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Protein-tyrosine Phosphatase SHP2 Is Positively Linked to Proteinase-activated Receptor 2-mediated Mitogenic Pathway*

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Proteinase-activated receptor-2 (PAR2), a new member of family of the G protein-coupled receptors, is activated by proteolytic cleavage of its extracellular amino terminus, a mechanism similar to that used by the thrombin receptor. It has been suggested that PAR2 has a potential role in the late phases of the acute inflammatory response and in tissue repair and/or skin-related disorders. Here we demonstrate that the agonist peptide (SLIGRL) stimulated c-*fos*-mediated mitogenic activation and tyrosine phosphorylation of cellular proteins. One of the tyrosine-phosphorylated proteins was identified as an Src homology-2 domain-containing protein-tyrosine phosphatase, SHP2. The stimulatory effect of the agonist peptide on early gene transcription was markedly blocked by pertussis toxin treatment whereas the induced tyrosine phosphorylation of SHP2 was completely abolished by the drug. More importantly, while expression of wild-type SHP2 enhanced the agonist-stimulatory mitogenic activity, overexpression of a catalytically inactive mutant of SHP2 strongly suppressed the stimulatory effect of the agonist peptide on both early gene transcription and DNA synthesis. These results suggest that SHP2 acts as a positive regulator linked to the PAR2-mediated mitogenic pathway coupled to a pertussis toxin-sensitive heterotrimeric G protein. Demonstration of SHP2 as a positive mediator in a G protein-coupled, receptor-mediated signaling adds to our understanding of the function of both SHP2 and PAR2 in the signaling pathway.

Many extracellular signals are transmitted to the interior of the cell by seven transmembrane-spanning receptors coupled to heterotrimeric guanine nucleotide-binding proteins (G protein; subunits, $\alpha\beta\gamma$). G protein-mediated signaling is perhaps the most conserved evolutionary mechanism by which eukaryotic cells respond to a wide variety of information reaching the cell surface. Thus, G protein-coupled receptors constitute the largest family of plasma membrane receptors. In contrast to the conventional model for receptor-ligand interaction, the thrombin receptor (1–4), which is activated by proteolytic cleavage of its extracellular amino terminus, defines a class of G protein-coupled receptors with a novel mechanism of activation. Recently, a second proteolytically activated receptor, designated proteinase-activated receptor-2 (PAR2), was identified (5–7). Like the thrombin receptor, PAR2 is activated by proteolytic cleavage of its extracellular amino terminus, thus exposing a new terminus sequence which functions as a "tethered" peptide ligand. A synthetic hexapeptide (SLIGRL) mimicking the tethered peptide ligand is able to activate the receptor (5, 6). Trypsin was also shown to be a potent activator of PAR2 (5, 6). Recent data suggest that PAR2 has a potential role in the later phases of the acute inflammatory response (8) and in tissue repair and/or skin-related disorders (9), although its physiological enzyme activator has yet to be identified.

It is known that protein-tyrosine phosphorylation plays a crucial role in regulating receptor signaling. The level of cellular protein-tyrosine-phosphorylation is determined by a balance between the activities of protein-tyrosine kinases (PTKs)[1] and protein-tyrosine phosphatases (PTPs). Recent studies on thrombin receptor-mediated signaling revealed that the receptor transmits both its coagulant and mitogenic effects via protein-tyrosine phosphorylation and mitogen-activated protein kinase activation in certain cell types (for a recent review, see Ref. 10). Both PTKs and PTPs were found to be involved in the transmission of thrombin receptor-mediated signaling. SHP1 (previously termed PTP1C, HCP, SHP, and SH-PTP1) (11–14), which is predominantly expressed in hematopoeitic cells, was found to couple to thrombin receptors in platelets and Dami cells (15, 16). SHP2 (previously also known as PTP2C, Syp, PTP1D, and SH-PTP2) (17–21), a widely expressed phosphatase, was reported to be a mediator in the mitogenic signaling induced by activation of the thrombin receptor in a Chinese hamster lung fibroblast cell line (CCL39) (22).

In comparison with the thrombin receptor, little is known regarding how other signaling molecules are involved in the PAR2-mediated pathway. In this report, we demonstrate that SHP2 acts as a positive regulator in PAR2-mediated mitogenic signal transduction through a pertussis toxin-sensitive heterotrimeric G protein.

