Ras-Dependent Activation of Fibroblast Mitogen-Activated Protein Kinase by 5-HT_{1A} Receptor *via* a G Protein $\beta\gamma$-Subunit-Initiated Pathway†

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ABSTRACT: Serotonin (5-HT) is a potent mitogen in many cells types, an action which is frequently mediated through pertussis toxin-sensitive G proteins. In the current study, we used pharmacological inhibitors and dominant negative signaling constructs to delineate elements which participate in the activation of MAPK, a growth-associated mitogen-activated protein kinase, by human G protein-coupled 5-HT_{1A} receptor transfected into CHO-K1 cells in a stable manner. The activation pathway does not directly involve phorbol ester-sensitive protein kinase C types, but does require (i) pertussis toxin-sensitive G protein $\beta\gamma$-subunits, (ii) a staurosporine- and genistein-sensitive protein kinase, (iii) phosphoinositide-3′-kinase activity, (iv) activation of Sos in a multimolecular complex that contains $p46^{Shc}$ and $p52^{Shc}$, and Grb2, (v) the GTPase $p21^{Ras}$, and (vi) the protein kinase $p74^{Raf-1}$. These data demonstrate that the 5-HT_{1A} receptor mediates MAPK activity by convergence upon a common activation pathway that is shared with receptor tyrosine kinases.

Serotonin (5-HT)[1] is a potent mitogen in many cells, and in some cases, the activation of cell growth by 5-HT is mediated through pertussis toxin-sensitive G proteins (Seuwen et al., 1988; Takuwa et al., 1989; Abdel-Baset et al., 1992). Given that receptors for other agonists such as bombesin and thrombin have been known to modulate cell growth through pertussis toxin-sensitive G proteins for a number of years, it is surprising that scant information exists regarding the effectors linking pertussis toxin-sensitive G proteins to cell growth. Only recently have the pertussis toxin-sensitive G proteins, $G_{i\alpha}$ and/or $G_{o\alpha}$, been definitively linked to the activation of cell growth. For example, an oncogenic constitutively active mutant of $G_{i\alpha 2}$, termed *gip2*, activates cellular proliferation and transformation (Hermouet et al, 1991; Pace et al., 1991; Gupta et al., 1992). Moreover, microinjection of neutralizing antibodies specific for $G_{i\alpha 2}$ (LaMorte et al., 1993) and $G_{o\alpha}$ (Baffy et al., 1994) block the synthesis of DNA in CCL36 and Balb/c 3T3 cells. More recent studies have implicated mitogen-activated protein kinases (MAPK's, also known as extracellular signal-regulated protein kinases or ERK's) (Marshall, 1995) in mitogenesis initiated by receptors which couple through pertussis toxin-sensitive G proteins, including α_2 adrenergic receptors and receptors for thrombin and lysophosphatidic acid (Chen et al., 1994; Crespo et al., 1994b; Hordijk et al., 1994). The activation of MAPK's by those receptors has only just begun to be connected to specific elements of highly conserved growth-signaling cascades.

The 5-HT_{1A} receptor is a prototypical G_i-linked receptor, which has been shown to modulate a large number of signaling pathways in mammalian cells exclusively through pertussis toxin-sensitive pathways. Nearly all of those pathways involve "classical" G protein-associated signals, such as the inhibition of adenylyl cyclase, activation of lipid hydrolysis, or modulation of ion channels (Fargin et al., 1989; Karschin et al., 1991; Liu & Albert, 1991). Biochemical studies have confirmed a nearly exclusive link of 5-HT_{1A} receptors with pertussis toxin-sensitive G proteins $G_{i\alpha}$ and $G_{o\alpha}$ (Fargin et al., 1991; Bertin et al., 1992; Raymond et al., 1993; Liu et al., 1994; Butkerait et al., 1995). Thus, the 5-HT_{1A} receptor is prototypical of receptors which might exert mitogenic effects *via* pertussis toxin-sensitive pathways. Because the pathways of MAPK activation stemming from integrins and receptor tyrosine kinases are known (Clark & Brugge, 1995; Marshall et al., 1995), we hypothesized that G protein-mediated activation of MAPK would share some

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[1] Abbreviations: βARK-1, β-adrenergic receptor kinase type 1; β1-CT, carboxyl terminal fragment of βARK-1; ERK, extracellular signal-activated kinase; FGF, fibroblast growth factor; G protein, guanine nucleotide-binding regulatory protein; genistein, 5,7-dihydroxy-3-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one; 5-HT, 5-hydroxytryptamine or serotonin; H-7, (±)-1-(5-isoquinolinesulfonyl)-2-methylpiperazine; MAPK, mitogen-activated protein kinase; MEK, mitogen/extracellular signal-activated kinase kinases; ΔmSos1, dominant negative Sos construct; NΔRaf, a dominant negative construct derived from $p74^{Raf-1}$; NRTK, non-receptor tyrosine kinases; PI-3K, Δp85 is a dominant negative p85 construct, phosphoinositide-3′-kinases; PKC, protein kinase C; PMA, phorbol 12-myristate, 13-acetate; Ras^{N17}, a dominant negative $p21^{Ras}$ construct; RTK, receptor tyrosine kinase; Shc, *src*- and collagen homology-containing protein; Sos-Pro, dominant negative Sos construct.

