

Src Homology 3 Binding Sites in the P2Y₂ Nucleotide Receptor Interact with Src and Regulate Activities of Src, Proline-rich Tyrosine Kinase 2, and Growth Factor Receptors*

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Jun Liu^{‡§}, Zhongji Liao[‡], Jean Camden[‡], Korey D. Griffin^{‡¶}, Richard C. Garrad^{||},
Laura I. Santiago-Pérez^{**}, Fernando A. González^{**}, Cheikh I. Seye[‡], Gary A. Weisman[‡],
and Laurie Erb^{‡‡}

From the [‡]Department of Biochemistry, University of Missouri-Columbia, Columbia, Missouri 65212,

^{||}Department of Biomedical Sciences, Southwest Missouri State, Springfield, Missouri 65804, and

^{**}Department of Chemistry, University of Puerto Rico, Rio Piedras, Puerto Rico 00931

Many G protein-coupled receptors activate growth factor receptors, although the mechanisms controlling this transactivation are unclear. We have identified two proline-rich, SH3 binding sites (PXXP) in the carboxyl-terminal tail of the human P2Y₂ nucleotide receptor that directly associate with the tyrosine kinase Src in protein binding assays. Furthermore, Src co-precipitated with the P2Y₂ receptor in 1321N1 astrocytoma cells stimulated with the P2Y₂ receptor agonist UTP. A mutant P2Y₂ receptor lacking the PXXP motifs was found to stimulate calcium mobilization and serine/threonine phosphorylation of the Erk1/2 mitogen-activated protein kinases, like the wild-type receptor, but was defective in its ability to stimulate tyrosine phosphorylation of Src and Src-dependent tyrosine phosphorylation of the proline-rich tyrosine kinase 2, epidermal growth factor receptor (EGFR), and platelet-derived growth factor receptor. Dual immunofluorescence labeling of the P2Y₂ receptor and the EGFR indicated that UTP caused an increase in the co-localization of these receptors in the plasma membrane that was prevented by the Src inhibitor PP2. Together, these data suggest that agonist-induced binding of Src to the SH3 binding sites in the P2Y₂ receptor facilitates Src activation, which recruits the EGFR into a protein complex with the P2Y₂ receptor and allows Src to efficiently phosphorylate the EGFR.

Src and proline-rich tyrosine kinase 2 (Pyk2)¹ are non-receptor tyrosine kinases that have been implicated as intermedi-

ates in the signaling pathway by which some G protein-coupled receptors (GPCRs) transactivate growth factor receptors (1–3). Although Src and Pyk2 activities are thought to be necessary for the transactivation of growth factor receptors by GPCRs, there are differing opinions in the literature about the role these kinases play in the activation of downstream mitogenic signaling. For example, inhibition of Src activity by cellular expression of C-terminal Src kinase was found to impair lysophosphatidic acid (LPA) and β 2-adrenergic receptor-mediated activation of MAP kinases in COS-7 cells (1, 4). In rat-1 fibroblasts, dominant-negative mutants of the epidermal growth factor receptor (EGFR) or Src were used to demonstrate that the EGFR and Src are important for linking GPCR activation with the activation of MAP kinases (5, 6). And in PC12 cells, a dominant-negative mutant of Pyk2 and the EGFR kinase inhibitor AG1478 inhibited GPCR-mediated MAP kinase activation (2, 7). In contrast, experiments performed with embryonic fibroblasts derived from Src^{−/−}, Pyk2^{−/−}, or Src^{−/−}Pyk2^{−/−} mice indicated that both Src and Pyk2 are essential for GPCR-mediated transactivation of the EGFR but are dispensable for GPCR-mediated activation of MAP kinases (3).

In the present study, we have expressed wild-type and mutant P2Y₂ nucleotide receptors in human 1321N1 astrocytoma cells to explore how this GPCR transactivates growth factor receptors and affects mitogenic signaling. The P2Y₂ receptor is a G_q/G₁₂-coupled receptor that is activated by extracellular ATP or UTP and is up-regulated in a variety of tissues in response to injury or stress (8, 9). For example, expression of the P2Y₂ receptor is dramatically increased in endothelial and smooth muscle cells by collar-induced stress to rabbit carotid arteries (9). Furthermore, *in situ* activation of the up-regulated P2Y₂ receptor in collared arteries promotes intimal hyperplasia by increasing smooth muscle cell proliferation and leukocyte infiltration (9). Therefore, the role of P2Y₂ receptors in atherosclerosis, inflammation, wound repair, and mitogenesis is of great interest, and the regulation of this receptor is likely to become an important therapeutic target. The results of the present study demonstrate that two consensus SH3 binding sites in the carboxyl-terminal tail of the P2Y₂ receptor bind directly to Src and are important for P2Y₂ receptor-mediated activation of several tyrosine kinases, including Src, Pyk2, and the growth factor receptors, EGFR and platelet-derived growth factor receptor (PDGFR), but not for other P2Y₂ receptor-mediated responses, including intracellular calcium mobilization or activation of MAP kinases. Activation of the P2Y₂ receptor also increased clustering of this receptor with the EGFR that was dependent upon Src kinase activity. These data suggest a mechanism for transactivation of the EGFR by the P2Y₂ recep-

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§ Supported by a pre-doctoral fellowship from the American Heart Association-Heartland Affiliate. Present address: Dept. of Pharmacology, Univ. of North Carolina, Chapel Hill, NC 27514.

¶ Present address: Santa Cruz Biotechnology, Inc., Santa Cruz, CA 95060.

‡‡ To whom correspondence should be addressed: M743 Medical Sciences Bldg, Univ. of Missouri-Columbia, Columbia, MO 65212. Tel.: 573-884-2065; Fax: 573-884-4597; E-mail: erbl@missouri.edu.

