5-Hydroxytryptamine 2B receptor regulates cell-cycle progression: Cross-talk with tyrosine kinase pathways

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In this paper, we present evidence that activation of 5-hydroxytryptamine 2B (5-HT2B) receptors by serotonin (5-HT) leads to cell-cycle progression through retinoblastoma protein hyperphosphorylation and through activation of both cyclin D1/cdk4 and cyclin E/cdk2 kinases by a mechanism that depends on induction of cyclin D1 and cyclin E protein levels. The induction of cyclin D1 expression, but not that of cyclin E, is under mitogen-activated protein kinase (MAPK) control, indicating an independent regulation of these two cyclins in the 5-HT2B receptor mitogenesis. Moreover, by using the specific platelet-derived growth factor receptor (PDGFR) inhibitor AG 1296 or by overexpressing a kinase-mutant PDGFR, we show that PDGFR kinase activity is essential for 5-HT2B-triggered MAPK/cyclin D1, but not cyclin E, signaling pathways. 5-HT2B receptor activation also increases activity of the Src family kinase, c-Src, Fyn, and c-Yes. Strikingly, c-Src, but not Fyn or c-Yes, is the crucial molecule between the Gα protein-coupled 5-HT2B receptor and the cell-cycle regulators. Inhibition of c-Src activity by 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP1) or depletion of c-Src is sufficient to abolish the 5-HT-induced PDGFR tyrosine kinase phosphorylation and MAPK activation, (ii) cyclin D1 and cyclin E expression levels, and (iii) thymidine incorporation. This paper elucidates a model of 5-HT2B receptor mitogenesis in which c-Src acts alone to control cyclin E induction and in concert with the receptor tyrosine kinase PDGFR to induce cyclin D1 expression via the MAPK/ERK pathway.

Serotonin (5-hydroxytryptamine, 5-HT) is involved in regulating cellular functions of central and peripheral nervous systems, endocrine and exocrine organs, as well as vascular and hematopoietic systems (1). These actions are mediated by numerous cognate receptors. For instance, 5-HT acts as a growth factor in a variety of cells, a function that is mediated by 5-HT1A, 5-HT1B, 5-HT2A, 5-HT2B, or 5-HT2C receptor subtypes (2–5). The 5-HT2B receptor belongs to the G protein-coupled receptor (GPCR) family. Binding of 5-HT to 5-HT2B receptor activates Gα4 protein, thereby stimulating phospholipase C, which initiates a rapid release of inositol trisphosphate and results in a rise in intracellular calcium levels (6). The signal transduction pathways activated downstream of the 5-HT2B receptor include the Ras and mitogen-activated protein kinase (MAPK of the MAPK/ERK subfamily) pathways (3). Despite numerous studies of 5-HT signal transduction pathways, the critical steps from signal integration to its proliferative action have not been elucidated.

The regulation of cell proliferation is a complex process controlled by external mitogenic factors. Once cells are induced to proliferate, passage through the mitotic cell cycle is directed by activation of a series of cyclins and their catalytic subunits, cyclin-dependent kinases (cdks) (7). Mitogenic factors act by stimulating both the proliferation of quiescent cells arrested in G0 and the progression of the cell cycle through the restriction point in late G1. Progression through G1 and the G1/S transition in mammalian cells is regulated by cdk4 and cdk6, which form complexes with cyclin D1, and by cdk2, which forms a complex with cyclin E (7). The G1 cyclin/cdk complexes drive the phosphorylation of retinoblastoma protein (pRb) that represents the limiting event in G1 progression (8). However, the mechanism whereby 5-HT triggers G1 progression has not been examined.

