferation), cells were incubated with EGF (100 ng/ml), washed extensively at the indicated times, and then allowed to progress for an additional 12 h in the presence of serum-free medium without EGF plus BrdUrd. For analysis of S-phase entry time, growth factors were added at time zero, and BrdUrd incorporation was monitored at the indicated times. As indicated in the figure, and as expected from previous work (see the introduction), the restriction point occurs approximately 1 to 2 h before S-phase entry. (B) Microinjection of anti-SHPTP2 antibodies during early, mid, and late G_1 of EGF-mediated mitogenesis. NIH 3T3 cells were rendered quiescent by serum deprivation, and anti-SHPTP2 (986) antibodies were microinjected into the cytoplasm either prior to EGF addition or at various times thereafter, as indicated in the scheme, in the presence of BrdUrd for 18 h. BrdUrd-positive, antibody-injected cells were detected as described in Materials and Methods, and the numbers of uninjected and injected BrdUrd-positive cells were quantitated. Results are means with SEM (*, $P < 0.05$) for three separate experiments, representing an accumulation of approximately 250 to 300 anti-SHPTP2 antibody-injected cells (shaded bars); the percentage of BrdUrd-positive, uninjected cells (open bars) was averaged over at least three separate fields per coverslip.

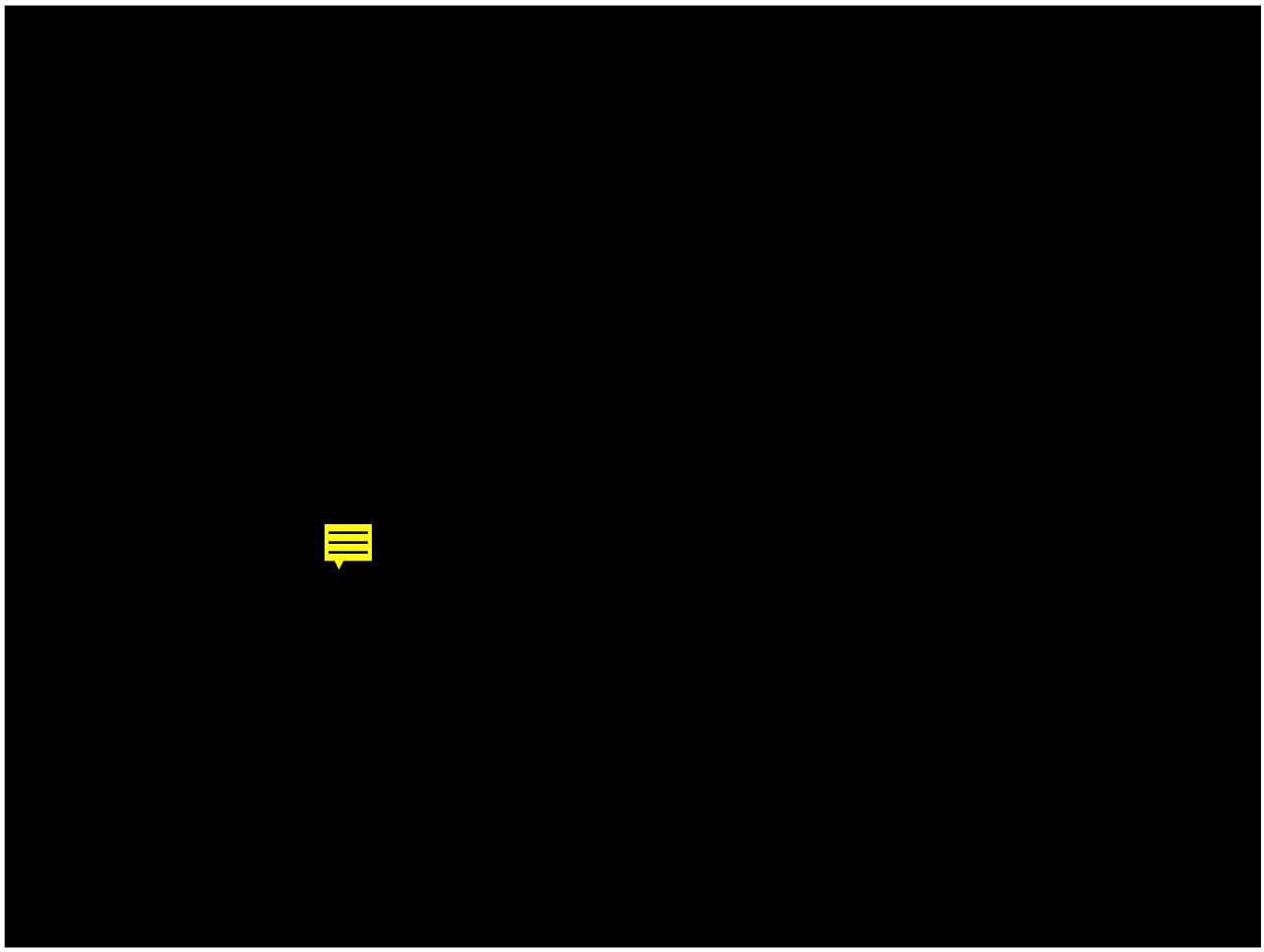

FIG. 10. Analysis of the effect of anti-SHPTP2 antibody microinjection into NIH 3T3 cells on EGF-mediated S-phase entry by BrdUrd staining. NIH 3T3 cells were microinjected with anti-SHPTP2 antibodies before or at various times after EGF stimulation. Coverslips of injected cells were prepared for double immunofluorescence photomicrography 18 h after EGF stimulation. Microinjected NIH 3T3 cells were identified with an anti-rabbit–TRITC secondary antibody (red), and BrdUrd-positive NIH 3T3 cells were identified with an anti-mouse–FITC secondary antibody (green). Antibody-microinjected cells that are BrdUrd positive show a yellow nuclear colocalization signal.