¹ The abbreviations used are: Pyk2, proline-rich tyrosine kinase 2 or focal adhesion-related kinase; GPCR, G protein-coupled receptor; LPA, lysophosphatidic acid; [Ca²⁺]_i, concentration of cytoplasmic free calcium; PP2, pyrazole pyrimidine-type 2 inhibitor; EGFR, epidermal growth factor receptor; Erk1/2, extracellular signal-regulated kinase (MAP kinase p42/p44); PBS, phosphate-buffered saline; HA, hemagglutinin; MAP, mitogen-activated protein; PDGFR, platelet-derived growth factor receptor; SH3, Src homology 3.

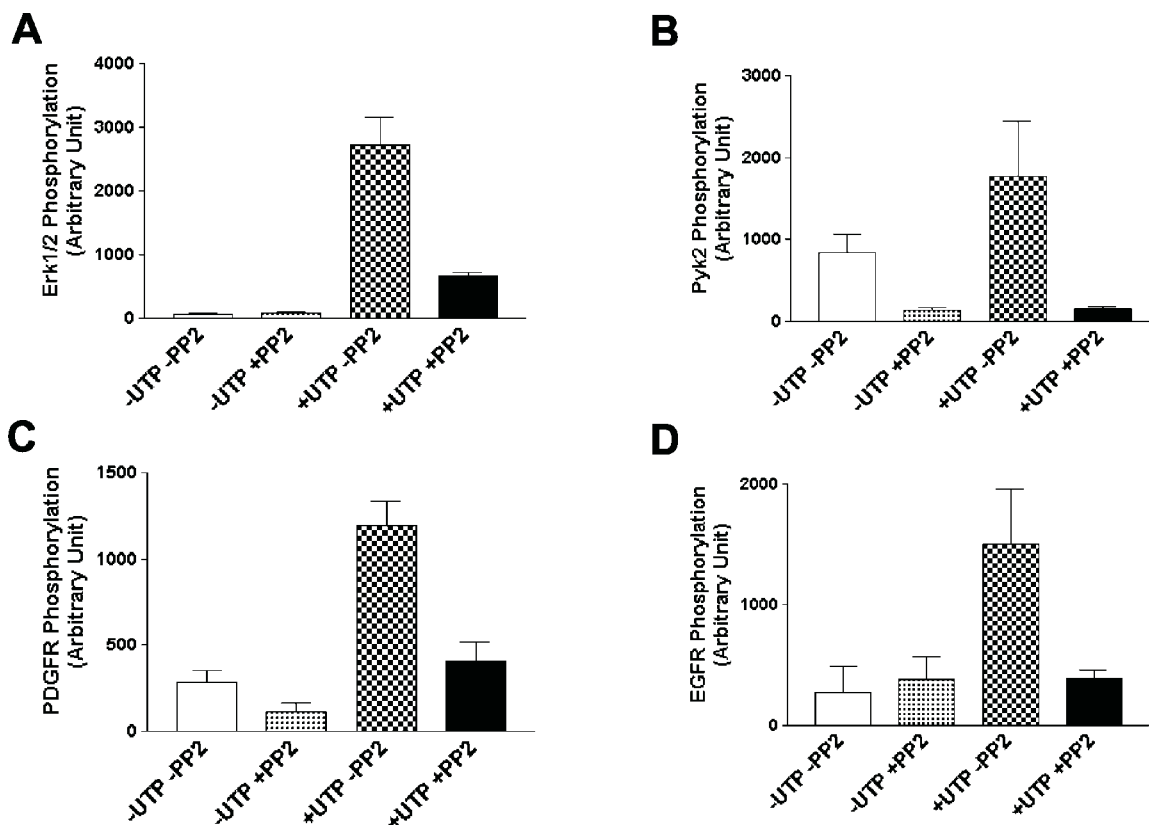


FIG. 1. Effect of the Src kinase inhibitor PP2 upon signaling pathways mediated by the P2Y₂ receptor. 1321N1 cells expressing the wild-type P2Y₂ receptor were treated with 10 μ M PP2 for 30 min and stimulated with 10 μ M UTP for 5 min. Cell lysates were prepared and analyzed by immunoblotting. Phosphorylation of Erk1/2 (A), Pyk2 (on Tyr⁸⁸¹) (B), PDGFR (on Tyr⁷¹⁶) (C), and EGFR (on Tyr⁹⁹²) (D) was detected with the appropriate antibodies, and the results were quantified and normalized as described under "Experimental Procedures." The data are the mean \pm S.E. of results from three experiments.

tor involving agonist-induced binding of Src to the SH3 binding sites in the P2Y₂ receptor.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Human 1321N1 astrocytoma cells lacking endogenous P2 receptor activities (10) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 5% fetal bovine serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Cells were maintained at 37 °C in a humidified atmosphere with 5% CO₂ and 95% air. The retroviral vector pLXSN was used for stable expression of the various human P2Y₂ receptor constructs in 1321N1 cells, as described previously (11).

Mutagenesis of Human P2Y₂ Receptor cDNA—Two primers were synthesized in the DNA Core Facility of the University of Missouri-Columbia (primer 1, 5'-AGGCTCGTACGCTTTGCCCGAGATGCCAAGGCTCGCCGAGGCTGGGCCTGCCGAGATC-3'; primer 2, 5'-CACACCCTAACTGACAC-3') and were used to generate (by PCR) cDNA encoding the P2Y₂ receptor deletion mutant. The PCR mixture contained 3.8 μ l of each primer (final amount, 20 pmol), 10 μ l of 10 \times Taq polymerase buffer with MgCl₂ (Roche Applied Science), 2 ng of template DNA, 1 μ l of TaqDNA polymerase (Roche Applied Science), 20 μ l of dNTP mixture (0.2 mM final concentration each of dATP, dCTP, dTTP, and dGTP), and sterile distilled water in a final volume of 100 μ l. The PCR parameters were: 30 cycles of 95 °C/1 min, 58 °C/1 min, and 72 °C/6 min. After verification of the PCR products by agarose gel electrophoresis, the products were purified using the PCR Wizard kit (Amersham Biosciences), digested with BsiWI and BamHI and inserted into pLXSN. The mutant cDNA was verified by DNA sequencing.