The link between upstream signaling circuitry and the cell-cycle machinery is of interest. Identified candidates for this link are Ras and MAPK (8, 9). Cyclin D1 and cyclin E expression are induced by growth factors, including estrogen and heregulin, possibly through Ras/MAPK pathways (10, 11). The functional link between receptor tyrosine kinase (RTK) pathways and cdks seems established; however, little is known about the G1 effectors of mitogenic signaling triggered by GPCRs. Recently, GPCRs have been shown to stimulate rapid tyrosine phosphorylation of RTKs, a phenomenon known as transactivation (12). Cytoplasmic tyrosine kinases of the Src family have been independently implicated in activation of the MAPK pathway by GPCR (13–15). However, the downstream targets and the role in biological responses of the cross-talk between GPCRs and tyrosine kinase activation have not been elucidated in the cell-cycle context.

We previously reported that expression of 5-HT2B receptors in the nontransformed mouse fibroblast LMTK− cells is mitogenic and transforming, because these cells form foci and induce tumors in nude mice. Also, the same receptor is overexpressed with a similar coupling to Ras in human carcinoid tumor cells (3). Because uncontrolled cell proliferation is the hallmark of cancer cells, studying of the complete signal-transduction system from the membrane to the cell-cycle machinery is essential to understand 5-HT-dependent transformation of this cell line. In this study, the mechanism by which 5-HT binding to the 5-HT2B receptor leads to activation of the cell-cycle machinery is examined. We present evidence for the differential involvement of Src and the platelet-derived growth factor receptor (PDGFR) in 5-HT2B-mediated cell-cycle progression.

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Abbreviations: 5-HT, 5-hydroxytryptamine; 5-HT2B, 5-hydroxytryptamine 2B; cdk, cyclin-dependent kinase; MAPK, mitogen-activated protein kinase; PDGFR, platelet-derived growth factor receptor; PP1, 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; GPCR, G protein-coupled receptors; pRb, retinoblastoma protein; RTK, receptor tyrosine kinase.

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Materials and Methods

Antibodies. All antibodies were from Santa Cruz Biotechnology except the anti-active MAPK from Promega, the anti-pRb from PharMingen, and the anti-phosphotyrosine PY20 from Upstate Biotechnology.

Cell Culture and Transfection. Mouse fibroblast LMTK- cells were transfected stably with an expression vector (pSG5) carrying the mouse 5-HT2B receptor cDNA by using Lipofectamine (GIBCO) as described previously (16) to generate LM6 cells. The plasmid (pcDNA3) carrying a mutant PDGFR-β (K634A) at the kinase activity site (17) was introduced into LM6 cells by using high-efficiency transferrin receptor-mediated Lipofectamine transfection (18). LM6 cells grown in 90-mm dishes were treated with 4 ml of serum- and antibiotic-free medium, including 50 µM Lipofectamine, 10 µg of total plasmid DNA, and 16 µM transferrin overnight, then medium including 10% serum was added for 24 h. At 48 h after transfection, cells were serum-starved for 36 h, then treated and lysed. With this protocol, transfection efficiency in LM6 cells reached 85% as measured by cotransfection of a β-galactosidase-containing construct.

To reduce endogenous protein expression, LM6 cells were transfected with phosphorothioate-protected oligonucleotides also by using transferrin-receptor-mediated Lipofectamine transfection (19). After transfection in similar conditions of oligonucleotides corresponding to mouse p42 and p44MAPK (20), c-Src (21), or Fyn (22), cells were serum-starved for 36 h, then treated and lysed. The efficiency of transfection was verified by Western blot analysis. For example, at least 70% of cells became transfected for MAPK antisense oligonucleotides within 5 h, and transfection reached ~95% 16 h after adding medium that included serum.

Cell-Cycle Analysis. Progression through the cell cycle was monitored by flow cytometric analysis of the DNA content of cell populations stained with propidium iodide and was carried out with a fluorescence-activated cell sorter (FACScan flow cytometer; Becton Dickinson). The percentage of cells within G1, S, and G2, and M phases was determined by using CELLQuest software (Becton Dickinson).

Thymidine Incorporation. Cells were seeded on 24-well plates at a density of 10³ cells per well, grown overnight, and serum-starved for 36–48 h. Quiescent cells were treated with 5-HT at different concentrations and time points, and 0.5 µCi (1 Ci = 37 GBq) of [³H]thymidine was added to the culture during the last 4 h of incubation. The free radioactive thymidine was washed away in 5% trichloroacetic acid, and the incorporated radioactive thymidine was quantified by scintillation counting.