30 min following EGF stimulation, but neither was detectable at 2 h or later during G_1 (Fig. 11B, upper panel). Reprobing the immunoblot with an antiserum that recognizes both isoforms showed that MAPK levels were comparable throughout the time course (Fig. 11B, lower panel). Previous work has shown that, immediately following growth factor stimulation, Raf-1 becomes hyperphosphorylated, leading to a retardation of its electrophoretic mobility on SDS-PAGE; activation of MAPK is necessary for this mobility shift (38). When a similar time course following EGF stimulation was performed, anti-Raf-1 immunoblots revealed several Raf-1 forms, presumably representing various Raf-1 phosphorylation species. At the earliest time point analyzed following EGF stimulation, Raf-1 underwent a marked retardation in electrophoretic mobility, as observed by the more slowly migrating phospho-Raf-1 species (Fig. 11C). By 6 h and for the entire duration of G_1, Raf-1 returned to a mobility similar to that in quiescent NIH 3T3 cells (Fig. 11C). These data also were consistent with absence of MAPK induction later in the cell cycle. Finally, we directly assessed the activities of both p44Erk1 and p42Erk2 throughout G_1. p44Erk1 and p42Erk2 were found to be activated approximately 10- and 20-fold, respectively, within 5 min following EGF stimulation (Fig. 11D, upper panel). By 1.5 h following EGF stimulation, the activities of p44Erk1 and p42Erk2 returned to basal levels, where they remained throughout the remainder of G_1, up to and including the restriction point (Fig. 11D, upper panel). Again, control immu-

TABLE 1. Blocking of EGF-induced S-phase entry late in G_1 by microinjection of SHPTP2 SH2[a] domains into NIH 3T3 cells

Time of injection	% BrdUrd-labeled cells[b]	
	Uninjected	Injected[c]
None (unstimulated cells)	9.1 ± 2.7	8.8 ± 3.6 (130)
1 h prior to stimulation	47.1 ± 5.9	19.3 ± 1.7[d] (130)
8 h after stimulation	45.7 ± 9.5	20.2 ± 7.8[d] (449)

[a] GST-SH2.
[b] Mean ± SEM for three separate experiments, determined as described in Materials and Methods. Microinjection of GST alone 1 h prior to or 8 h after EGF stimulation did not alter BrdUrd labeling in the presence or absence of EGF.
[c] Identified by coinjection of GST-SH2 with FITC-dextran. The total numbers of cells are indicated in parentheses.
[d] Significantly different ($P < 0.05$) from the corresponding value for uninjected cells.