In Vitro Peptide Binding Assay—Three peptides were synthesized in the Protein Core Facility of the University of Missouri-Columbia. The Src binding peptide (RALPPLPRY) was reported to have high affinity for Src (K_d = 7.8 μ M) (12). The P2Y₂ PXXP peptide (AKPPTGPSPAT-PARRR) comprised the consensus SH3 binding sites in the carboxyl-terminal tail of the human P2Y₂ receptor (amino acid numbers 320–335). The P2Y₂ C-tail peptide (IGDVLGSSSEDFRRTSTPAGSENTKD) comprised a sequence from the carboxyl-terminal tail of the human P2Y₂ receptor that lacks the proline-rich region (amino acid numbers

349–374). Each peptide was coupled to activated CH-Sepharose 4B (Amersham Biosciences) according to the manufacturer's directions. Purified Src (Calbiochem) was incubated overnight at 4 °C with 60 μ l of 50% Sepharose slurry plus 200 μ l of bovine serum albumin blocking solution (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.01% Tween 20, 5% bovine serum albumin, 0.02% NaN₃) and 800 μ l of Cantley lysis buffer (4.5 M NaCl, 0.9 M Tris-HCl, pH 7.4, 90 mM MgCl₂, 90 mM CaCl₂, 10% glycerol, 1% Nonidet P-40, 0.0018% NaVO₄). The next day, Sepharose-bound proteins were washed three times: first in washing buffer 1 (1 mM CaCl₂, 1 mM MgCl₂, 1% Nonidet P-40 in phosphate-buffered saline (PBS), pH 7.4), then in washing buffer 2 (100 mM Tris-HCl, pH 7.5, 0.5 M LiCl), and finally in washing buffer 3 (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA). The proteins were solubilized by incubation for 1.5 min at 95 °C in 2 \times Laemmli's sample buffer containing 100 mM dithiothreitol. The proteins were electrophoresed on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with rabbit polyclonal anti-c-Src antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:400 dilution.

Measurement of the Intracellular Calcium Concentration—The intracellular free calcium concentration ([Ca²⁺]_i) was measured by dual excitation spectrofluorometric analysis of cell suspensions loaded with fura-2 (Sigma), as described previously (13), and cells were assayed in HEPES-buffered saline (10 mM HEPES, pH 7.4, 130 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 5 mM glucose). Concentration-response data for UTP were analyzed with the Prism curve-fitting program (GraphPad Software Inc., San Diego, CA).

Detection of Tyrosine Phosphorylation of Src, Erk1/2, Pyk2, EGFR, and PDGFR—Cells were grown to 80% confluence in 6-well tissue culture plates and incubated at 37 °C in medium without serum for 24 h before the experiments. After stimulation (as described in the figure legends), the cells were washed with ice-cold PBS and lysed in 0.3 ml of Laemmli's sample buffer containing 100 mM dithiothreitol. The lysates were heated in boiling water for 1.5 min, sonicated for 2 s, resolved by SDS-PAGE, and transferred to nitrocellulose membranes. Immunoblotting of phosphorylated proteins on the membranes was performed by incubation overnight at 4 °C in TBS (10 mM Tris-HCl, pH 7.4, 120 mM NaCl) containing 5% non-fat milk and 1:1000 dilution of rabbit poly-

clonal anti-phospho-Src (Tyr⁴¹⁶) antibody (Cell Signaling Technology, Beverly, MA), 1:1200 dilution of mouse monoclonal anti-phospho-Erk1/2 antibody (Cell Signaling Technology), 1:1000 dilution of rabbit polyclonal anti-phospho-Pyk2 (Tyr⁸⁸¹) antibody (BIOSOURCE International, Camarillo, CA), 1:1000 dilution of rabbit polyclonal anti-phospho-EGFR (Tyr⁹⁹²) antibody (Cell Signaling), or 1:1000 dilution of rabbit polyclonal anti-phospho-PDGFR (Tyr⁷¹⁶) antibody (Upstate Biotechnology, Lake Placid, NY). Then, the nitrocellulose membranes were washed four times in TTBS (TBS containing 0.5% Tween 20), incubated for 1 h at room temperature in TBS with 1:2000 dilution of HRP-conjugated goat anti-mouse or anti-rabbit IgG, and washed three times in TTBS. Tyrosine phosphorylated proteins were visualized on x-ray film with the Western blotting Luminol reagent system (Santa Cruz Biotechnology). Chemiluminescent protein bands detected on x-ray films were quantitated by using a computer scanner and Quantity One software (Bio-Rad). For normalization of the signals, the membranes were stripped of antibodies by incubation at 60 °C for 30 min in stripping buffer (62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, and 2% SDS), washed with TTBS, reprobed with 1:3000 dilution of rabbit anti-Erk1 antibody (Santa Cruz Biotechnology), and the proteins were visualized as above.

Dual Fluorescence Labeling—Cells stably expressing amino-terminal hemagglutinin (HA) epitope-tagged P2Y₂ receptor constructs were grown to 60% confluence on coverslips in 35-mm dishes. Cells were serum-starved overnight followed by incubation with rabbit anti-HA antibody (1:100; Santa Cruz Biotechnology) and mouse anti-human EGFR antibody (1:100; Santa Cruz Biotechnology) for 1 h at 37 °C. Cells were washed three times with PBS and then incubated with 1:200 dilution of goat anti-rabbit Alexa FluorTM 594-conjugated IgG and goat anti-mouse Oregon GreenTM 488-conjugated IgG antibodies (Molecular Probes, Eugene, OR) in serum-free medium for 1 h at 37 °C. The cells were washed in PBS and allowed to re-equilibrate in serum-free medium for 1 h prior to subsequent stimulation with 100 μM UTP for 5 min. Cells were then promptly fixed in 4% paraformaldehyde for 10 min, washed three times in PBS, rinsed in H₂O, and mounted on glass slides in ProLong Antifade reagent (Molecular Probes).