Cell Lysis, Immunoprecipitation, and Immunoblotting. After treatment of quiescent cells with 5-HT, cells were lysed in an immunoprecipitation buffer containing protease inhibitors. Antibody/antigen complexes were detected with an enhanced chemiluminescence kit (Amersham Pharmacia) per manufacturer’s instructions. For immunoprecipitations, lysates containing equal amounts of protein were incubated with the appropriate antibody overnight at 4°C, 25 µg/ml of protein A/G-Sepharose beads (Amersham Pharmacia) were added for 1 h, and the immune complexes were washed two times with immunoprecipitation buffer. Loading homogeneity was verified by stripping and reprobing the blots according to the enhanced chemiluminescence kit’s recommendations. Densitometric analysis was performed with an image analyzer (GS-700; Bio-Rad).

Cyclin/cdk-Associated Kinase Assay. For cyclin E/cdk2 kinase activity, the lysate (500 µg) was immunoprecipitated with cyclin E antibody, and histone H1 (2.5 µg) was used as a substrate. For cyclin D1/cdk4 kinase assay, the lysate (500 µg) was immunoprecipitated with cyclin D1 antibody, and glutathione S-transferase–retinoblastoma protein (GST-Rb; 10 µg) was used as a substrate according to the information found in ref. 23.

Statistical analyses were performed according to the Kruskal–Wallis test. The chosen significance criterion was P < 0.05. All values given are representative of at least two duplicated independent experiments.

Results

5-HT2B Receptor Activation Stimulates S Phase Entry, pRb Phosphorylation, and G1 Cyclin Kinase Activity. We determined the kinetics of thymidine incorporation of quiescent fibroblasts expressing 5-HT2B receptor (LM6). A peak of DNA synthesis was observed after 16 h of 5-HT treatment, at which time the rate of thymidine incorporation was increased 5-fold over uninduced cells (Fig. 1A). Activation of thymidine incorporation reached a maximum at 1 µM 5-HT and could be blocked by ritanserin (1 µM), a selective antagonist of 5-HT2B receptors (data not shown). Flow cytometric analysis of serum-starved cells stimulated with 5-HT for 0–16 h showed that >60% of cells had begun replicating their DNA by the time the peak of thymidine incorporation occurred.
Expression of cyclin D1 protein increased within 2 h after stimulation and achieved its maximum level between 6 and 8 h. A maximum was reached between 12 and 16 h, almost complete disappearance of the hypophosphorylated form in an increase of hyperphosphorylated pRb with within 4 h and in the subsequent progressive hyperphosphorylation of pRb by 8 h. A maximum was reached between 12 and 16 h, when over 80% of pRb was hyperphosphorylated. The kinetics of pRb phosphorylation preceded the time of entry into S phase. Cyclin D1/cdk4 activity increased significantly after 4 h of 5-HT treatment (1 μM), reaching a maximum at 8 h (Fig. 2A Middle). After stimulation, cyclin E/cdk2 activity increased between 4 and 8 h, peaking at 12 h (Fig. 2A Bottom). The time point when cyclin E/cdk2 activity peaks corresponds to the G1/S phase transition. The initial hyperphosphorylation of pRb is coincident with the peak in cyclin D1/cdk4 activation at 8 h. Subsequent progressive hyperphosphorylation of pRb could be caused by cyclin E/cdk2 activation, which reached a maximum at 12 h. This finding is consistent with progressive hyperphosphorylation of pRb by cyclin D1- and cyclin E-dependent kinase complexes.