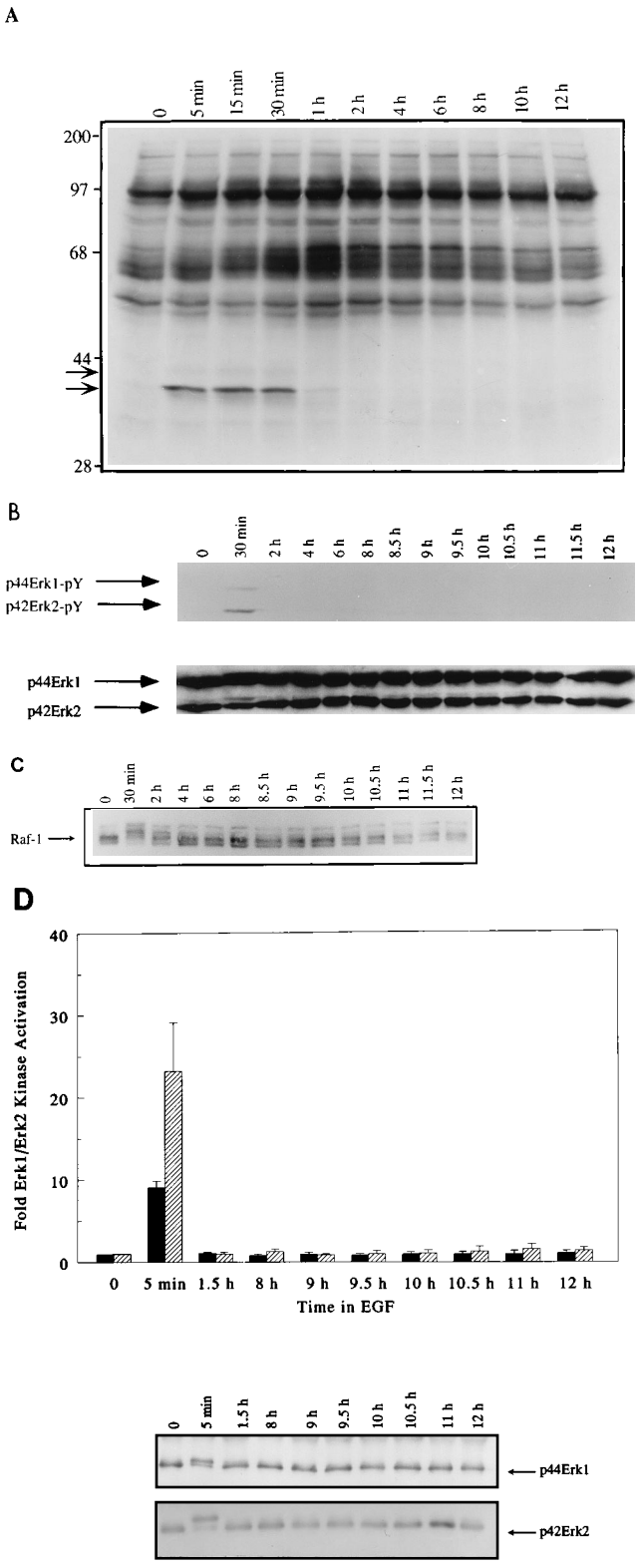

FIG. 11. Time course of EGF-mediated tyrosyl phosphorylation, Raf-1 hyperphosphorylation, and MAPK activation during G_1. (A) NIH 3T3 cells rendered quiescent by serum deprivation were stimulated with EGF (100 ng/ml) for the indicated times. Whole-cell lysates were prepared at the indicated time points and resolved by SDS–10% PAGE, and phosphotyrosyl proteins were identified by immunoblotting with antiphosphotyrosine antibody 4G10. Arrows, MAPKs. (B, upper panel) Unstimulated and stimulated NIH 3T3 cells prepared as for panel A, only with an expanded number of points at later cell cycle stages, were immunoblotted with anti-phospho-MAPK antibodies. Note initial activation of MAPKs but no activation at later time points. (B, lower panel) Reprobe of the blot in the upper panel with polyclonal anti-MAPK antibodies. (C) Cell lysates prepared as for panel B were resolved by SDS–8% PAGE and immunoblotted with anti-Raf-1 polyclonal antibodies. Raf-1 undergoes a mobility shift in response to EGF at 30 min, but its mobility then returns to baseline; no shift is observed at later time points. (D) EGF-stimulated NIH 3T3 cells were lysed at the indicated times, and equal amounts of protein from each time point were immunoprecipitated with an antibody to either p44Erk1 or p42Erk2. The activities of the MAPKs were assessed by immune-complex kinase assay using MBP as substrate. The activities of p44Erk1 (solid bars) and p42Erk2 (hatched bars) represent means with SEM for three separate experiments (upper panel). Immune complexes from one such experiment were resolved by SDS–10% PAGE, transferred to Immobilon, and immunoblotted with an antiserum that recognizes both p44Erk1 and p42Erk2 (lower panel). Equivalent amounts of p44Erk1 and p42Erk2 are present in these immune complexes at all time points.