Confocal Microscopy—Confocal microscopy was performed on a Bio-Rad Radiance 2000 system coupled with an Olympus IX70 microscope using a 60× water-immersion lens (numerical aperture 1.3). The data were processed with LaserSharp 2000 software. Co-localization experiments with Alexa FluorTM 594-conjugated IgG to detect the HA-tagged P2Y₂ receptor and Oregon Green 488-conjugated IgG to detect the EGFR were performed at 590 nm excitation/617 nm emission and 496 nm excitation/524 nm emission wavelengths for the respective antibodies. Yellow pixels, representing co-localization of the P2Y₂ receptor and EGFR, were quantified in single cells using Meta Imaging Series version 4.5 (Universal Imaging Corporation, Downingtown, PA). In brief, the images representing the P2Y₂ receptor and the EGFR were normalized for their intensities before single cells were selected. The number of yellow pixels representing co-localization *versus* the number of pixels representing total P2Y₂ receptors was recorded. The extent of co-localization was expressed as a percentage of the total P2Y₂ receptors detected.

Immunoprecipitation of the P2Y₂ Receptor—Cells expressing HA-tagged P2Y₂ receptor constructs were grown to 80% confluence in 100-mm tissue culture plates and transiently transfected for 5 h with 24 μg of c-Src cDNA inserted in vector pUSEamp (Upstate Biotechnology) using LipofectAMINE 2000 transfection reagent (Invitrogen) at a DNA:LipofectAMINE ratio of 1:5. The cells were then serum-starved for 24 h and stimulated in the presence or absence of 100 μM UTP. The cells were washed with cold PBS and incubated in 0.4 ml of lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, and Complete protease inhibitor mixture (Roche Applied Science)) for 10 min at 4 °C. The lysates were centrifuged at 12,000 × g for 5 min, and a Lowry assay was performed on the supernatant to determine protein concentration. Then, 200 μg of the supernatant was incubated with 50 μl of anti-HA antibody conjugated to agarose beads (Roche Applied Science) overnight at 4 °C. The beads were washed three times with lysis buffer, resuspended in 80 μl of 2× Laemmli's sample buffer containing 100 mM dithiothreitol and heated in boiling water for 5 min to solubilize and dissociate the proteins. The solubilized proteins (30 μl/lane) were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblotting of Src on the membranes was performed by incubation overnight at 4 °C in TBS (10 mM Tris-HCl, pH 7.4, 120 mM NaCl) containing 5% non-fat milk and 1:1000 dilution of mouse monoclonal anti-Src antibody (Upstate Biotechnology) or rabbit polyclonal anti-phospho-Tyr⁴¹⁶ Src antibody (Cell Signaling Technology). Then, the nitrocellulose membranes were washed four times in TTBS, incubated for 1 h at room temperature in TBS with 1:1000 dilution of HRP-

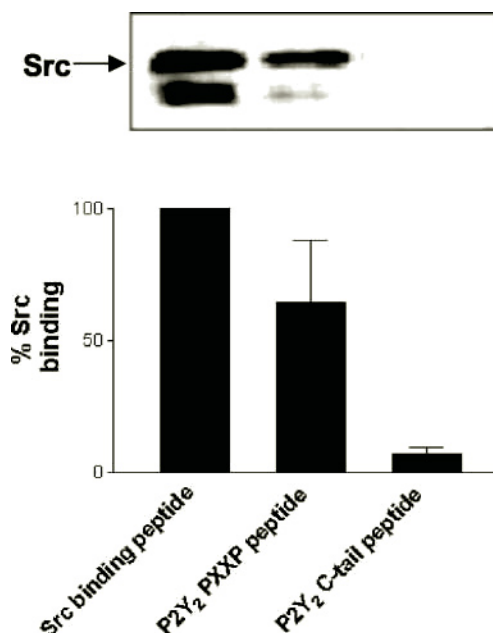


FIG. 2. In vitro binding of Src to the P2Y₂ receptor PXXP motifs. The P2Y₂ PXXP peptide (AKPPTGSPATPARRR) that mimics the consensus SH3 binding sites in the P2Y₂ receptor, the P2Y₂ C-tail peptide (IGDVLGSSSEDFRRTESTPAGSENTKD) that mimics a carboxyl-terminal region of the P2Y₂ receptor lacking prolines, and a positive control Src binding peptide (RALPLPRY) (12) were coupled to activated CH-Sepharose 4B. Binding of these peptides to purified Src was detected by immunoblotting with anti-Src antibody. A representative blot and the mean ± S.E. of results from three experiments are shown.

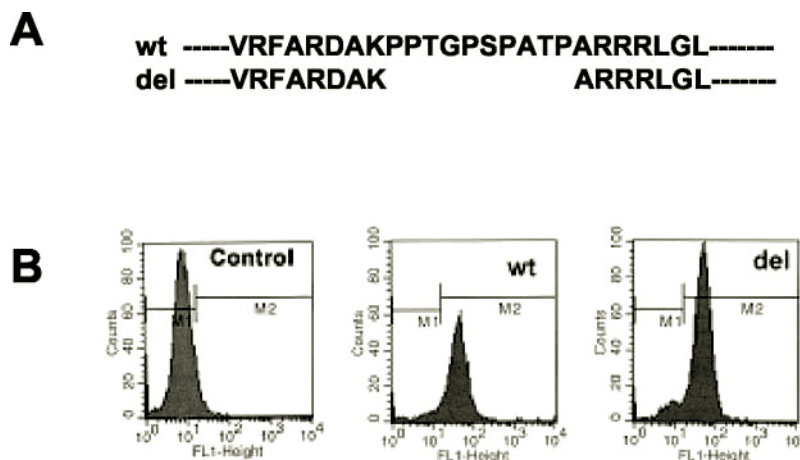
conjugated goat anti-mouse IgG antibody, and washed three times in TTBS. Proteins were visualized on x-ray film with the Luminol reagent as described above.