EXPERIMENTAL PROCEDURES

Materials and Cell Culture—293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. IEC-6 cells were grown in DMEM supplemented with 5% fetal bovine serum. M1 cells were maintained in 1:1 mixture of DMEM and Ham's F-12 medium with 5% fetal calf serum and 5 μM dexamethasone. Antibodies used were rabbit anti-SHP2 polyclonal antibody generated as described previously (23), mouse anti-SHP2 monoclonal antibody (anti-PTP-1D) (Transduction Laboratories Inc.), mouse anti-hemagglutinin (HA) monoclonal antibody 12CA5 (Boehringer Mannheim), and mouse

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[1] The abbreviations used are: PTK, protein-tyrosine kinase; PTP, protein-tyrosine phosphatase; EGF, epidermal growth factor; PMA, phorbol 12-myristate 13-acetate; DMEM, Dulbecco's modified Eagle's medium; HA, hemagglutinin; RT-PCR, reverse transcription-polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; SH2, Src homology-2; SRE, serum-responsive element.

anti-phosphotyrosine monoclonal antibody (Upstate Biotechnology Inc.). Nitrocellulose membrane Hybond-C, anti-mouse IgG-horseradish peroxidase, and anti-rabbit IgG-horseradish peroxidase along with enhanced chemiluminescence (ECL) were obtained from Amersham. Protein A-Sepharose CL-4B was from Pharmacia Biotech Inc. Pertussis toxin and bovine trypsin type III were purchased from Sigma. G418 was from Life Technologies, Inc. Peptide Ser-Leu-Ile-Gly-Arg-Leu-OH (SLIGRL) was synthesized by the solid-phase method and purified by high performance liquid chromatography.

Detection of PAR2 Gene Expression and Isolation of PAR2 cDNA—Nine cell lines (293, A10, A431, CCL39, DAM 1, Hism, IEC-6, M1, and MIA PaCa) were used for screening the expression of PAR2 gene by reverse transcription-polymerase chain reaction (RT-PCR) using five pairs of oligonucleotide primers based on the reported mouse PAR2 cDNA sequence (6). The full-length PAR2 cDNA coding region was isolated from M1 and IEC-6 cells by PCR. The forward primer CACAAGCTTATGCGAAGTCTAGCCTGGC contains an *Hin*dIII restriction site. The reverse primer GACCCCGGGTCAGTAGGAGGTTTTAACACT contains a *Sma*I restriction site. The amplified fragment was digested with *Hin*dIII and *Sma*I and cloned into pcDNA3 expression vector between the *Hin*dIII and *Eco*RV sites. The resultant plasmid was designated PAR2-pc. The PAR2 coding sequence was also inserted into pAC TAG2 expression vector (24) to express a fusion protein in which the three repeats of 9 amino acids of hemagglutinin (HA) were added to the carboxyl terminus of PAR2 for detection of the fusion protein by anti-HA antibody (12CA5). The resultant plasmid was termed PAR2-HA. The expression constructs were transfected into 293 cells by calcium phosphate co-precipitation, and clonal cell lines were isolated by selection in 800 μg/ml G418.

Coexpression of SHP2 and SHP2 C459S with PAR2 in 293 Cells—Native SHP2 and catalytically inactive SHP2 with a Cys-459 → Ser mutation (SHP2 C459S) were constructed into the pRc/CMV expression vector as described previously (23). 293 cells with stable expression of PAR2 were transfected with 1 μg of pHYG (a vector that efficiently expresses hygromycin-B phosphotranferase) and 20 μg of pRc/CMV containing SHP2 or SHP2 C459S cDNA by calcium phosphate precipitation. The cells were grown in DMEM containing 200 μg/ml hygromycin B for selection. Colonies were isolated 3 weeks after transfection. Several cell lines (clones) overexpressing SHP2 or SHP2 C459S were identified by immunoblotting with anti-SHP2 antibodies. Four clones of the each constructs were chosen for the experiments detailed in this paper.

Western Blot—Protein samples were separated on SDS-PAGE gels and blotted to nitrocellulose membranes. The membranes were blocked with 5% dry milk in Tris-buffered saline for 2 h at room temperature or overnight at 4 °C, and then incubated for 2 h with specified antibodies: mouse anti-phosphotyrosine (1:5000 dilution), mouse anti-SHP2 monoclonal antibody (1:2000 dilution), or 12CA5 (0.5 μg/ml). After washing for 1 h, the membranes were incubated with horseradish peroxidase-linked secondary antibody for 1 h and washed for an additional 1 h. Immunoreactive protein bands were revealed by chemiluminescence with ECL detection kit according to the manufacturer's instructions (Amersham Corp.).

Immunoprecipitation of SHP2 and Membrane Preparation—Cells were lysed in buffer A (50 mM HEPES, pH 7.2, 150 mM NaCl, 1 mM EGTA, 2 mM EDTA, 10 mM NaF, 1.5 mM $MgCl_2$, 10 mM sodium pyrophosphate, 1% Triton X-100, 10% glycerol, 2 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Cell lysates were clarified by centrifuging at 14000 rpm at 4 °C for 10 min. Fifty μl of 50% slurry protein A-Sepharose CL-4B was added to 1 ml of cell lysate and incubated for 30 min with gentle shaking. The precleared lysates were incubated for 2 h with 5 μl of anti-SHP2 and then incubated for 2 h with 50 μl of 50% slurry protein A-Sepharose CL-4B. The immunoprecipitates were washed four times with lysis buffer A.