noblots with anti-MAPK antibodies revealed that similar levels of p44Erk1 and p42Erk2 were present in the immune complexes subjected to activity assays (Fig. 11D, lower panel). Taken together, we conclude that, unlike the immediate-early events in which SHPTP2 is required for MAPK activation in response to EGF, the requirement for SHPTP2 for S-phase entry during the later stages of G_1 does not involve regulation of the MAPKs.

DISCUSSION

We have further elucidated the role of SHPTP2 in growth factor-induced mitogenesis. Transient transfection studies indicate that SHPTP2 is required for activation of the MAPK pathway immediately upon EGF stimulation of growth factor-depleted cells. This does not appear to reflect a general requirement by all mitogens for SHPTP2, because similar experiments reveal that SHPTP2 function is dispensable for PDGF- and serum-induced transactivation of Elk-1 by MAPK. Analysis of SHPTP2 mutants reveals that the PTP domain and, most likely, PTP activity are required for the role of SHPTP2 in EGF-induced MAPK activation, whereas tyrosyl phosphorylation of SHPTP2 appears to be dispensable. Microinjection studies indicate that, in addition to its immediate-early functions, SHPTP2 is required later in G_1 for EGF- but not PDGF- or serum-induced mitogenesis (at saturating growth factor concentrations). Since EGF does not appear to activate MAPK at these later times, our results suggest that the signal transduction pathways regulated by SHPTP2 at different times following growth factor stimulation may be distinct.

Two different PTP domain mutants of SHPTP2 (PTP2CS and PTP2ΔP) were found to impair EGF-induced MAPK activation in transiently transfected 293 cells, although PTP2ΔP was a less potent inhibitor. These mutants most likely act in a dominant negative fashion to interfere with the function of endogenous SHPTP2, a notion firmly supported by the rescue experiment in Fig. 3B. The rescue experiments also indicate that PTP2YF is expressed in transiently transfected cells; indeed immunofluorescent staining of transiently transfected 293 and COS-1 cells revealed that all transfected SHPTP2 expression constructs were expressed at levels substantially higher than endogenous SHPTP2 (3). The effect of the SHPTP2 mutants is specific, as overexpression of a catalytically impaired version of SHPTP2's closest relative, SHPTP1, does not inhibit the EGF-induced MAPK pathway. Thus, the inhibitory effect of PTP2CS and PTP2ΔP cannot be explained merely by overexpression of either a nonspecific PTP or an SH2-containing protein. Instead, these data imply that SHPTP2 is a required element for immediate-early events in the EGF signal transduction pathway. Similar conclusions were reached in earlier

studies of the effects of transient (79), stable (49), or inducible (41) overexpression of PTP2CS on insulin/IGF-1 signaling. While the manuscript was in preparation, Zhao et al. reported that stable expression of PTP2CS in 293 cells blocked EGF-induced MEK and MAPK induction (83). Since, in principle, PTP2CS can "trap" substrates (5, 42, 66), it is difficult to be certain from studies of its effects alone whether SHPTP2 has a positive or negative function in a given signal transduction pathway. However, our finding that the effects of PTP2CS and PTP2ΔP are qualitatively similar, together with the earlier finding of Yamauchi et al. (79) that transient overexpression of the SH2 domains of SHPTP2 has effects similar to those of PTP2CS overexpression on insulin-induced c-*fos* induction, strongly suggests that SHPTP2 phosphatase activity is required for a positive function in the immediate-early response to EGF and insulin. The apparently lower potency of PTP2ΔP compared with PTP2CS in our assays could reflect lower stability of PTP2ΔP protein and/or PTP2CS's additional ability to trap substrates and thus interfere with EGF signaling in multiple ways; further studies will be required to resolve these issues.