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of the same signaling elements. Those elements include receptor and non-receptor tyrosine kinases (RTK's and NRTK's) such as the platelet-derived growth factor receptor and Src, respectively; phosphoinositide 3′-kinases (PI-3K's); multifunctional "docking" proteins such as Shc (*src* homology−and collagen homology−containing protein); Grb2, an adaptor protein; Sos, an activator of the proto-oncogene product of Ras; $p21^{Ras}$; a small monomeric G protein, and a number of members of a kinase cascade, including some protein kinase C types, $p74^{Raf-1}$ and closely related kinases and mitogen-activated protein kinase kinases (also called mitogen/extracellular signal-activated kinase kinases or MEK). The purposes of the current work were 2-fold. First, we wanted to determine whether the 5-HT_{1A} receptor could activate mammalian MAPK, and second, to determine whether the 5-HT_{1A} receptor shares with RTK's some of the signaling elements that lead to activation of MAPK.

MATERIALS AND METHODS

Materials. CHO-K1 cells expressing 5-HT_{1A} receptors (∼1 pmol of receptor/mg of protein) were obtained as previously described (Gettys et al., 1994). [γ^{32}P]ATP was from DuPont NEN (Boston, MA). Lipofectamine was obtained from Life Technologies (Gaithersburg, MD). Cell culture supplies were purchased from Life Technologies, the Comprehensive Cancer Center at Duke University, or Corning Costar (Cambridge, MA). Genistein, daidzein, staurosporine, and H-7 were from LC Laboratories (Woburn, MA). Wortmannin, pertussis toxin, and myelin basic protein were from Sigma (St. Louis, MO), and LY294002 was from Biomol (Plymouth Meeting, PA).

DNA Constructs. DNA constructs were obtained from the following sources: minigene encoding the carboxyl terminal residues 495−689 of bovine β-adrenergic receptor kinase 1 (βARK-1) in mammalian expression vector [pRK-βARK-1-(495−689)] from one of our laboratories as previously reported (Koch et al., 1994); hemagglutinin-tagged $p44^{MAPK}$ (HA-ERK1) from Dr. J. Pouysségur (Nice, France); and dominant negative $p21^{Ras}$, Ras^{N17}, from Drs. D. Aultschuler and M. Ostrowski (Columbus, OH). ΔmSos-1 was obtained from Dr. M. Sakaue (Kobe, Japan), and Sos-Pro was created as previously described (van Biesen et al., 1995). Dominant negative $p74^{Raf-1}$, NΔRaf (Schaap et al., 1993), was from Dr. L. T. Williams (San Francisco), and a dominant negative mutant of p85 (Δp85) was from Dr. M. Sakaue (Hara et al., 1994).

Antibodies. Protein A-agarose, rabbit polyclonal anti-rat MAPK (ERK-1 carboxyl terminus), and anti-human Shc antibodies were from Upstate Biotechnology Inc. (Lake Placid, NY). Mouse monoclonal IgG1 anti-Grb2 was from Transduction Laboratories (Lexington, KY), and protein G-Plus agarose was from Oncogene Science, Inc. (Cambridge, MA). The PhosphoPlus MAPK antibody kit was obtained from New England Biolabs, Inc. (Beverly, MA). Rat monoclonal anti-$p21^{Ras}$ immunoglobulin was obtained from Oncogene Science (Uniondale, NY). 12CA5 antibody was from Boehringer Mannheim (Indianapolis, IN). Goat-anti rabbit peroxidase-immunoglobulin conjugate was from Sigma. Enhanced chemiluminescence reagents (ECL kit) were from Amersham (Arlington Heights, IL).

Expression of Constructs in CHO-K1 Cells. Stable expression of about 1 pmol of 5-HT_{1A} receptor/mg of protein was achieved as previously described (Raymond et al., 1993). Transient expression of other constructs was achieved in those cells by transfection in the presence of Lipofectamine (2 h in serum- and antibiotic-free medium). Two days prior to assays, cells were transfected with DNA as follows (per million cells): 2 μg of pRK-βARK-1-(495−689); 0.1 μg of HA-ERK1; 1 μg of dominant negative $p21^{N17Ras}$ and NΔRaf; and 1 μg each of dominant negatives Sos-Pro, ΔmSos-1, and Δp85. Transfection efficiency was evaluated using an anti-HA monoclonal antibody or a β-galactosidase marker; about 50−70% of the cells were detected by anti-HA antibody, and ≥85% with the β-galactosidase marker.