293 cells stably expressing PAR2 with HA tag (PAR2-HA) were rinsed once in cold TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5), once in cold H_2O, and then harvested in 1 ml of 0.5 × TE buffer (5 mM Tris, 0.5 mM EDTA, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Na_3VO_4). After incubation at 4 °C for 5 min, the swollen cells were disrupted by passing them seven times through a 26-gauge needle, and nuclei were removed by centrifugation at 3,000 rpm for 10 min at 4 °C. The supernatant was centrifuged at 14,000 rpm for 30 min at 4 °C. The pellets were resuspended in 0.5 ml of buffer A. PAR2-HA was immunoprecipitated using 12CA5 monoclonal antibody (anti-HA) with the same conditions as for the SHP2 immunoprecipitation.

Transient Transfection and Luciferase Activity Assay—293, M1, and IEC-6 cells were transfected using calcium phosphate co-precipitation with a vector containing c-*fos* promoter-driven luciferase gene (SRE-Luc). The efficiency of transfection for SRE-Luc was normalized with a reference plasmid as described before (25). Twenty hours following transfection, the cells were aliquoted into several dishes. Six hours later, the cells were starved for a period of 16 h in a medium without serum and then stimulated for 6 h with 100 μM SLIGRL. Cells in a 60-mm dish were lysed with 200 μl of cell lysis buffer (Promega Inc.), and luciferase activity was assayed using the kit from Promega as reported previously (25). Twenty μl of the 200-μl cell lysis was mixed with 100 μl of substrate (Promega) followed by counting for 10 s in a luminometer (Lumat LB9501d). Luciferase activity is expressed as the ratio of SLIGRL-stimulated to unstimulated samples.

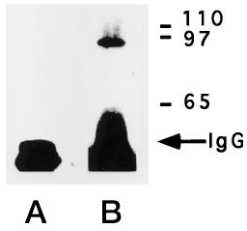

FIG. 1. **Expression of PAR2-HA in 293 cells.** 293 cells were transfected with PAR2-HA and clonal cell lines were isolated by selection in G418. Membranes were prepared from control cells (*A*) or cells transfected with PAR2-HA plasmid (*B*). PAR2-HA was immmunoprecipitated and immunoblotted by anti-HA (12CA5). Molecular size standards are shown in kDa.

Assay of DNA Synthesis by [^{3}H]Thymidine Incorporation—Cells growing in 24-well plates were starved for 24 h in serum-free DMEM/F-12 medium and then stimulated with SLIGRL in the same medium containing [^{3}H]thymidine (5 μCi/ml, 72 Ci/mmol) for 16 h. The cells were fixed, washed three times with cold 10% trichloroacetic acid, and then recovered with 0.5 ml of 0.1 N NaOH. After neutralization with acetic acid, the radioactive content in the cell extracts was measured by liquid scintillation spectrometry.

RESULTS

Isolation of PAR2 cDNA and Stable Expression of Mouse PAR2 cDNA in 293 Cells—To clone the complementary DNA of the PAR2 gene, a number of cell lines were screened for the expression of PAR2 gene by RT-PCR using several pairs of primers based on the mouse PAR2 cDNA sequence (6). Expression of the PAR2 gene was observed in M1, a transgenic mouse kidney cortical duct cell line, and in IEC-6, a normal rat epithelial small intestine cell line. The full-length PAR2 cDNA clone was isolated from both M1 and IEC-6 cells by RT-PCR amplification. The nucleotide sequence was confirmed by sequencing several cDNA clones independently amplified by RT-PCR. Two amino acid residues predicted from the amplified cDNA sequence in M1 cells are different from those reported by Nystedt *et al.* (6): both Lys-133 and Ser-135 in the previous reported sequence have been replaced by alanine, suggesting that polymorphism of sequence in mouse PAR2 gene is present. The amino acid sequence of PAR2 from rat epithelial small intestine cells (IEC-6) shares 93% identity with the mouse PAR2 and 85% identity with the human PAR2.

To investigate the function of PAR2, the cDNA amplified from M1 cells was cloned into the expression vector of pAC-Tag and the resultant plasmid was termed PAR2-HA. In this construct, a sequence coding for three repeats of 9 amino acids taken from HA was joined to the COOH terminus of PAR2 sequence, allowing the expressed PAR2-HA fusion protein to be detected with an anti-HA antibody (12CA5). The PAR2-HA plasmid was transfected into 293 cells, and the G418-resistant cell populations were selected. Expression of tagged PAR2 protein was detected by immunoblotting with the anti-HA antibody. As shown in Fig. 1, the apparent relative molecular mass of the tagged PAR2 on SDS-PAGE is approximately 90 kDa. The calculated molecular mass of the HA-tagged PAR2 protein is, however, approximately 45 kDa. The difference between the

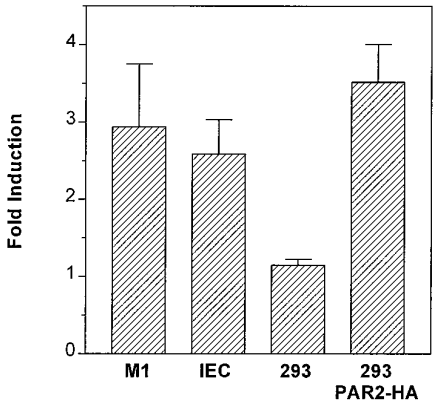

FIG. 2. **Induction of c-*fos*-luciferase expression by SLIGRL.** M1, IEC-6, 293 cells and 293 cells stably transfected with pAC TAG2 vector containing PAR2 cDNA tagged by HA gene (PAR2-HA) were transiently transfected with SRE-Luc vector, which contains a c-*fos* promoter-driven luciferase gene. Cells were stimulated with 100 μM SLIGRL for 6 h following a 16-h starvation. Luciferase activity was then assayed as described under "Experimental Procedures." Luciferase activity was shown as the ratio of SLIGRL-stimulated to unstimulated samples. Results are the means ± S.E. of at least three experiments.