**5-HT2B Receptor Stimulation Increases Cyclin D1 and Cyclin E Expression Levels.** Expression of cyclin D1 protein increased within 2 h after stimulation and achieved its maximum level between 6 and 8 h (Fig. 2B Left). Cyclin E expression levels increased more slowly, beginning at 2 h after stimulation and peaking at 12 h (Fig. 2B Right). The profiles of cyclin D1 and cyclin E expression levels induced by 5-HT correlate with the kinetics of cyclin D1- and cyclin E-associated kinase activity (Fig. 2A). The levels of other cyclins, such as cyclin A (data not shown), or cdk2, cdk4, and p27kip protein levels remained unchanged during this period (Fig. 2C).

**MAPK Regulates Cyclin D1 but Not Cyclin E Levels in the 5-HT2B Receptor Mitogenic Signaling and Is Regulated by Tyrosine Kinases.** In MAPK (ERK1/ERK2) antisense oligonucleotide-transfected cells (Fig. 3A), 5-HT (1 μM) was able to increase cyclin E expression to the same extent as seen in the sense oligonucleotide-transfected cells, whereas cyclin D1 stimulation was abolished (Fig. 3B). PD 098059, a specific inhibitor of the enzyme MEK-1 (25), at a concentration of 15 μM, which maximally inhibits MAPK activation (Fig. 3C), significantly inhibited 5-HT-induced cyclin D1 but not cyclin E levels. Genistein, a general tyrosine kinase inhibitor (15 μM) inhibited 5-HT-induced MAPK activity as assessed by the relative ERK1/ERK2 activity (Fig. 3C). In the same assay, both the PDGFR-specific inhibitor tyrphostin AG 1296 (0.1 μM) (26) and the Src family kinase inhibitor 4-amino-5-(4-methylphenyl)-7-(4-butylo)pyrazolo[3,4-d]pyrimidine (PP1; 1 μM) (27) reduced 5-HT-induced MAPK activity (Fig. 3C). By measuring thymidine incorporation, in addition to genistein (15 μM), AG 1296 (0.1 μM) significantly reduced 5-HT-induced DNA synthesis by 85% and 65%, respectively, indicating that activation of PDGFR could regulate cell-cycle machinery (data not shown).

**5-HT2B Receptor Activation Triggers Tyrosine Phosphorylation of PDGFR.** Time-course experiments revealed that in the absence of PDGF, 5-HT (1 μM) induces PDGFR-β tyrosine phosphorylation, reaching its maximum within 10 min in the absence of variation in the level of PDGFR protein (Fig. 4A). Ligand-
induced phosphorylation of PDGFR by 5-HT could be blocked completely by AG 1296 at 0.1 μM (Fig. 4B). Overexpression of a kinase mutant PDGFR-β (K634A) diminished PDGFR phosphorylation by 5-HT (data not shown), indicating that 5-HT2B receptor stimulation transactivates PDGFR. c-Src depletion (Fig. 4C) or c-Src inhibition by PP1 (1 μM) (Fig. 4D) abolished 5-HT-induced PDGFR phosphorylation without changing PDGFR protein levels.

**5-HT2B Receptor Stimulation Activates Src Family Kinase Activity, and c-Src Activation Is Required for Thymidine Incorporation.** Stimulation of LM6 cells by 5-HT (1 μM) raises the phosphorylation level of c-Src within 5 min (Fig. 5A), whereas this phosphorylation was completely blocked by PP1 (1 μM), which also blocked PDGFR phosphorylation induced by 5-HT (Fig. 4D). No change in the level of Src protein was observed during this assay (Fig. 5A). In a Src kinase assay (24), 5-HT provoked a 1.5-fold increase in c-Src kinase activity at 1 min, reaching to the maximum (8-fold increase) at 3 min, which was sustained after 10 min of stimulation (Fig. 5B). This increase could be blocked by PP1 (1 μM) but not AG 1296 (1 μM) (data not shown). Other Src family kinases (Fyn and c-Yes) expressed in fibroblast cells could substitute for c-Src. Therefore, we also assayed Fyn and c-Yes activity in response to 5-HT. Fyn activity rose when 5-HT2B activation reached the maximum at 10 min (Fig. 5B); c-Yes activity was elevated later than c-Src activity was, c-Src antisense transfection (but not sense) inhibited MAPK activity and thymidine incorporation, whereas Fyn antisense or sense transfection did not alter either activity (Fig. 5C and D).