MAPK Assays. For most studies, MAPK activity was measured in immune complexes using myelin basic protein as the substrate (van Biesen et al., 1995). Cells were treated with various agents and then harvested in 200 μL of RIPA buffer [(in mM) 50 Tris, 150 NaCl; 10 NaF, 10 sodium pyrophosphate, 0.1 phenylmethylsulfonyl fluoride; pH 8.0, supplemented with 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS; and soybean trypsin inhibitor, benzamidine, and leupeptin at 5 μg/mL] containing either 6.5 μg of 12CA5 antibody or 5 μg of polyclonal anti-ERK1.[2] The polyclonal antibody is also known to cross-react with ERK-2; therefore the activity in immune complexes represents the combined effects of ERK-1 and ERK-2. Cells were scraped, and the supernatants were harvested by centrifigation. Immune complexes containing either HA-tagged $p44^{MAPK}$ or endogenous ERK1 and 2 were precipitated with protein A sepharose, washed twice with ice cold RIPA buffer, then twice with kinase buffer [(in mM) 20 Hepes, 10 mM $MgCl_2$; 1 dithiothreitol; pH 7.4]. Complexes were resuspended in 40 μL of kinase buffer supplemented with 10 μg of myelin basic protein, 20 μM ATP, and 0.2 μCi of [γ^{32}P]ATP, incubated at ambient temperature for 30 min, and then quenched by addition of 40 μL of 2× Laemmli sample buffer. Samples were resolved by SDS−PAGE with 4−20% pre-cast gels (Novex, San Diego, CA). Gels were quantitated either by ImageQuant software on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) or with Kodak X-OMat film and a model GS-670 densitometer using Molecular Analyst software (Bio-Rad, Hercules, CA). Ultrapure preparations of myelin basic protein were used and yielded either one or two phosphorylated bands which corresponded with the major (or only) bands identified by Coomassie staining. In experiments where two ∼19 kDa bands were detected, there was no difference in the increase in agonist-induced phosphorylation and both bands were pooled for densitometric evaluation.

In some initial experiments, MAPK activity was assessed using a phosphorylation state specific MAPK antibody (New England Biolabs) which specifically recognizes tyrosine[204]-phosphorylated (but not non-phosphorylated) ERK1 and ERK2 and which does not react with closely related p38 MAPK or jun-kinases or stress-activated protein kinases (JNK/SAPK's). The phospho-MAPK antibody was used at 1:1000 dilution, whereas the control antibody, which recognizes equally well the phosphorylated and non-phosphorylated MAPK, was used at 1:500 dilution as per the manufacturer's recommendations.

[2] Both antibodies produced equivalent results, so data from both have been pooled.

p21^{Ras} Assay. Quiescent cells in six-well plates were washed twice with phosphate-free DMEM and then labeled in 1 mL of the same medium in the presence of 100 μCi of ^{32}P orthophosphate for 2 h. Cells were stimulated with 5-HT or vehicle for 2 min, placed on ice, and washed twice with ice cold PBS. Cells were then lysed in a buffer containing (in mM) 50 Tris, pH 7.5, 150 NaCl, 20 $MgCl_2$, supplemented with 1% NP-40 and soybean trypsin inhibitor, benzamidine, and leupeptin at 5 μg/mL as well as with 10 μg of anti-p21^{Ras} immunoglobulin, vortexed, and incubated on ice for 30 min. Lysates were centrifuged at 16 000*g* and the supernatants transferred to fresh tubes, then centrifuged at 100 000*g*. Supernatants were incubated for 1 h with 30 μL of protein G-Plus agarose, pelleted by centrifugation, resuspended, and washed in lysis buffer four times and then once with PBS. Beads were resuspended in 20 μL of 2 mM EDTA, 2 mM dithiothreitol, and 0.2% SDS, heated at 95 °C for 5 min, and then cooled on ice. 10 μL of each sample was spotted onto PEI cellulose plates, placed in a thin-layer chromatography chamber, and chromatographed for 2 h. To control for lane to lane variability, each sample served as its own control as follows. After exposure to a phosphor screen (12−48 h), the percentage of GTP was calculated by dividing the integrated values from the GTP spots by the sum of the values from the corresponding GTP and GDP spots.

Immunoprecipitation. Quiescent cells (10 000 000 per sample) were exposed for 5 min to vehicle or 5-HT and were then scraped into 500 μL of RIPA buffer and placed on ice for 15 min. The lysates were precleared by addition of protein A agarose. After 15 min, samples were centrifuged at 15 000*g*. The supernatants were harvested, added to 10 μg of mouse anti-Grb2 IgG, and incubated at 4 °C for 2 h. Immune complexes were harvested with protein A-agarose or protein G-Plus agarose, washed thrice with RIPA buffer, and then run on SDS−PAGE gels for immunoblot with rabbit anti-Shc or anti-Grb2 as described in the next section.