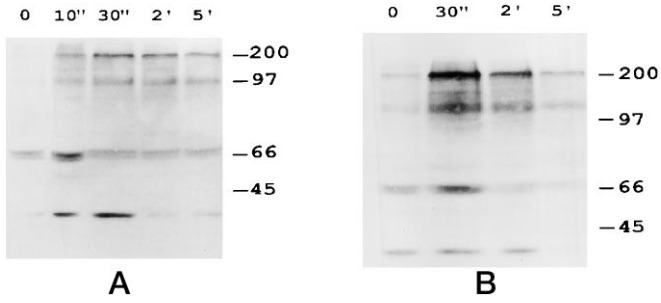

FIG. 3. **Tyrosine phosphorylation in response to SLIGRL and trypsin stimulation.** 293 cells stably transfected with PAR2-HA plasmid were treated for various periods of time with 100 μM SLIGRL (*A*) or 10 nM trypsin (*B*) after starvation for 24 h. The cell lysates containing equal amounts of total protein were subjected to SDS-PAGE and analyzed by Western blotting with anti-phosphotyrosine antibody. Molecular size standards are shown in kDa.

calculated molecular mass and detected size on SDS-PAGE suggests that the PAR2 protein is probably glycosylated, as has been observed in other G protein-coupled receptors, such as in the thrombin receptor (26).

PAR2-mediated Mitogenic Response—To investigate whether PAR2 mediates the mitogenic pathway, the luciferase reporter gene driven by the c-*fos* promoter was used to detect its activation in response to ligand stimulation. The c-*fos* promoter contains a well characterized serum-responsive element (SRE) whose activity can be stimulated upon mitogenic activation of appropriate receptors. The plasmid construct containing the c-*fos* promoter-driven luciferase gene (SRE-Luc) was transiently transfected in M1, IEC-6, and 293 cells, as well as 293 cells stably transfected with the plasmid PAR2-HA. As shown in Fig. 2, the PAR2 tethered peptide ligand SLIGRL significantly stimulated SRE-regulated luciferase activity in M1 and IEC-6 cells, but not in 293 cells. This result, consistent with RT-PCR detection, shows that PAR2 is indeed expressed in M1 and IEC-6 cells, but not in 293 cells. However, when 293 cells were stably transfected with PAR2-HA plasmid, SLIGRL greatly increased the luciferase activity in these cells, suggesting that the cloned PAR2-HA was functionally expressed in these transfected cells. The concentration of SLIGRL required for half-maximal stimulation (EC_{50}) was approximately 10 μM. The expression of the c-*fos*-driven luciferase reporter gene was also stimulated by 0.1 nM trypsin in transfected 293 cells (data not shown). The functional expression of PAR2 in transfected 293 cells was also confirmed by both ligand SLIGRL- and trypsin-induced intracellular calcium mobilization (data not shown).

Protein-tyrosine Phosphorylation in Response to PAR2 Activation—The ligand-induced activation of c-*fos* promoter implies that PAR2 may mediate mitogenic responses in cells. This result prompted us to examine whether PAR2-mediated signaling involves protein-tyrosine phosphorylation or dephosphorylation of signaling proteins. Whole cell extracts were prepared from 293 cells stably transfected with PAR2-HA. The phosphotyrosine content of basal extracts as well as for SLIGRL or trypsin-stimulated extracts was analyzed by immunoblotting with anti-phosphotyrosine antibody. Fig. 3 shows that both SLIGRL and trypsin induced a rapid and transient tyrosine phosphorylation of at least four major proteins whose relative molecular masses are approximately 200, 120, 70, and 30 kDa, respectively. SLIGRL or trypsin did not stimulate protein-tyrosine phosphorylation in untransfected 293 cells (data not shown), suggesting that the tyrosine phosphorylation of proteins occurred specifically in response to the activation of PAR2.