Although earlier biochemical studies implicated SHPTP2 in multiple growth factor signal transduction pathways (see the introduction), the domains of SHPTP2 required in these pathways have remained unclear. Studies of the effects of PTP2CS on insulin/IGF-1 signal transduction (see above) suggested that PTP activity was essential for SHPTP2's ability to promote GTP loading of Ras (49), activation of MAPK (41, 49, 79), induction of c-*fos* transcription (79), and mitogenesis (21, 78) in response to insulin/IGF-1. SHPTP2 is not detectably tyrosyl phosphorylated in response to insulin (28), arguing against a general requirement for tyrosyl phosphorylation in SHPTP2 function. However, upon PDGF or EGF stimulation of fibroblasts or epithelial cells (4, 15, 26, 30, 76) or treatment of hematopoietic cells with a number of cytokines (69, 70, 77), SHPTP2 is tyrosyl phosphorylated at one or two sites within its C terminus (3, 4). Following PDGF or cytokine stimulation, these sites can recruit the Grb2-Sos complex (4, 33, 70, 77). These findings led to a model in which SHPTP2 serves as an adapter to help couple receptor activation to the Ras pathway (4, 30, 33, 47). In this "adapter" model, one or more C-terminal tyrosyl residues would be essential for SHPTP2's role as a positive signaling component. Our results strongly argue that, as is the case for insulin/IGF-1 signaling, SHPTP2 catalytic activity is essential for the immediate-early response to EGF. Moreover, several lines of evidence argue that SHPTP2's ability to serve as an adapter is not essential for its role in immediate-early signaling events. First, the effects of PTP2ΔP (and, most likely, PTP2CS; see above) are not due to its inability to become tyrosyl phosphorylated, since PTP2ΔP, which migrates below endogenous SHPTP2, was found to be tyrosyl phosphorylated in response to EGF and PDGF (Fig. 3A) (3). Second, unlike the inhibitory effects of PTP2CS and PTP2ΔP following EGF stimulation, overexpression of PTP2YF had little effect on EGF- or PDGF-induced MAPK activation in 293 cells (Fig. 2B and 5). If the adapter model were operative, such a mutant might have been expected to behave in a dominant negative mode. Equally important, PTP2YF is able to rescue the block in EGF-induced MAPK activation produced by PTP2CS (Fig. 3B). There may be a small difference in the potencies of wild-type SHPTP2 and PTP2YF, as suggested by the decreased ability of PTP2YF to potentiate EGF-induced signaling (Fig. 2A). Nevertheless, our results imply that tyrosyl phosphorylation of SHPTP2 is not essential for SHPTP2's positive signaling role in the MAPK pathway. PTP2YF is also able to rescue the block in normal *Xenopus* development produced by injection of PTP2ΔP into early embryos (68), arguing that tyrosyl phosphorylation of SHPTP2 is dispensable in this nonmitogenic pathway.

Although not absolutely required for EGF or PDGF growth factor signaling, tyrosyl phosphorylation of SHPTP2 probably is important for at least some functions of SHPTP2. One of the tyrosyl phosphorylation sites in SHPTP2 (Y-542 in human SHPTP2) is conserved in *Drosophila melanogaster* (*csw*) and *Xenopus laevis*; the other (Y-580), although absent in *csw*, is conserved in *X. laevis* and mammals. It seems likely that these sites have some as yet unidentified function. As indicated above, our results suggest that tyrosyl phosphorylation may slightly enhance the ability of wild-type SHPTP2 to potentiate EGF-induced immediate-early responses. This effect, although modest, was consistently observed and statistically significant ($P < 0.05$). We cannot exclude the possibility that this difference was due to lower stability of PTP2YF protein, causing it to accumulate to lower levels than wild-type SHPTP2 in transfected cells. Even if PTP2YF and PTP2WT are expressed comparably, it is not clear whether the decreased potentiating capacity of PTP2YF reflects its inability to recruit and bind Grb2 or other, as yet undefined effects of tyrosyl phosphorylation on, for example, PTP activity. Tyrosyl phosphorylation could be more important in hematopoietic pathways, in which SHPTP2 is tyrosyl phosphorylated in response to several cytokines yet has not been shown to associate stably with the cognate receptors in vivo (3, 70, 77). Alternatively, tyrosyl phosphorylation could be required for later events in growth factor signaling and/or for one or more nonmitogenic effects of growth factors. What is clear from our studies is that the integrity and, most likely, the activity of the SHPTP2 PTP domain, rather than its ability to serve as an adapter, are essential for EGF-induced immediate-early gene responses.