Immunoblots. For immunoblots, cells were scraped into Laemmli buffer, boiled for 3 min, and subjected to SDS−PAGE under reducing conditions with 4−20% pre-cast gels (Novex, San Diego, CA). After semidry transfer to polyvinylidine difluoride membranes, the membranes were blocked with a Blotto buffer (5% defatted dried milk in 10 mM Tris, 150 mM NaCl, 1% Tween-20, pH 8.0). The membranes were incubated with the Blotto containing anti-Shc IgG (1:5000) for 2 h at 4 °C, washed thrice in Blotto, and then incubated with goat-anti rabbit peroxidase-immunoglobulin conjugate (1:10 000) for 1 h at ambient temperature. After extensive washing in 10 mM Tris, 150 mM NaCl, 1% Tween-20, pH 8.0, immunoreactive bands were visualized using an enhanced chemiluminescence technique (ECL).

RESULTS

We used two distinct methods to show that the 5-HT$_{1A}$ receptor activates MAPK. First, we used a phosphorylation state-specific antibody to detect phospho-MAPK. That antibody, which specifically recognizes phosphotyrosine^{204} of ERK1 and ERK2, detected a time-dependent, nearly 5-fold increase in immunoreactivity after stimulation with 10 μM 5-HT (Figure 1, top panel). A control antibody for ERK2, which recognizes phosphorylated and non-phosphorylated

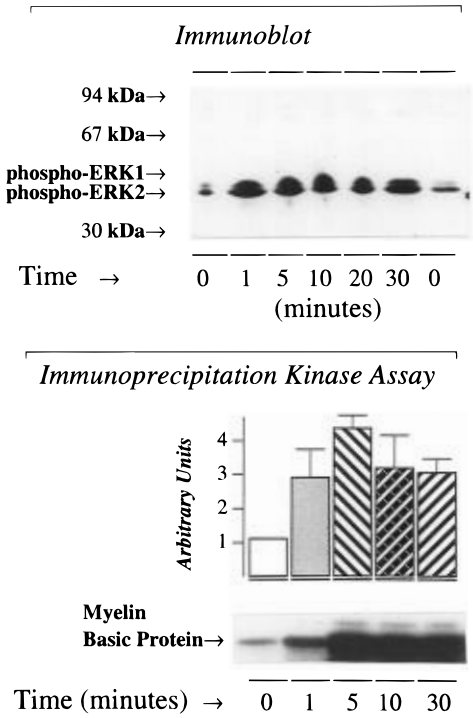

FIGURE 1: 5-HT$_{1A}$ receptor activates MAPK in CHO-K1 cells. *Top panel.* Phosphorylation state-specific antibody detected a time-dependent, ∼5-fold increase in phospho-MAPK immunoreactivity after stimulation with 10 μM 5-HT. The same blot was stripped and reprobed with antibody for ERK2, which recognizes phosphorylated and nonphosphorylated ERK2 equally well, showing no increase (not shown). The experiment shown is one of three. *Bottom panel.* Substrate assay of MAPK immunoprecipitates revealed a 4−5-fold increase in phosphorylation of myelin basic protein after 5-HT treatment. The activity peaked at ∼5 min. Data are means of three values ± standard errors.

ERK2 equally well, did not show any increase after 5-HT treatement (not shown). Second, we used a substrate-based phosphorylation assay to assess MAPK activity. After MAPK was purified from cell lysates by immunoprecipitation, samples were assayed for the ability to phosphorylate the substrate, myelin basic protein. This method revealed an ∼4-fold increase in phosphorylation of myelin basic protein after 5-HT treatment (bottom panel of Figure 1), which could be attenuated by preincubation with 10 μM spiperone, a 5-HT$_{1A}$ receptor antagonist (Figure 2A). The activity was dependent on time, peaking by 5 min (Figure 1, bottom panel), and on the concentration of 5-HT, with an EC_{50} of about 10 nM ($n = 3$ in duplicate, not shown).

To investigate the involvement of the various signaling elements in the 5-HT-mediated activation of MAPK, we performed a series of studies in which we attempted to attenuate 5-HT-induced signaling by using either pharmacological inhibitors or dominant negative mutants of the various hypothetical signaling elements. In Figure 2A, we show results of studies designed to investigate the role of G proteins in the 5-HT signal. The importance of pertussis toxin-sensitive G proteins was confirmed by elimination of the effect of 5-HT by prior pertussis toxin treatment (200 ng/mL overnight). CHO-K1 cells express primarily $G_{i\alpha 2}$ and $G_{i\alpha 3}$ (Gettys et al., 1994) and lesser amounts of $G_{o\alpha}$ (van Biesen et al., 1996), and these would be most likely to mediate the signal. The results show that pertussis toxin-sensitive G proteins are important in mediating the 5-HT signal but do not allow us to distinguish between α- and