**c-Src Is Required for Both Cyclin D1 and Cyclin E Induction in 5-HT2B Receptor Mitogenic Signaling.** AG 1296 at the concentration that totally inhibits PDGFR-β phosphorylation (0.1 μM) reduced cyclin D1 expression levels induced by 5-HT stimulation, whereas induction in cyclin E expression remained unchanged (Fig. 6A). Similar data were obtained when LM6 cells were pretreated with a higher concentration of AG 1296 (1 μM). Moreover, when the kinase mutant PDGFR (K634A) was overexpressed, the cyclin D1, but not cyclin E, expression level was significantly reduced on stimulation (Fig. 6B). These data indicate that the signaling pathway downstream of PDGFR does not regulate cyclin E expression; however, PDGFR plays a key role in regulating 5-HT-induced cyclin D1 expression. PP1 at the concentration that totally inhibits c-Src kinase activity (1 μM) abolished both cyclin D1 and cyclin E levels induced by stimulation (Fig. 6A). Similar results in response to 5-HT were obtained in a c-Src-depleted cell line. However, in the Fyn-depleted cell line, neither cyclin E nor cyclin D1 expression levels were changed in response to 5-HT (Fig. 6B). Supporting evidence for these findings is the inhibition by AG 1296 of the initial hyperphosphorylation of pRb within the first 8 h after 5-HT2B receptor stimulation, a period in which pRb mainly is regulated by activation of cyclin D1/cdk4 kinase. However, 12 h after stimulation, progressive hyperphosphorylation of pRb through cyclin E/cdk2 kinase activity was blocked by PP1 but not by AG 1296 (Fig. 6C).

**Discussion**

In the absence of growth factors, 5-HT is potent at inducing cell-cycle progression of L cells expressing the 5-HT2B receptor (LM6) (Fig. 1). Stimulation of 5-HT2B receptor induces pRb hyperphosphorylation by activating both cyclin D1/cdk4 and
Based on the initial observation that treatment of LM6 cells with a selective PDGFR kinase inhibitor, AG 1296, or a Src family kinase inhibitor, PP1, reduced 5-HT-induced MAPK activation, pRb hyperphosphorylation, and tyrosine kinases seemed to be involved in the 5-HT2B mitogenic signaling. Tyrosine kinases were proposed to mediate MAPK activation by 5-HT1A–5-HT2A (5, 30) and angiotensin II receptors. Furthermore, the fact that activation of 5-HT2B receptor stimulates PDGFR-β phosphorylation in the absence of PDGF demonstrates the existence of cross-talk between the Gq-coupled receptor and RTKs (Fig. 4). The requirement for the PDGFR kinase activity in the 5-HT2B receptor mitogenic pathway is demonstrated either by using the PDGFR kinase inhibitor AG 1296 or by overexpressing a kinase mutant PDGFR in LM6 cells in which PDGFR phosphorylation was abolished. G{i/}}{{g}i} coupled receptors activate the ERK pathway via both tyrosine kinase-dependent and -independent pathways. Receptors for endothelin, angiotensin II, lysophosphatidic acid, and muscarinic acetylcholine receptor have been shown to stimulate ligand-independent tyrosine phosphorylation of PDGFR (31), insulin-like growth factor-1 receptor-β subunit (32), epidermal growth factor receptor, and ErbB-2 (12). Lysophosphatidic acid and thrombin receptors have been shown to mediate ERK1/2 activation and transactivation of the epidermal growth factor receptor with pertussis toxin-sensitive G proteins (33). Because pertussis toxin was ineffective in blocking GTPase activation triggered by 5-HT in the same cell line (6), we excluded the Gi protein from 5-HT2B mitogenic signaling. In addition, activation of 5-HT2B receptor seems preferentially to transactivate PDGFR, because 5-HT does not phosphorylate the ErbB-2 receptor also present on LM6 cells, whereas endogenous epidermal growth factor receptor is not expressed (34). Therefore, ligand-independent activation of RTKs by GPCRs occurs in a cell-specific manner. Preferential coupling to or specific expression of the RTKs could explain this specificity. Treatment of LM6 cells with PDGFR kinase inhibitor AG 1296 or overexpression of a kinase mutant PDGFR abolished MAPK activity and cyclin D1 expression in response to 5-HT (Figs. 4 and 6). These data suggest the requirement of the PDGFR kinase activity for transduction of the 5-HT2B receptor-induced MAPK activity and cyclin D1 expression. Thus, in the 5-HT2B receptor mitogenic signaling, both PDGFR serves as a signal transducer of MAPK activity and cyclin D1 but not cyclin E expression. This study demonstrates that 5-HT2B receptor activation regulates cell-cycle progression by inducing cyclin E and cyclin D1 expression levels.