The precise target(s) of SHPTP2 which must be dephosphorylated to permit activation of the MAPK pathway in response to EGF or insulin, and thus, SHPTP2's precise position in these pathways, remains undefined. Overexpression of catalytically inactive SHPTP2 blocks Ras-GTP loading (49), MAPK activation (41, 49, 79), and transcriptional activation of c-*fos* (79) in response to insulin/IGF-1. These data support the notion that SHPTP2 is required for insulin/IGF-1-induced Ras activation or for a parallel pathway induced by insulin. In agreement with this notion, the dominant negative effects of PTP2CS on EGF-induced Elk-1 activation are rescued by activated Ras and Raf (Fig. 5) (3). We titrated the level of the activated Ras expression construct used in our cotransfection experiments such that further stimulation of the MAPK pathway was observed upon EGF addition. Coexpression of the SHPTP2 dominant negative mutants clearly blocked EGF-stimulable MAPK activation without impairing the ability of activated c-Ras to activate downstream signaling components, thus placing SHPTP2 upstream or parallel to Ras in the EGF pathway (Fig. 5). Members of the *src* family of nontransmembrane PTKs are particularly attractive candidates for SHPTP2 targets, as they are negatively regulated by tyrosyl phosphorylation (53), are activated in response to growth factors (29), and have been shown to act upstream of Ras (55). In agreement with this notion, PTP2CS does not inhibit Elk-1 activation in response to transfection of an activated c-*src* (Y-527→F mutation) expression vector (3). However, there is as yet no direct evidence that Y-527 of c-Src (and/or cognate residues in other *src* family PTKs) is dephosphorylated upon EGF or insulin stimulation and/or that interference with SHPTP2 function impairs *src* family PTK activation in response to growth factors.

Our results further imply that SHPTP2 function is required for growth in response to only some mitogens. Both transient

transfection studies with 293 and COS-1 cells (Fig. 6) (3) and microinjection experiments with NIH 3T3 cells (Fig. 8) indicated that SHPTP2 is not absolutely essential for PDGF- or serum-induced immediate-early transcriptional activation and/or S-phase entry. Our results are consistent with previous studies of PDGFR mutants expressed in heterologous cell types. Such studies revealed that (at saturating concentrations of PDGF) there is no difference in the proliferative capacity of TRMP cells expressing the wild-type PDGFR or a PDGFR mutant that cannot bind SHPTP2 (74). Secondly, HepG2 cells expressing PDGFR mutants that retain only their SHPTP2 and c-Src binding sites fail to progress into S phase in response to PDGF, whereas mutants retaining either phospholipase C-γ1 or PI-3 kinase sites are sufficient to induce mitogenesis (75). Taken together, these data support the conclusion that SHPTP2 is not a major contributor to mitogenesis downstream of the PDGFR. Since there appeared to be a slight rightward shift in PDGF dose-response in heterologous cells expressing the SHPTP2 binding-site mutant of the PDGFR (74), SHPTP2 could have a weak modulatory effect on PDGFR-induced mitogenesis. It should be noted that if SHPTP2 does have a potentiating effect on PDGFR signaling that is too weak to be detected by our assays, our data do not exclude a role for tyrosyl phosphorylation of SHPTP2 (and/or Grb2 binding) in this process. We did not observe impairment of PDGF-induced S-phase entry at low doses of PDGF in our microinjection experiments (3), but our assay may not be sensitive enough to detect small differences. While the manuscript was in preparation, Rivard et al. (56) reported that a phosphatase domain mutant of SHPTP2 inhibits c-*fos* reporter gene expression in response to PDGF or serum in CCL39 cells, a Chinese hamster lung fibroblast cell line. Our data do not support such a role for SHPTP2 in PDGF- or serum-induced mitogenesis in 293, COS-1, or NIH 3T3 cells. In addition, earlier microinjection studies (21, 78), along with the ability to isolate stable PTP2CS mutant-expressing cell lines in serum-containing media (49, 83), argue strongly that SHPTP2 function is not required for serum-induced mitogenesis in multiple cell types. It remains possible, however, that, in addition to differential requirements for SHPTP2 function in different growth factor signal transduction pathways, there are subtle differences in SHPTP2 requirements for a given growth factor in different cell types and/or in different species. We have shown previously that SHPTP2 function is required for some (e.g., GLUT 1 expression and mitogenesis) but not all (e.g., GLUT 4 translocation) aspects of insulin action (21). Thus, SHPTP2 appears to be a selective modulator of growth factor-induced signaling pathways.