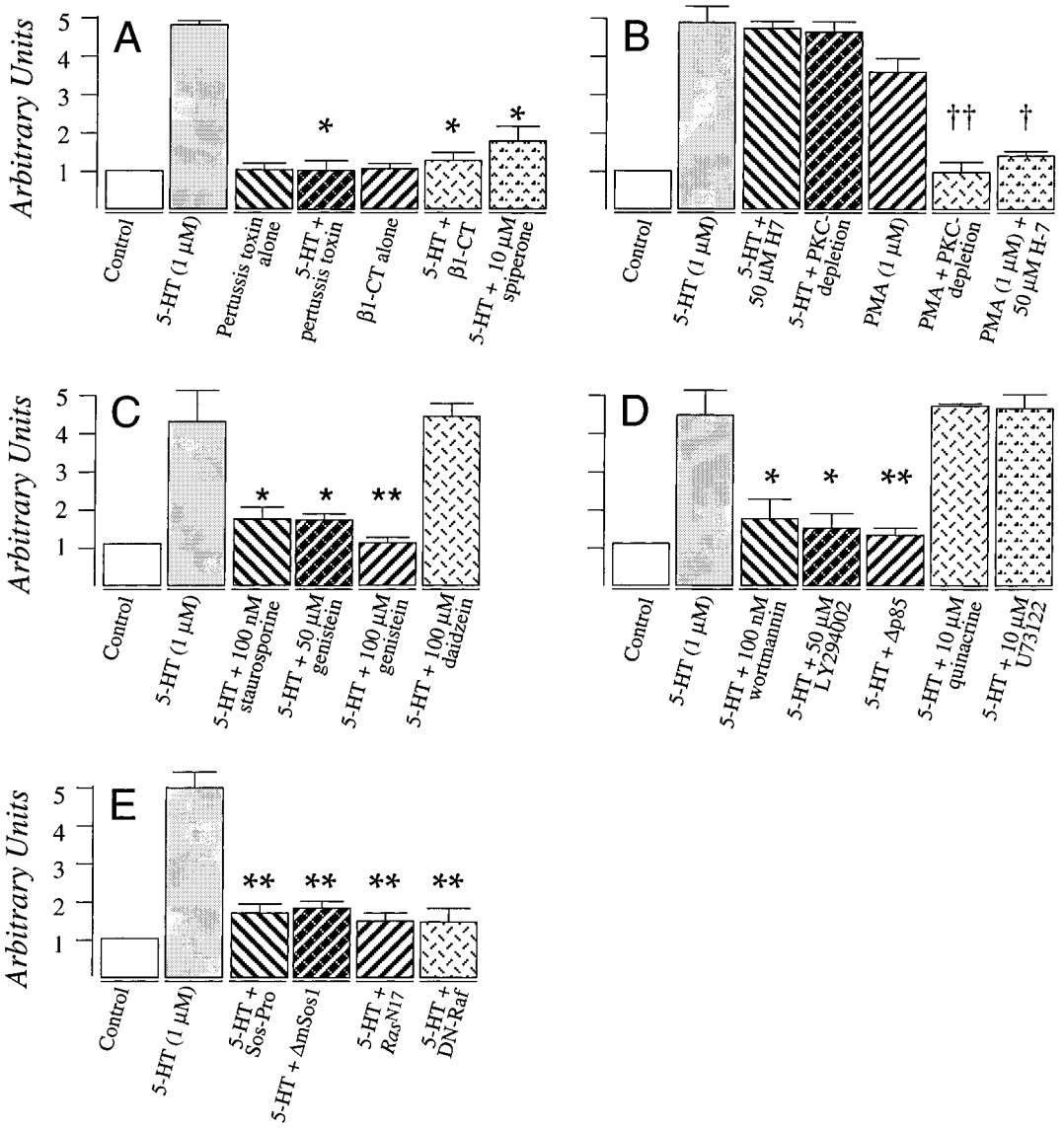

FIGURE 2: A. Role of pertussis toxin-sensitive G proteins in the activation of MAPK. Pertussis toxin treatment and sequestration of $\beta\gamma$ subunits with β1-CT block 5-HT-activated MAPK activity, while having no effect on basal activity. B. Role of phorbol ester-sensitive PKC in the activation of MAPK. PKC-depletion ($n = 3$) and H-7 ($n = 2$) had no effect on basal or 5-HT-stimulated MAPK activity. C. Effects of kinase inhibitors on MAPK activation. D. Effects of PI-3 kinase blockade on MAPK activation. E. Effects of dominant negative constructs on MAPK activation. Data in panels A−E were analyzed by one-tailed, paired *t*-test with Bonferonni correction. $^{*}p < 0.05$ and $^{**}p < 0.01$ when compared with 5-HT-stimulated values, and $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ when compared with PMA-stimulated values. Experiments were performed at least three times in duplicate with the exception of H-7 and staurosporine, which were performed twice in duplicate.

$\beta\gamma$-subunits as the direct downstream mediators. In order to test the role of $\beta\gamma$-subunits, we used a minigene construct containing the carboxyl terminus of βARK-1 (β1-CT), which is a known sequesterer of $\beta\gamma$-subunits (Koch et al., 1994). Transfection with β1-CT markedly attenuated the ability of 5-HT to activate MAPK. The effect of β1-CT is a specific one, because the same treatment did not attenuate 5-HT-activated proton efflux from the same cells (M. Garnovskaya, K. Chuprun, and J. R. Raymond, unpublished data). Neither pertussis toxin treatment nor transfection with β1-CT significantly affected the basal MAPK activity (Figure 2A). Those results suggest that $\beta\gamma$-subunits released from pertussis toxin-sensitive G proteins are downstream effectors of the 5-HT_{1A} receptor.