RESULTS

Role of Src Tyrosine Kinase in P2Y₂ Receptor Signaling—Consistent with a role for Src in signaling pathways coupled to the wild-type P2Y₂ receptor expressed in human 1321N1 astrocytoma cells, the Src inhibitor PP2 decreased phosphorylation of a number of signaling molecules induced by the P2Y₂ receptor agonist UTP. PP2 partially inhibited the UTP-induced activation of the MAP kinases Erk1/2 (Fig. 1A), consistent with the Src-dependence of Erk1/2 phosphorylation by many GPCRs (1, 14). Pyk2 was phosphorylated on Tyr⁸⁸¹ (Fig. 1B) but not on Tyr⁴⁰² (data not shown) upon UTP stimulation of 1321N1 cells expressing P2Y₂ receptors, similar to Pyk2 activation mediated by the P2Y₂ receptor in PC12 cells (15), and Tyr⁸⁸¹-Pyk2 phosphorylation was completely inhibited by PP2 (Fig. 1B). In UTP-treated 1321N1 cells expressing P2Y₂ receptors, PP2 also inhibited phosphorylation of the PDGFR-β on Tyr⁷¹⁶ (Fig. 1C), a reported Grb2 binding site (16), and phosphorylation of the EGFR on Tyr⁹⁹² (Fig. 1D), consistent with phosphorylation of EGFR mediated by the P2Y₂ receptor in PC12 cells (2).

In Vitro Binding of Src to Peptides Matching the Consensus SH3 Binding Sites of the P2Y₂ Receptor—A region in the carboxyl-terminal tail of the human P2Y₂ receptor (AKPPTGSPATPARRR) contains two PXXP motifs (P, proline; X, any amino acid) that are consensus binding sequences for SH3 domains of many proteins, including Src and PI3 kinase (17). To determine whether the consensus SH3 binding sites in the P2Y₂ receptor interact with Src, *in vitro* Src binding assays were performed with synthetic peptides. Results shown in Fig. 2 indicate that Src binds to a synthetic peptide (P2Y₂ PXXP peptide) matching the carboxyl-terminal region of the P2Y₂ receptor containing the SH3 binding sites. In contrast, a peptide (P2Y₂ C-tail peptide) designed from the carboxyl-terminal

FIG. 3. Sequence and expression of a P2Y₂ receptor deletion mutant lacking the PXXP motifs. A, sequence of the PXXP motifs in the carboxyl terminus of the human P2Y₂ receptor (*wt*). A mutation that deletes the PXXP motifs from the P2Y₂ receptor is shown (*del*). B, cell surface expression of HA-tagged *wt* or *del* P2Y₂ receptors in 1321N1 cells was determined by flow cytometry. Untransfected 1321N1 cells were used as a negative control.



IP: HA-epitope of P2Y₂ receptor

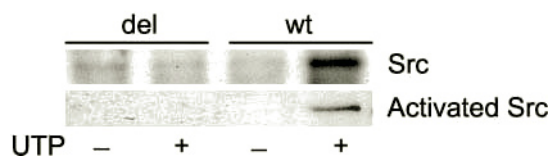


FIG. 4. Agonist-dependent binding of Src to the P2Y₂ receptor. 1321N1 cells expressing HA-tagged wild-type (*wt*) or mutant (*del*) P2Y₂ receptors were transiently transfected with human c-Src cDNA, and the cells were incubated with or without 100 μ M UTP for 5 min, as indicated. Immunoprecipitation of HA-tagged receptors was performed, and co-precipitated Src was detected by immunoblot analysis with specific antibodies against total Src or activated Src (phosphorylated on Tyr⁴¹⁶). The data are representative of results from three experiments. IP, immunoprecipitation.

region of the P2Y₂ receptor that lacks the SH3 binding domains did not show appreciable Src binding. A peptide (Src binding peptide) selected from a combinatorial peptide library and known to have high binding affinity for Src (12) was used as a positive control. The band below p60 Src (Fig. 2) is believed to be a degradation product of Src.

Sequence and Expression of a P2Y₂ Receptor Lacking the PXXP Motifs—To assist in determining the function of the P2Y₂ receptor PXXP motifs, a deletion mutant lacking the PXXP motifs was constructed (Fig. 3A, *del*). Also, a hemagglutinin (HA) epitope tag was incorporated at the amino terminus of the P2Y₂ receptor constructs for immunological analysis (18). Cell surface expression of the deletion mutant was verified by using flow cytometry, which indicated that the deletion mutant was expressed at a level equivalent to the wild-type P2Y₂ receptor (Fig. 3B).

Association of Src with the Activated P2Y₂ Receptor—To further address the possibility that the PXXP motifs in the P2Y₂ receptor interact with Src, immunoprecipitation experiments were performed. The results showed that upon UTP stimulation, Src co-precipitated with the wild-type P2Y₂ receptor but not with the deletion mutant (Fig. 4, top), suggesting that activation of the P2Y₂ receptor allows Src to bind to the PXXP motifs in this receptor. Furthermore, the Src protein that co-precipitated with the wild-type P2Y₂ receptor was phosphorylated at Tyr⁴¹⁶ (Fig. 4, bottom), an autophosphorylation site that stimulates Src kinase activity (19).

Deletion of the PXXP Motifs Does Not Affect P2Y₂ Receptor-mediated Calcium Mobilization or MAP Kinase Phosphorylation—The deletion mutant was used to investigate the role of the PXXP motifs on downstream signaling of the P2Y₂ receptor. The results indicated that the deletion mutant mediated UTP-