SHP2 Is Phosphorylated on Tyrosine Residues upon Ligand Stimulation—The rapid and transient induction of tyrosine phosphorylation of cellular proteins in response to ligand stimulation implies that putative PTKs and PTPs participate in a PAR2-mediated signaling pathway. Since vanadate is a potent inhibitor of PTPs, we therefore examined its effect on the activation of the c-*fos* promoter by the peptide ligand SLIGRL. As shown in Fig. 4, while vanadate had no effect on the c-*fos* promoter-dependent luciferase activity in non-stimulated cells, it almost completely abolished the ligand-stimulated activation of luciferase expression, suggesting that PTP(s) may participate in PAR2-mediated mitogenic signaling. Since one band of the ligand-stimulated tyrosine-phosphorylated proteins migrated at a position corresponding to around 70 kDa (Fig. 3), we speculated that the 68-kDa SHP2, among others, may have been tyrosine-phosphorylated. To verify this assumption, SHP2 was immunoprecipitated from ligand-stimulated 293 cells stably expressing PAR-HA by a polyclonal antibody specific to the phosphatase. The immunoprecipitates were resolved by SDS-PAGE and subjected to immunoblotting either with anti-phosphotyrosine or with mouse anti-SHP2 monoclonal antibody. As shown in Fig. 5, upon ligand stimulation, SHP2 was indeed rapidly tyrosine-phosphorylated. However, the tyrosine-phosphorylated SHP2 underwent rapid dephosphorylation in less than 5 min. Stimulation with ligand did not affect the amount of SHP2 in the precipitates, but increased the phosphatase activity of SHP2 by approximately 30% when compared with that precipitated from unstimulated cells (data not shown) as has been observed previously (20).

Both the Tyrosine Phosphorylation of SHP2 and the Activation of the c-fos Promoter Are Coupled to PAR2 through a Pertussis Toxin-sensitive G Protein—To determine whether the ligand-stimulated tyrosine phosphorylation of SHP2 in PAR2-mediated signaling is coupled to a pertussis toxin-sensitive G protein, PAR2-HA-transfected 293 cells were treated with pertussis toxin for 16 h. Cells were then incubated in the presence or absence of the ligand. Cell lysates were immunoprecipitated by anti-SHP2 antibody. As shown in Fig. 6, pertussis toxin treatment completely inhibited the ligand-induced tyrosine phosphorylation of SHP2. It should be pointed out that the total abolition of tyrosine phosphorylation of SHP2 by pertussis toxin was observed throughout the entire period of ligand stimulation, *i.e.* from 0.5 min to 15 min. The effect of pertussis toxin treatment on the ligand-induced activation of the c-*fos* promoter was also investigated. Consistent with previous results

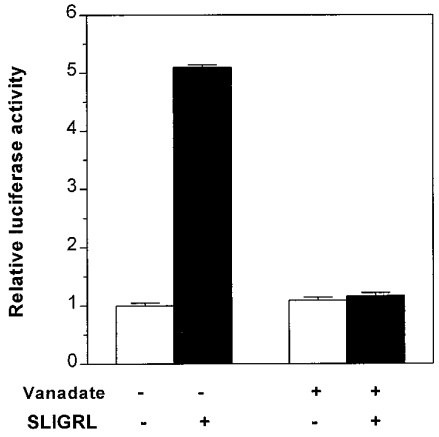

FIG. 4. **Vanadate inhibits the SLIGRL-induced expression of c-*fos*-luciferase.** 293 cells with stably expressed PAR2-HA were transiently transfected with the SRE-Luc vector. After starvation, cells were treated with 100 μM SLIGRL in the presence or absence of 1 mM sodium orthovanadate for 6 h and luciferase activity was assayed as described previously. The data are presented as means ± S.E. of three independent experiments.

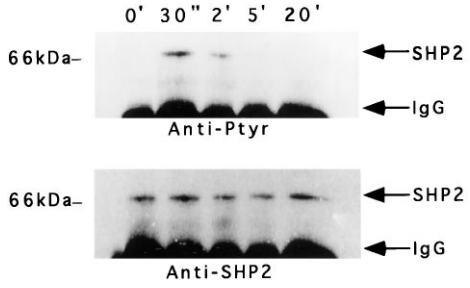

FIG. 5. **SLIGRL induces tyrosine phosphorylation of SHP2.** 293 cells stably expressing PAR2 were starved and stimulated by SLIGRL (100 μM). SHP2 was immunoprecipitated with anti-SHP2 rabbit polyclonal antibodies and immunoblotted with either anti-phosphotyrosine monoclonal antibody or anti-SHP2 monoclonal antibody.

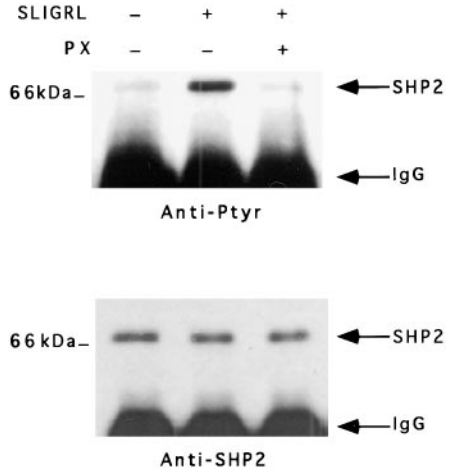

FIG. 6. **Pertussis toxin inhibits the SLIGRL-induced phosphorylation of SHP2.** 293 cells stably expressing PAR2 were starved for 8 h by growing in serum-free DMEM medium and then 16 h in the presence or absence of 100 ng/ml pertussis toxin before stimulation with 100 μM SLIGRL. Cells were lysed, and SHP2 was immunoprecipitated with rabbit anti-SHP2 polyclonal antibodies and immunoblotted with anti-phosphotyrosine monoclonal antibody or anti-SHP2 monoclonal antibody.