Because G proteins can activate MAPK through phorbol ester-sensitive PKC types, and the 5-HT_{1A} receptor can activate PKC in some cell types (Fargin et al., 1989), we tested the involvement of PKC's in MAPK activation by the 5-HT_{1A} receptor. Figure 2B shows that the ability of 5-HT to stimulate MAPK [(4.8 ± 0.3)-fold] was unaffected by either prior PKC depletion achieved by prolonged exposure (16 h) to 1 μM PMA or concurrent exposure to 50 μM of the nonspecific kinase inhibitor, H-7. The effectiveness of that treatment to eliminate PKC-derived signals was tested in several ways. First, the same treatment completely eliminated the ability of transfected PKC-linked M_1 muscarinic acetylcholine receptor to activate MAPK (van Biesen et al., 1996). Additionally, pretreatment completely blocked the ability of 1 μM PMA to either activate cells as determined by microphysiometry (not shown, $n = 3$) or to activate MAPK (Figure 2B). Thus, it is unlikely that phorbol ester-sensitive PKC types play a role in activation of MAPK by the 5-HT_{1A} receptor.

Because activation of MAPK's typically requires activation of phosphorylation cascades, we examined the roles of two other types of kinases in the effect of 5-HT, those being tyrosine kinases and PI-3K's. Tyrosine kinases comprise a large family of cell surface receptors (RTK's) and non-

receptor type kinases (NRTK's) which share the ability to phosphorylate tyrosine residues, and in many cases a susceptibility to blockade of the phosphorylation reaction by genistein. A growing family of PI-3K's exert physiological effects by phosphorylating (among other substrates) phosphatidylinositol 4,5-bisphosphate to yield phosphatidylinositol 3,4,5-trisphosphate (Stephens et al., 1993). For this set of studies, we relied primarily on pharmacological kinase inhibitors. Staurosporine (100 nM) a broad spectrum kinase inhibitor with effects on both serine/threonine and tyrosine kinases, was able to effectively suppress the effect of 5-HT upon MAPK [(4.6 ± 0.2) vs (1.6 ± 0.3)-fold increase, n = 2 in duplicate], suggesting that a kinase is involved in the pathway. Moreover, because H-7 treatment and PKC-depletion have no effect on the signal (previous paragraph), the effect of staurosporine is most likely secondary to inhibition of a tyrosine kinase. Genistein (50 and 100 μM), a broad-spectrum tyrosine kinase inhibitor markedly attenuated the ability of 5-HT to activate MAPK, whereas the structurally similar but inactive compound, daidzein, had no effect, suggesting the involvement of a tyrosine kinase in this pathway (Figure 2C).

Two structurally distinct PI-3K inhibitors, wortmannin (100 nM) and LY294002 (50 μM), each inhibited 5-HT activation of MAPK in concentrations at which each is reported to be highly specific for PI-3K's (Nakanishi et al., 1992; Powis et al., 1994; Vlahos et al., 1994) (Figure 2D). Because of a recent report that suggested that wortmannin can inhibit some phospholipase C and A activities (Cross et al., 1995), we also tested two inhibitors of those phospholipases. Neither the basal activity nor the ability of 5-HT to activate MAPK was affected by preincubation with 10 μM U73122 or quinacrine (n = 3 in duplicate for each), suggesting that phospholipases C and A_2 are not involved in the 5-HT-mediated activation of MAPK. Moreover, an inactive mutant form of the p85 subunit of PI-3K was able to markedly attenuate the 5-HT effect. Therefore, this set of experiments supports an important role for tyrosine phosphorylation events and PI-3K(s) as critical participants in the ability of the 5-HT_{1A} receptor to activate MAPK.

Figure 2E illustrates the results of experiments in which several dominant negative constructs were used to probe the involvement of Sos (Sos-Pro and ΔmSos1), $p21^{Ras}$ (*Ras*N17), and $p74^{Raf-1}$ (NΔRaf). 5-HT activated MAPK by (4.9 ± 0.8)-fold, and both Sos constructs significantly attenuated that activation. Similar results were obtained using *Ras*N17 and NΔRaf. These results suggest that Sos, $p21^{Ras}$, and $p74^{Raf-1}$ are essential mediators of 5-HT-stimulated MAPK activity.

In order to confirm the involvement of $p21^{Ras}$ and of a Grb2-Sos multimolecular complex in 5-HT-mediated activation of MAPK, we performed two further biochemical assays. First, we measured the amounts of $p21^{Ras}$ in the GTP- and GDP-bound states. Those studies (not shown) showed that 5-HT increased the fraction of $p21^{Ras}$-GTP from 9 ± 1% to 15 ± 1% (n = 5 in duplicate or triplicate, $p < 0.05$). When CHO-K1 cells were stimulated with basic fibroblast growth factor (100 ng/mL), the fraction of $p21^{Ras}$-GTP increased from 9 ± 1% to 16 ± 2% (n = 2). Therefore, 5-HT activates Ras to a similar extent as does the ligand for an endogenous Ras-activating fibroblast growth factor receptor. These results complement the dominant negative studies by demonstrating that the 5-HT_{1A} receptor activates $p21^{Ras}$, confirming its involvement in the activation of MAPK.