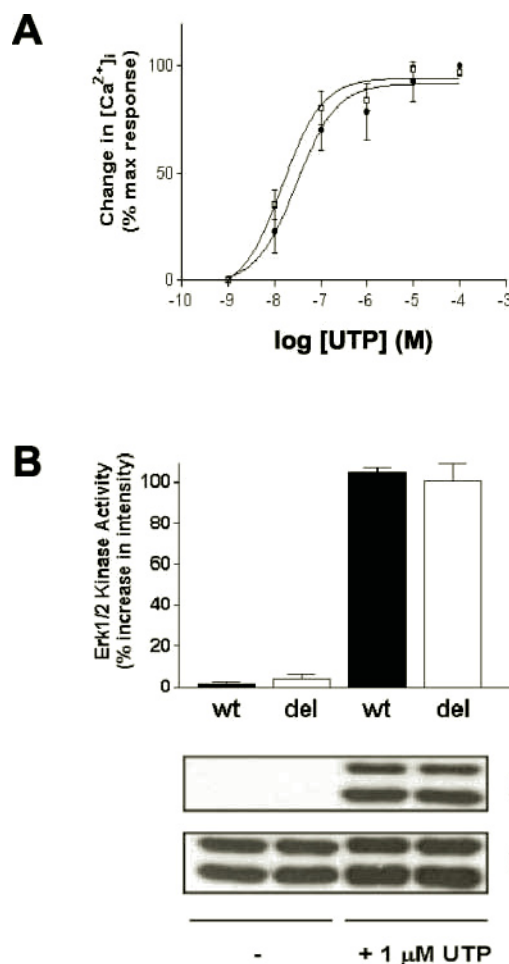


FIG. 5. Deletion of the P2Y₂ receptor PXXP motifs does not affect receptor-mediated calcium mobilization or Erk1/2 phosphorylation. A, $[Ca^{2+}]_i$ was measured in 1321N1 cells expressing *wt* (\square) or *del* (\bullet) P2Y₂ receptors after incubation with the indicated concentration of UTP. The results are expressed as a percentage of the maximal increase in $[Ca^{2+}]_i$ elicited by 100 μ M UTP for each receptor construct. The data are the mean \pm S.E. of results from three to four experiments. B, 1321N1 cells expressing the indicated receptor constructs were incubated with or without 1 μ M UTP for 5 min. Phosphorylated Erk1/2 and total Erk1/2 were detected by immunoblot analysis. A representative blot and the mean \pm S.E. of data from three experiments normalized to total Erk1/2 are shown.

induced calcium mobilization (Fig. 5A), phosphorylation of the MAP kinases, Erk1/2 (Fig. 5B), and phosphorylation of the stress-activated kinases p38 and JNK1/2/3 (data not shown), similar to the wild-type receptor.

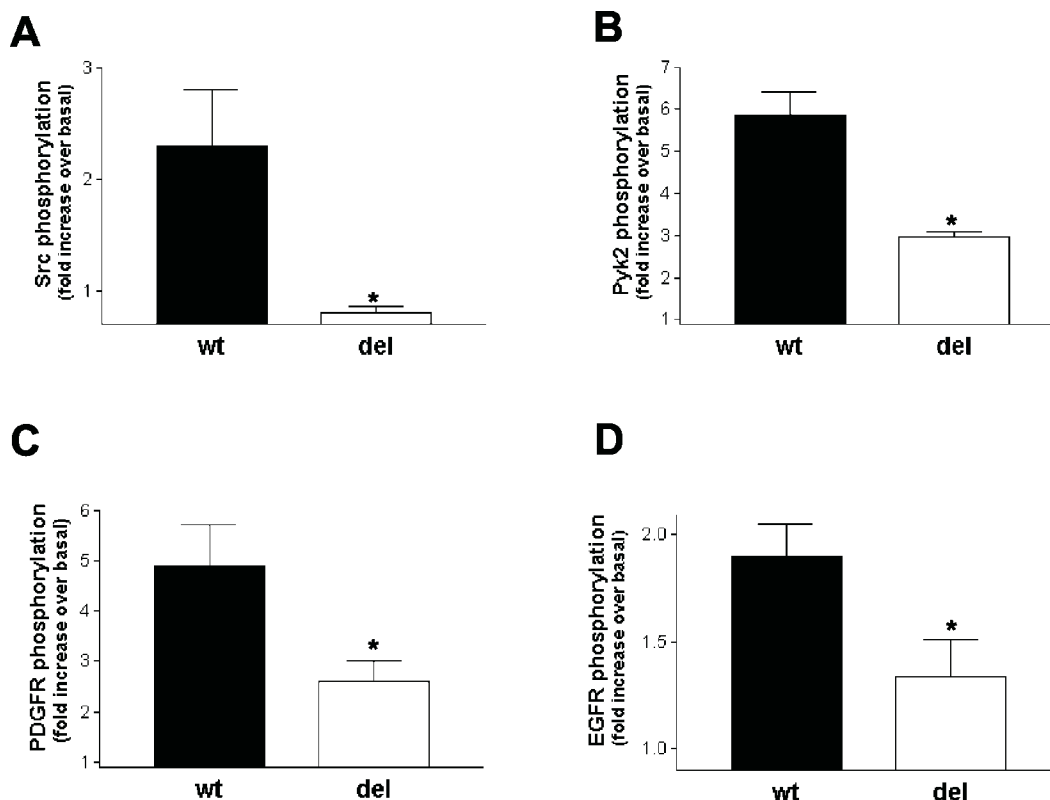


FIG. 6. The PXXP motifs of the P2Y₂ receptor are required for phosphorylation of Src, Pyk2, PDGFR, and EGFR. 1321N1 cells expressing wt or del P2Y₂ receptors were incubated with 1 μ M UTP for 5 min. Cell lysates were prepared and analyzed by immunoblotting. Phosphorylation of Src (on Tyr⁴¹⁶) (A), Pyk2 (on Tyr⁸⁸¹) (B), PDGFR (on Tyr⁷¹⁶) (C), and EGFR (on Tyr⁹⁹²) (D) was detected with the appropriate antibodies, and the results were quantified and normalized as described under "Experimental Procedures." The data are the mean \pm S.E. of results from three to four experiments. *, $p < 0.05$, according to analysis of variance in a randomized complete block design.

Phosphorylation of Src, Pyk2, PDGFR, and EGFR Is Mediated by the PXXP Motifs of the P2Y₂ Receptor—In comparison to the wild-type P2Y₂ receptor, Src phosphorylation induced by UTP was decreased by 85% for the deletion mutant (Fig. 6A). In addition, UTP-induced phosphorylation of Pyk2 (Fig. 6B), PDGFR (Fig. 6C), and EGFR (Fig. 6D) was decreased by ~50% for the deletion mutant compared with the wild-type P2Y₂ receptor.