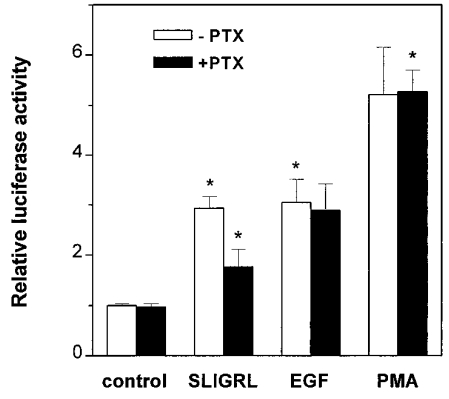

FIG. 7. **Pertussis toxin inhibits SLIGRL-induced c-*fos* promoter-driven luciferase expression.** 293 cells with stably expressed PAR2 were transiently transfected with SRE-Luc reporter plasmids. At 20 h after transfection, the cells were aliquoted and starved by growing in serum-free medium in the presence or absence of 50 ng/ml pertussis toxin for 16 h. Cells were then treated with 100 μM SLIGRL, 10 nM EGF, or 20 nM PMA for 6 h, and luciferase activity was assayed as described previously. The data are presented as means ± S.E. of three independent experiments. *, $p < 0.05$.

(Fig. 2), in PAR2-transfected 293 cells, the peptide ligand SLIGRL stimulated the c-*fos* promoter-driven luciferase activity by nearly 3-fold, a level comparable to the activation observed with EGF stimulation (Fig. 7). However, whereas pertussis toxin suppressed SLIGRL-induced activation of the c-*fos* promoter by 60%, it had no significant effect on the EGF-induced c-*fos*-luciferase expression or on the PMA-stimulated c-*fos* promoter (Fig. 7). These experiments suggest that both the ligand-stimulated phosphorylation of SHP2 on tyrosine residue(s) and ligand-stimulated c-*fos*-luciferase activation in PAR2-mediated signaling are coupled to a pertussis toxin-sensitive G protein.

Overexpression of Catalytically Inactive Mutant SHP2 Inhibited PAR2-mediated Activation of c-fos Promoter—To further clarify the role of SHP2 in PAR2-mediated mitogenic signaling, we investigated the effect of the wild-type and the catalytically inactive mutant of SHP2 on SLIGRL-induced activation of the c-*fos* promoter. As shown in Fig. 8*A*, while the overexpression of wild type SHP2 exerted no significant effect on the SLIGRL-stimulated luciferase activity, the overexpression of a catalytically inactive mutant of SHP2 resulted in a reduction of ligand-stimulated luciferase activity by approximately 50%. In contrast, the expression of the mutant phosphatase had no effect on the PMA-stimulated c-*fos* activation, indicating that the inhibitory effect of mutant SHP2 on the c-*fos*-dependent luciferase activity is specific to SLIGRL ligand stimulation. To investigate whether the inhibitory effect of the catalytically inactive mutant SHP2 on the ligand-stimulated luciferase activity can be overcome by co-overexpression of wild-type SHP2, cells were cotransfected by both the wild-type SHP2 and the catalytically inactive mutant (SHP2C459S) with different ratios of the two plasmid DNAs. The inhibitory effect of the catalytically inactive mutant SHP2 was partially overcome by cotransfection of the wild type SHP2 with the mutant SHP2C459S at a ratio of 4:1 (data not shown). However, when the ratio of the two DNAs increased to 10:1 (wild type:mutant), the inhibitory effect of the mutant on the c-*fos*-dependent luciferase activity was totally abolished (Fig. 8*B*). It should be noted that the abolishing of the inhibitory effect of the mutant SHP2 with the wild type SHP2 was not due to the reduction of the mutant plasmid DNA in the mixture because the same amount of the mutant DNA (2 μg) when mixed with 20 μg of pRc/CMV vector also exerted approximately 50% inhibition of the ligand-stimulated luciferase activity (Fig. 8*B*). These results suggest that the inhibitory effect of the catalytically inactive mutant on the ligand-stimulated luciferase activity was due to a specific inhibition of the endogenous SHP2 activity. Thus, the inhibitory effect of the mutant SHP2 on the activa-