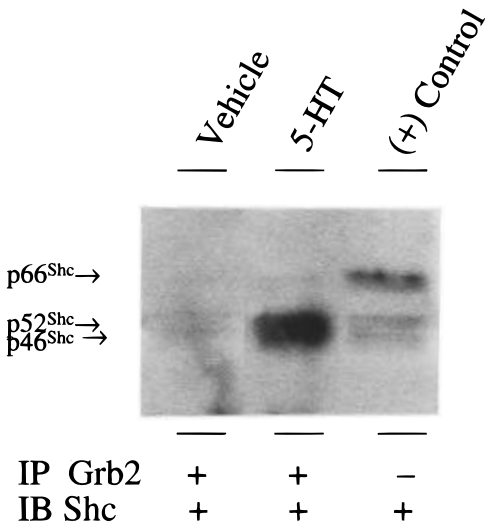

FIGURE 3: Co-immunoprecipitation of Shc with Grb2. Experiments were performed five times with similar results. Each time, the amounts of $p46^{Shc}$ and $p52^{Shc}$ species in the Grb2 immunoprecipitates were increased. When the blots were stripped and reprobed for Grb2, equal amounts of Grb2 were present in the precipitates (not shown). Positive control was derived from 20 μg of PC12 cell lysates. IB means immunoblot, and IP means immunoprecipitation.

In the second assay, we immunoprecipitated Grb2 and assessed the precipitates for the presence of Shc, which is thought to play a critical role in transducing $\beta\gamma$-mediated activation of MAPK (Touhara et al., 1995; van Biesen et al., 1995). As shown in Figure 3, 5-HT markedly increased the amount of Shc in Grb2 precipitates, particularly of the $p46^{Shc}$ and $p52^{Shc}$ species. Similar results were obtained five times, and when the blots were stripped and reprobed for Grb2, equal amounts of Grb2 were present in the precipitates (not shown). These studies demonstrate that the 5-HT_{1A} receptor induces the formation of a multimolecular complex which includes Grb2 and $p46^{Shc}$ and $p52^{Shc}$, a step that is a prerequisite for Sos activation.

DISCUSSION

The current studies elucidate an activation pathway of MAPK that is triggered by stimulation of the human 5-HT_{1A} receptor in fibroblasts. Because 5-HT is a known mitogen that acts through pertussis toxin-sensitive G proteins, more specific knowledge of pathways leading from 5-HT receptors to the activation of MAPK's will be important for understanding how 5-HT and other ligands activate cell growth, proliferation, and mitogenesis. Although the 5-HT_{1A} receptor has been shown to couple to and/or activate several pertussis toxin-sensitive α-subunits in CHO-K1 and other cells, in the current case the G protein $\beta\gamma$-subunits appear to mediate the MAPK activation (Figure 2A). This is in distinct contrast to 5-HT_{1A} receptor-mediated activation in the same cells of another growth-associated effector, the type 1 sodium-proton antiporter, which appears to depend upon the G protein α-subunits and not the $\beta\gamma$-subunits (M. Garnovskaya and J. R. Raymond, unpublished data). The requirement of $\beta\gamma$-subunits for the 5-HT_{1A} receptor to activate MAPK is similar to that of other receptors (lysophosphatidic acid, D_2 dopamine, α_2-adrenergic, and A_1 adenosine) which activate MAPK's (ERK1 and/or ERK2) primarily *via* pertussis toxin-sensitive G proteins in COS-7 (Crespo et al., 1994a; Faure et al., 1994; van Biesen et al., 1995). Our current results in CHO-K1

cells were not predictable, however, because cell-specific regulation of MAPK activity has been described (Faure et al., 1994), and because the receptor for platelet-activating factor stimulates MAPK in CHO-K1 cells through a pertussis toxin-sensitive pathway that bypasses $p21^{Ras}$ (Hawes et al., 1995), and is unaffected by $\beta\gamma$ sequestration (van Biesen et al., 1996). Thus, it appears that two distinct pertussis toxin-sensitive pathways lead to activation of MAPK's in CHO-K1 cells, one of which requires $p21^{Ras}$ and G protein $\beta\gamma$-subunits, and the other operating independently of those signaling elements.

Our studies also support the involvement of several signaling elements typically associated with signals propagated by RTK's, including tyrosine kinase and PI-3 kinase activities. The identity of the tyrosine kinase(s) involved remains elusive. The most likely candidates probably derive from the *Src* family or closely related NRTK's because (i) those kinases are thought to be upstream regulators of MAPK's (Pawson, 1995), (ii) *Src* kinases have a well-established function in cell growth, (iii) recent studies have shown that pertussis toxin-sensitive G proteins activate *Src* and *Fyn* kinases in fibroblasts and platelets (Chen et al., 1994), and (iv) $\beta\gamma$-subunits activate Tsk and Btk NRTK's in HEK293 cells (Langhans-Rajasekaran et al., 1995).