Co-localization of the EGFR with the Activated P2Y₂ Receptor—To determine whether the P2Y₂ receptor associates with growth factor receptors, we used immunofluorescence to analyze the distribution of P2Y₂ receptors relative to EGFRs. The results indicated that UTP caused co-localization of the wild-type P2Y₂ receptor and EGFR in the plasma membrane (Fig. 7A). Furthermore, this UTP-induced co-localization was inhibited by pretreatment of the cells with PP2 (Fig. 7B), suggesting a role for Src kinase in mediating co-localization of the P2Y₂ receptor and EGFR. UTP did not induce co-localization of the deletion mutant with the EGFR (data not shown), further suggesting that Src bound to the PXXP motifs in the P2Y₂ receptor is responsible for mediating co-localization of these receptors.

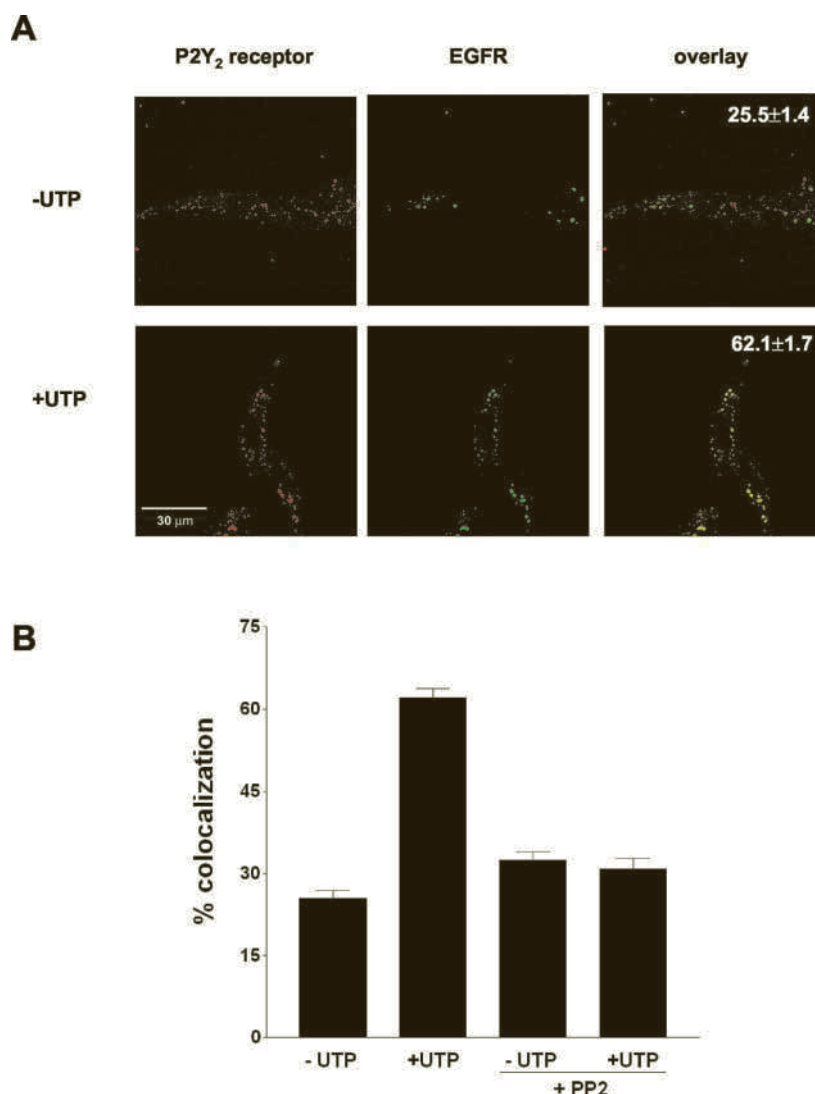
DISCUSSION

SH3 binding sites (PXXP motifs) serve important functional roles in many GPCRs. For example, a recent study has shown that PXXP motifs in the third intracellular loop and the carboxyl-terminal tail of the β_3 adrenergic receptor interact directly with Src and are required for Erk1/2 activation (14). PXXP motifs in the β_1 adrenergic receptor interact with endophilins, SH3 domain-containing proteins, and are involved in receptor internalization and receptor coupling to G proteins (18, 19). In the dopamine D4 receptor, PXXP motifs have been

implicated in the control of receptor internalization as well as coupling to adenylyl cyclase and MAP kinase (20). Our findings indicate that the PXXP motifs in the carboxyl-terminal tail of the P2Y₂ receptor interact directly with Src and are required for activation of Src, Pyk2, and growth factor receptors.

Although transactivation of growth factor receptors by GPCRs has been reported to cause Src-dependent activation of Erk1/2 in some cells (5, 6), the results of the present study show that the PXXP motifs of the P2Y₂ receptor are not required for activation of Erk1/2 but are required for transactivation of growth factor receptors. One explanation for this discrepancy could be that multiple pathways couple the P2Y₂ receptor to Erk1/2 activation and inhibition of one pathway (e.g. EGFR transactivation mediated by Src binding to PXXP motifs in the P2Y₂ receptor) can be compensated for by an increase in Erk1/2 activity by means of another pathway. Another explanation could be that other proteins in a complex with the P2Y₂ receptor via different domains than the PXXP motifs are also responsible for interacting with Src, and it is this interaction that allows Src to activate Erk1/2. In support of this hypothesis, an RGD integrin binding motif in the first extracellular loop of the P2Y₂ receptor was found to be important for coupling this receptor to $\alpha_v\beta_3/\beta_5$ integrins, as well as to Erk1/2 activation (21). Because Src is also an intermediate in the $\alpha_v\beta_3$ signaling pathway (22), this may explain why the Src inhibitor, PP2, inhibited P2Y₂ receptor-mediated Erk1/2 activation in 1321N1 cell transfectants (Fig. 1), whereas deletion of the PXXP motifs in the P2Y₂ receptor had no effect on Erk1/2 activation (Fig. 5B). The inhibitory effect of PP2 on P2Y₂ receptor-mediated Erk1/2 activity in this study is consistent with effects of Src kinase inhibitors on GPCR-mediated Erk1/2 activity (23, 24) and with inhibition of Src activity by over-expression of C-