Preincubation of LM6 cells with PD 098059 at a concentration that completely inhibits MAPK activation also blocks 5-HT-induced thymidine incorporation. In MAPK-depleted cells, 5-HT still is able to induce cyclin E expression, whereas cyclin D1 induction is abolished, indicating that the MAPK pathway is necessary for 5-HT-dependent regulation of cyclin D1 but not of cyclin E levels (Fig. 3). Interestingly, MAPKs are required to pass the G1 restriction point in fibroblasts, and inhibition of MAPK activity abolishes cyclin D1 promoter activity (29). Inhibition of the MAPK by PD 098059 blocks heregulin-induced cell-cycle progression that is caused by cyclin D1 induction in T-47D human breast cancer cells (10). Therefore, our data indicate that, in 5-HT2B receptor mitogenic signaling, only cyclin D1 expression is regulated by MAPK; however, another pathway independent of MAPK activation regulates cyclin E expression.
induced PDGFR phosphorylation (Fig. 4). This finding indicates that c-Src targets phosphorylation sites of the PDGFR that modulate its activation. Moreover, inactivating PDGFR by a specific inhibitor, AG 1296, or overexpressing the kinase-mutant PDGFR did not affect Src kinase activation by 5-HT, confirming that it is not upstream of Src in 5-HT2B mitogenic signaling. The kinetics of c-Src kinase activation by 5-HT also show Src kinase activation earlier than PDGFR phosphorylation. The observed 5-HT-induced Src kinase activity suggests that c-Src acts as a kinase rather than as a scaffolding protein. Each of the Src family kinases (c-Src, Fyn, and c-Yes) that we examined was activated by 5-HT. However, c-Src activation in response to 5-HT is stronger and appears earlier than that of either c-Yes or Fyn (Fig. 5). Both the Fyn and c-Yes kinase activity after 5-HT2B stimulation do not correlate with PDGFR transactivation. Depletion of c-Src but not of Fyn abolished 5-HT2B receptor-induced thymidine incorporation, MAPK activation, and cyclins D1 and E expression. PP1 totally inhibited 5-HT-induced initial and progressive hyperphosphorylation of pRb (Fig. 6), which is regulated by cyclin D1- and cyclin E-dependent kinase activity, respectively. These data strongly support the notion that c-Src is the key factor in 5-HT2B receptor-induced cell-cycle progression. Fyn and c-Yes do not substitute for c-Src, but might be involved in other biological effects of the 5-HT2B receptor. Anti-p60-c-Src but not c-Yes or Fyn antibodies blocked the Gq-coupled angiotensin II receptor-stimulated tyrosine phosphorylation (36), lending further support to a specialized role for c-Src in the Gq-dependent mitogenic process.

Our data support a model for the 5-HT mitogenic signaling in which c-Src is the crucial molecule that links the Gq-coupled 5-HT2B receptor and the cell-cycle regulators. c-Src alone controls cyclin E induction and transactivates RTK PDGFR to induce cyclin D1 expression via the MAPK/ERK pathway (Fig. 7).

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