Previous studies have shown that activation of MAPK by G protein-coupled receptors was inhibited by the PI-3K inhibitor, wortmannin (Ferby et al., 1994; Sakanaka et al., 1994). In the current study, the sensitivity of 5-HT_{1A} receptor-mediated MAPK activity to LY294002 and wortmannin and to dominant negative Δp85 strongly implicates a PI-3K in the signaling. It is also not clear whether PI-3K activity is directly involved in the MAPK pathway or whether it functions more in a permissive role. The precise location of the PI-3K activity in the pathway has also not been completely delineated. $p21^{Ras}$ has recently been shown to be a possible regulator of PI-3-kinase (Rodriguez Viciana et al., 1994; Zhang et al., 1995), which would suggest that PI-3K is downstream of $p21^{Ras}$. In contrast, a PI-3K has also been shown to be a regulator of $p21^{Ras}$, suggesting a possible location upstream of $p21^{Ras}$ (Hu et al., 1995; Touhara et al., 1995; Hawes et al., 1996). The current experiments do not allow us to distinguish between those two possibilities. Moreover, the subtype of PI-3K involved in 5-HT1A receptor signaling in CHO-K1 cells is not known. In that regard, myeloid cells, rat osteosarcoma cells, and platelets have recently been shown to have cytosolic PI-3K activities that can be activated by G protein $\beta\gamma$-subunits (Stephens et al., 1994; Thomason et al., 1994; Morris et al., 1995; Stoyanov et al., 1995; Zhang et al., 1995). The $\beta\gamma$-activated PI-3K activity described by Stephens et al. was not stimulated by α-subunits (Stephens et al., 1994), nor did $\beta\gamma$-subunits activate p85α/p110β isoforms of PI-3K when expressed in insect cells (Morris et al., 1995). In contrast, another type of 110 kDa PI-3K was recently cloned which was shown to be activated both by $\beta\gamma$- and α-subunits of G proteins (Stephens et al., 1994). Any of those PI-3K's should currently be considered as potential candidates for mediating the effect on MAPK by 5-HT_{1A} receptor activation.

The involvement of a multimolecular complex containing Grb2, $p46^{Shc}$ and/or $p52^{Shc}$, and Sos is suggested by studies with dominant negative Sos constructs, which block activation of MAPK by the 5-HT_{1A} receptor (Figure 2E) and also by the co-immunoprecipitation of $p46^{Shc}$ and $p52^{Shc}$ with Grb2, which is markedly increased by 5-HT (Figure 3). In that respect, this pathway is similar to one described for α_2-adrenergic and lysophosphatidic acid receptors in Cos-7 cells (van Biesen et al., 1995), both of which involve pertussis toxin-sensitive G proteins. In a similar vein, the phospholipase C-linked thyrotropin-releasing hormone receptor in GH3 pituitary cells was shown to induce tyrosine phosphorylation of $p52^{Shc}$ and to increase its association with Grb2 (Ohmichi et al., 1994). Endothelin in astrocytes was also previously shown to activate Raf$-$1, to cause tyrosine phosphorylation of $p52^{Shc}$, and to induce association of Grb2 with $p52^{Shc}$ (Cazaubon et al., 1994). In those two studies, the nature of the G proteins involved in those actions was not elucidated so a direct comparison with the current study is not possible. The involvement of $p21^{Ras}$ is supported both by studies with a dominant negative *Ras* construct (Ras^{N17}, Figure 2E), and by 5-HT-initiated conversion of $p21^{Ras}$—GDP to $p21^{Ras}$—GTP. While the studies using the dominant negative construct (NΔRaf) derived from $p74^{Raf-1}$ support its involvement, it remains possible that other Raf species such as A-Raf or B-Raf might also be involved as has been shown for the interleukin-8 receptor (Knall et al., 1996).

In summary, the current work provides evidence that MAPK can be stimulated through the transfected human 5-HT_{1A} receptor *via* a pathway that shares many of the mediators of growth signals initiated by RTK's. The activation pathway involves (i) pertussis toxin-sensitive G protein $\beta\gamma$-subunits, (ii) genistein-sensitive tyrosine kinase, (iii) and a PI-3K activity. Moreover, the pathway appears to involve (iv) activation of Sos in a multimolecular complex that likely contains Shc and Grb2, (v) $p21^{Ras}$, and (vi) $p74^{Raf-1}$. We hypothesize that the 5-HT_{1A} receptor mediates MAPK activity by convergence upon a common activation pathway shared with RTK's. The current studies underscore an emerging realm of overlap in growth-related signals previously thought to be compartmentalized between G protein-coupled receptors and tyrosine kinase-type growth factor receptors.

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