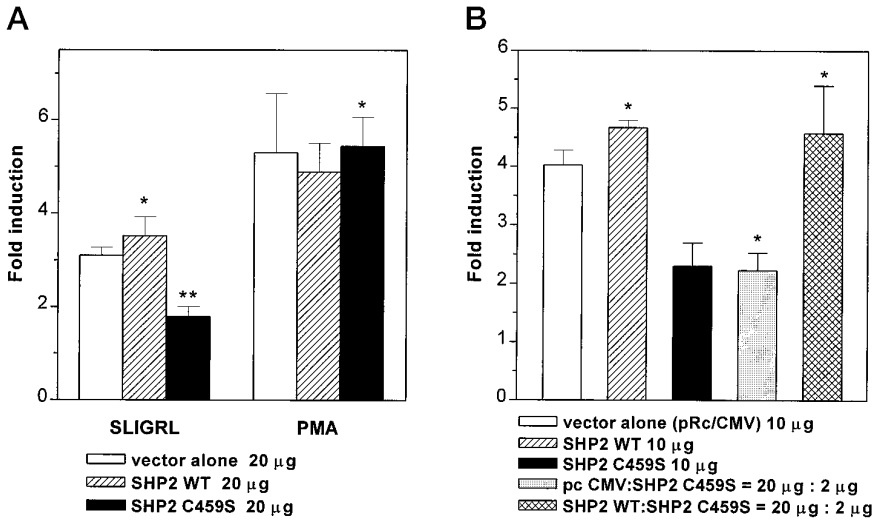

FIG. 8. **Effects of overexpression of wild-type and catalytically inactive SHP2 on SLIGRL-stimulated c-*fos* promoter activation.** 293 cells with stably expressed PAR2 were transiently transfected with 1 μg of SRE-Luc reporter plasmids plus various amount of DNA of each vectors as indicated. The efficiency of transfections was normalized with a reference plasmid (25). Cells were stimulated with 100 μM SLIGRL in *panels A* and *B* or 20 nM PMA in *panel A* after starvation, and luciferase activity was assayed as described under "Experimental Procedures." Luciferase activity was shown as the ratio of SLIGRL-stimulated to unstimulated samples. The data are presented as means ± S.E. from three independent experiments each performed in triplicate. *, $p < 0.05$; **, $p < 0.01$.

tion of the c-*fos* promoter is likely caused by its dominant negative competition with the endogenous SHP2.

Overexpression of Catalytically Inactive Mutant SHP2 Suppressed PAR2-stimulated DNA Synthesis—The functional significance of SHP2 in PAR2-mediated signaling was further investigated by determining the effect of overexpression of the wild-type and the catalytically inactive mutant SHP2 on SLIGRL-induced DNA synthesis. To do this, cells stably expressing PAR2 were cotransfected with the vector alone, wild type SHP2, and mutant SHP2C459S, respectively. For both the wild type SHP2- and mutant SHP2C459S-transfected cells, four stable clones obtained from two independent transfections, all of which expressed approximately 5-fold of the exogenous wild type SHP2 or mutant SHP2C459S when compared with that of untransfected cells (data not shown), were chosen for further characterization. The ligand-stimulated DNA synthesis was examined in all clones by incorporation of [^{3}H]thymidine into DNA. Results were consistently comparable when different clones were used. Fig. 9 is representative of one set of data generated by two different clones. DNA synthesis was stimulated by the ligand maximally at a concentration of approximately 50 μM in all cells transfected with PAR2, but not in parental 293 cells, which do not express PAR2. Most notably, while overexpression of wild type SHP2 exerted no significant effect on ligand-stimulated DNA synthesis, overexpression of mutant SHP2 strongly inhibited SLIGRL-induced [^{3}H]thymidine incorporation by more than 40% as compared with control cells during a 16-h period, demonstrating that the catalytically inactive SHP2 has a strong inhibitory effect on the ligand-stimulated DNA synthesis.

The dramatic inhibitions of both the ligand-induced c-*fos*-dependent luciferase activity and the ligand-stimulated DNA synthesis by overexpression of the catalytically inactive mutant phosphatase demonstrate that SHP2 is a positive regulator of PAR2-mediated signal transduction.

DISCUSSION

Recent studies have suggested that protein-tyrosine phosphorylation plays an important role in regulating G protein-coupled receptors. PAR2 is a newly identified G protein-coupled receptor, which, like the thrombin receptor, is activated by a proteolytic mechanism. Upon stimulation with its ligand, PAR2, expressed either endogenously or exogenously, activates a mitogenic pathway, resulting in protein-tyrosine phosphorylation and an increase of early gene transcription through the activation of the c-*fos* promoter. Vanadate, a potent inhibitor of PTPs, completely blocked the stimulatory activation of the c-*fos*

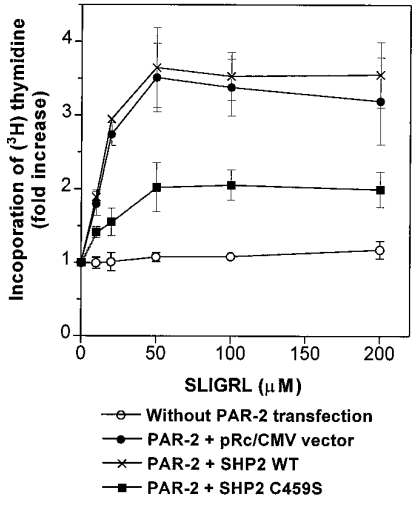

FIG. 9. **Effects of overexpression of wild-type and catalytically inactive SHP2 on SLIGRL-stimulated DNA synthesis.** Quiescent 293 cells stably expressing PAR2 or coexpressing PAR2 with wild type SHP2 or PAR2 with catalytically inactive SHP2 mutant were stimulated with SLIGRL for 16 h in the presence of [^{3}H]thymidine (5 μCi/ml, 72 Ci/mmol) and [^{3}H]thymidine incorporation was assayed. Results are expressed as the ratio of SLIGRL-stimulated to unstimulated samples. The data are presented as means ± S.E. of two independent experiments with two different clones each performed in triplicate.