In addition to establishing a requirement for SHPTP2 for immediate-early events in response to growth factor action, we have found that SHPTP2 function is required at later stages of EGF-induced S-phase entry. Microinjection of either anti-SHPTP2 antibodies or GST-SH2 domains of SHPTP2 inhibited S-phase entry up to 8 h after EGF stimulation of quiescent NIH 3T3 cells. Anti-SHPTP2 antibodies microinjected 12 h poststimulation had no effect on BrdUrd incorporation (Fig. 9B and 10D). Antibody-induced inhibition was not due to nonspecific (i.e., toxic) effects of injection, since there was no inhibition of EGF-induced S-phase entry by matched preimmune antibodies nor of PDGF-induced S-phase entry by immune anti-SHPTP2 antibodies. The restriction point in these cells occurred at approximately 10 h (Fig. 9A). Thus, SHPTP2 function appears to be required for EGF-induced S-phase entry up to and perhaps including the restriction point. Whether SHPTP2 is required at a discrete time(s) (e.g., near the restriction point) or, instead, must be continuously active throughout

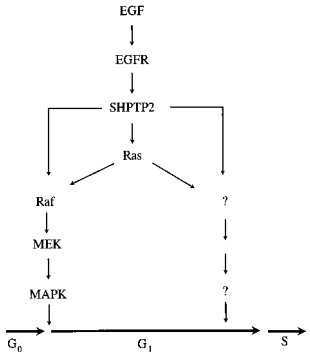

FIG. 12. Model for SHPTP2 signaling in EGF-mediated mitogenesis. See the text for details.

G_1 for EGF to induce S-phase entry remains to be determined. Both the antibodies and the GST-SH2 fusion protein that were microinjected should inhibit binding of the SH2 domains of SHPTP2 with its upstream phosphotyrosyl target(s). Thus, SH2 domain function is likely required for the later actions of SHPTP2. Whether PTP activity and/or its C-terminal tyrosyl phosphorylation sites are required for SHPTP2's role in late G_1 remains to be established.

Analogous microinjection studies have explored the temporal requirements for Ras and *src* family PTKs in signaling by other growth factors. Ras is required during both early (13) and late (up to 8 h) (46) periods of the cell cycle in response to serum in BALB/3T3 cells. Also, microinjection of antibodies that recognize the C termini of multiple *src* family PTKs up to the restriction point blocks S-phase entry in response to PDGF in NIH 3T3 cells (72). Not all downstream targets of RPTKs are continuously required for S-phase entry, as microinjection of antibodies against the catalytic subunit (p110) of PI-3K inhibited mitogenesis only for up to 6 h following PDGF stimulation of NIH 3T3 cells (57). The different requirements for signaling components for early and late growth factor-induced events suggests that initial and late RPTK signaling pathways may be distinct. Accordingly, although SHPTP2 function is required for MAPK activation as part of the immediate-early response to EGF and insulin, we found no evidence for MAPK activation around the restriction point (Fig. 11), when SHPTP2 function also is required. We observed subtle differences in the pattern of phosphotyrosyl proteins observed immediately after EGF stimulation and during later cell cycle stages (Fig. 11A). These observations with EGF signaling in NIH 3T3 cells are reminiscent of earlier studies of tyrosyl phosphorylation during G_1 of the cell cycle. Upon stimulation of BALB/3T3 cells with either serum or fibroblast growth factor, the initial tyrosyl phosphorylation events are followed by a second wave of tyrosyl phosphorylation observed near the G_1/S boundary (45, 81). The molecular details of the RPTK-induced late pathways required for S-phase entry remain largely undefined (Fig. 12). If SHPTP2 lies upstream of Ras in immediate-early pathways, SHPTP2 may contribute to the required Ras activation (13, 46) later in the cell cycle as well. In that case, the downstream consequences of Ras activation must be different around the restriction point, since no significant MAPK activation occurs at this time. A possible explanation is provided by the recent report that Raf interacts with cdc25A (18). Alternatively, SHPTP2 could regulate distinct parallel pathways at different times following growth factor stimulation. Further studies are required to define the role of SHPTP2 in these later events.