FIG. 7. UTP-induced co-localization of P2Y₂ and EGFR receptors is dependent upon Src kinase activity. 1321N1 cells expressing HA-tagged P2Y₂ receptors and endogenous EGFR were treated with or without 10 μ M PP2 for 30 min and incubated with or without 10 μ M UTP for 5 min, as indicated. **A**, the locations of P2Y₂ receptors and EGFR in individual cells were detected by confocal microscopy and immunofluorescence as described under "Experimental Procedures." The combined fluorescence in the overlay, indicative of co-localization, is shown in *yellow*, and the number of yellow pixels per cell (*upper right*) is the mean \pm S.E. for 39–52 individual cells from six experiments. **B**, the effect of the Src kinase inhibitor PP2 on UTP-induced co-localization of P2Y₂ receptors and EGFR was expressed as a percentage of total P2Y₂ receptors detected. The data shown are the mean \pm S.E. of results from 39–52 individual cells from a total of four to six experiments.



terminal Src kinase in COS-7 cells that impaired LPA receptor-mediated Erk1/2 activation (25). Likewise, the inability of the mutant P2Y₂ receptor lacking the PXXP motifs to affect Erk1/2 signaling in this study is consistent with other studies showing that expression of a dominant-negative Src mutant did not affect LPA receptor-mediated Erk1/2 activation in COS-7 cells (26), and that Erk1/2 activation was not impaired in fibroblasts from Src^{-/-} mice in response to LPA receptor activation even though transactivation of the EGFR was inhibited (3). Together, these results suggest that not only do the mechanisms of Erk1/2 activation by GPCRs vary somewhat between different cell and receptor types but that caution should be taken when interpreting results from experiments with kinase inhibitors that may not be entirely specific and with over-expression experiments that may alter the normal composition of signaling complexes.

Analysis of 160 human protein sequences listed in a Class A rhodopsin-like GPCR data base indicated that ~30% of these receptors contain one or more PXXP motif(s) in the third intracellular loop or the carboxyl-terminal tail. It was noted that the percent distribution of PXXP motifs is fairly equal among G α i-, G α s-, and G α q-coupled receptors. Because $\beta\gamma$ -subunits of G proteins, which can interact with any α -subunit, have been shown to stimulate Src kinase activity (25, 27), complex formation between an SH3 domain-containing protein and certain GPCRs containing PXXP motifs may facilitate the effects of $\beta\gamma$ -subunits on Src kinase activity. In support of this idea, our

results in 1321N1 cells have shown that activation of the wild-type P2Y₂ receptor, but not the P2Y₂ receptor lacking the PXXP motifs, causes phosphorylation of Src at Tyr⁴¹⁶ (Fig. 6A), an autophosphorylation site that stimulates Src kinase activity (28). Therefore, we speculate that recruitment of Src to the cell membrane by binding to the PXXP motifs in the P2Y₂ receptor may be necessary to mediate the effects of $\beta\gamma$ -subunits of G proteins on Src activation.

Our results also indicate that activation of wild-type P2Y₂ receptors, but not P2Y₂ receptors lacking the PXXP motifs, causes phosphorylation of Pyk2 at Tyr⁸⁸¹ (Fig. 6B). Several Pyk2 tyrosine residues have been reported to be either autophosphorylated (at Tyr⁴⁰²) or phosphorylated by Src (at Tyr⁵⁸⁰ and Tyr⁸⁸¹) (3, 29). We found that the Src kinase inhibitor PP2 inhibited P2Y₂ receptor-mediated phosphorylation of Pyk2 at Tyr⁸⁸¹ (Fig. 1B), further suggesting that phosphorylation of Tyr⁸⁸¹ requires Src kinase activity. Src-dependent phosphorylation of Pyk2 causes the formation of an SH2 binding site in Pyk2 that has been shown to enhance association of Pyk2 with several SH2 domain-containing proteins including Src (3) and the adaptor protein Grb2 (29). Besides having a role in GPCR-mediated transactivation of growth factor receptors (1, 3), activation of Pyk2 has been associated with cytoskeletal reorganization and cell proliferation (29, 30). Thus, P2Y₂ receptor-mediated Pyk2 activation, which is dependent upon the presence of the PXXP motifs in the receptor, is postulated to have similar physiological consequences.

This study shows that the agonist UTP causes the wild-type P2Y₂ receptor to co-localize with the EGFR, and that this co-localization is inhibited by the Src inhibitor PP2 (Fig. 7). Our previous studies have shown that the P2Y₂ receptor also co-localizes with $\alpha_v\beta_3/\beta_5$ integrins, although this interaction was found to occur independently of agonist stimulation (21). Other reports indicate that $\alpha_v\beta_3$ integrins interact with growth factor receptors such as EGFR, PDGFR, VEGFR, and the insulin receptor (22, 31, 32), and that Src can form a complex with $\alpha_v\beta_3$ integrins, Pyk2, and the PDGFR (22, 28, 33). Thus, we speculate that the P2Y₂ receptor may be a component of a large signaling complex containing integrins and proteins associated with integrins such as Src, focal adhesion kinase, Pyk2, EGFR, PDGFR, and the actin cytoskeleton. A better understanding of the nature of these multi-protein interactions and signaling events involving P2Y₂ receptors will likely identify intervention points for selectively controlling P2Y₂ receptor activities that mediate the development of intimal hyperplasia in arteries (9, 34) and inflammatory responses in the cardiovascular and nervous systems (34, 35), leading to new treatments for atherosclerosis, Alzheimer's disease, and other disorders.

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