promoter, suggesting that PTP(s) may have been involved in modulating the PAR2-mediated pathway. Of several SLIGRL-stimulated tyrosine-phosphorylated proteins detected, we have identified one as the Src homology-2 (SH2) domain-containing PTP, SHP2. Once tyrosine-phosphorylated, SHP2 rapidly underwent dephosphorylation (Fig. 5).

Pretreatment of the cells with pertussis toxin resulted in inhibition of both the tyrosine phosphorylation of SHP2 and the activation of the c-*fos* promoter by ligand stimulation, indicating that PAR2 is coupled to a pertussis toxin-sensitive heterotrimeric G protein. However, while pertussis toxin completely blocked the ligand-activated tyrosine phosphorylation of SHP2, this drug could not entirely suppress the ligand-induced activation of the c-*fos* promoter. Approximate 40% of the ligand-stimulated early gene transcription was not inhibited by pertussis toxin treatment (Fig. 7), indicating that the remaining activation (40%) of the c-*fos* promoter in the drug-treated cells was produced in PAR2-mediated mitogenic pathways through functional coupling to other G proteins insensitive to pertussis toxin in 293 cells.

SHP1, a structurally related PTP, was also tyrosine-phosphorylated in the thrombin receptor mediated-pathway (20, 21), but its potential function in the signaling is still unknown. In this report, we found that in addition to being tyrosine-phosphorylated in response to SLIGRL-mediated stimulation, SHP2 also appears to actively regulate PAR2-mediated signaling. Overexpression of wild-type SHP2 slightly increased the ligand-stimulated activation of the c-*fos* promoter and DNA synthesis, whereas expression of its catalytically inactive mutant strongly suppressed both the ligand-stimulated activation of early gene transcription and DNA synthesis, suggesting that the intrinsic phosphatase activity of SHP2 can function as a positive regulator in a G protein-coupled receptor-mediated signaling. In a number of growth factor-activated pathways in which SHP2 has been reported as a positive regulator, the catalytically inactive mutant of SHP2 displayed a similar suppressive effect on mitogen activated-pathways (22, 23, 27–31). In these receptor-PTK-mediated pathways, interaction of SHP2 with the receptors has been well established. Upon activation of the growth factor receptors, SHP2 was tyrosine-phosphorylated and its SH2 domains bound to specific tyrosine-phosphorylated sites of the ligand-activated receptors, such as Tyr-954 of the EGF receptor, Tyr-1009 of the platelet-derived growth factor receptor, and Tyr-546/Tyr-895/Tyr-1172/Tyr-1222 of insulin receptor substrate-1 (32–37). However, in the PAR2-mediated pathway, we were unable to detect an association of SHP2 with the activated PAR2 by co-immunoprecipitation. Consistent with this, we were also unable to detect the tyrosine phosphorylation of PAR2 with an anti-phosphotyrosine antibody. Therefore, in contrast to receptor-PTK-mediated signaling, in the PAR2-activated pathway, SHP2 does not appear to interact directly with PAR2. It may interact with other signaling proteins, which are recruited by the activated PAR2 during its functional coupling to G protein(s). A PTK is likely activated in response to the ligand stimulation, resulting in tyrosine phosphorylation of SHP2 and other proteins. The nature of this specific tyrosine kinase is yet to be identified.

Although our data suggest that SHP2 functions as a positive regulator in PAR2-mediated signaling, the precise mechanism by which the inactive mutant SHP2 is capable of attenuating SLIGRL ligand-activated mitogenic signaling is presently unknown. It is known that the c-*fos* promoter is activated by various signals primarily through a kinase cascade linked to Ras-dependent pathways. It is also known that PMA, a potent activator of protein kinase C, induces mitogen-activated protein kinase activation in a Ras-independent manner (38). Since the mutant SHP2 strongly suppressed the ligand-stimulated c-*fos* promoter activity and DNA synthesis, but the inactive phosphatase did not interfere with PMA activation of the gene, it appears that the mutant SHP2 targets an upstream molecule in the Ras mitogen-activated protein kinase pathway, rather than downstream effector(s). One possible target for SHP2 is a potent regulator in signaling, which is regulated by tyrosine phosphorylation. This regulator protein may be dephosphorylated by SHP2 on phosphotyrosine site(s) which negatively regulate(s) its function, resulting in its activation, such as the Src family of kinases. Identification of such physiological substrate(s) of SHP2 in cells would lead to the elucidation of how the phosphatase positively regulates these signal transduction pathways.

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Protein-tyrosine Phosphatase SHP2 Is Positively Linked to Proteinase-activated Receptor 2-mediated Mitogenic Pathway

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