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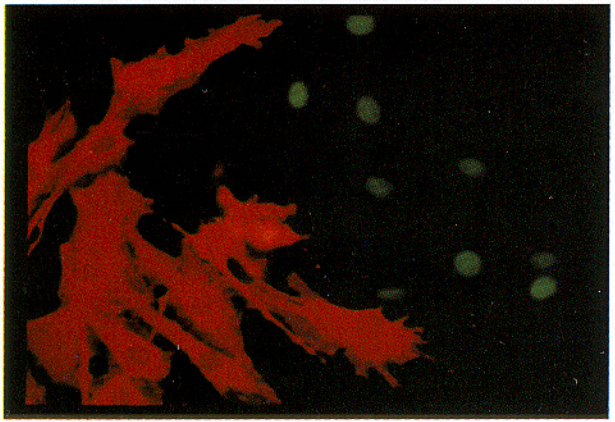
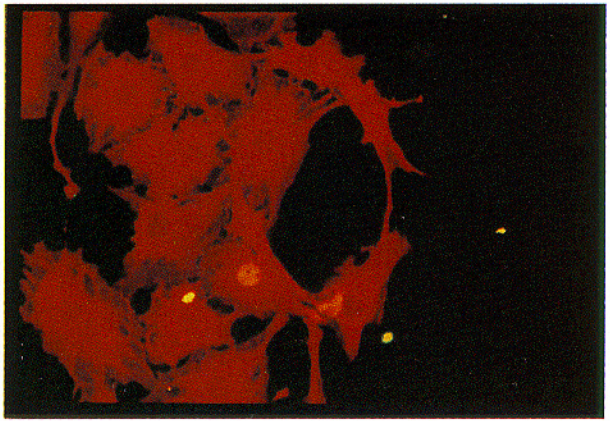
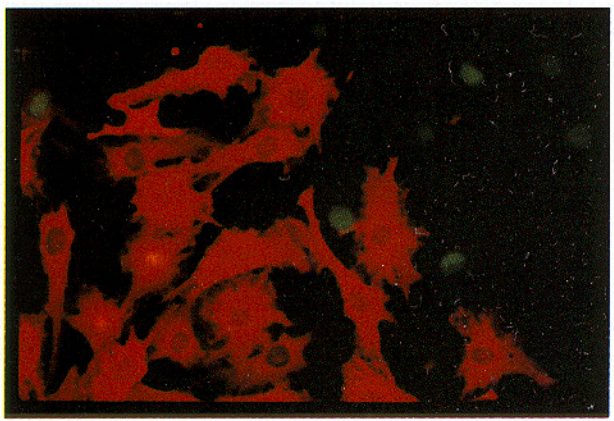


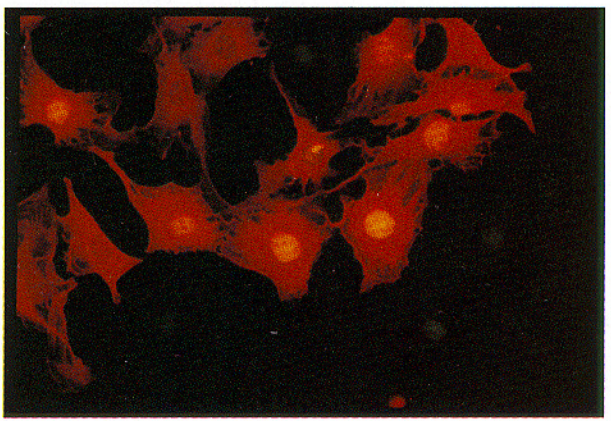
A. Antibody injected 1 h before EGF stimulation



B. Antibody injected 4 h after EGF stimulation



C. Antibody injected 8 h after EGF stimulation



D. Antibody injected 12 h after EGF stimulation