Ras Involvement in Signal Transduction by the Serotonin 5-HT2B Receptor*

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The family of serotonin 5-HT2 receptors stimulates the phospholipase C second messenger pathway via the α subunit of the Gq GTP-binding protein. Here, we show that agonist stimulation of the 5-HT2B receptor subtype stably expressed in the mouse fibroblast LMTK− cell line causes a rapid and transient activation of the proteoglycan product p21ras as measured by an increase in GTP-bound Ras in response to serotonin. Furthermore, 5-HT2B receptor stimulation activates p42/44MAPK in a GTP-dependent phosphorylation. The MAP kinase activation is correlated with a stimulation of cell division by serotonin. In addition to this mitogenic action, transforming activity of serotonin is mediated by the 5-HT2B receptor since its expression in LMTK− cells is absolutely required for foci formation and for these foci to form tumors in nude mice. Finally, we detected expression of the 5-HT2B receptor in spontaneous human and Mastomys natalensis carcinoid tumors and, similar to the 5-HT2B receptor transfected cells, the Mastomys tumor cells are also responsive to serotonin with similar coupling to p21ras activation.

Serotonin (5-hydroxytryptamine, 5-HT) is one of the best known examples of a neurotransmitter that mediates a wide variety of physiological effects, including peripheral and central actions, through the binding to multiple receptor subtypes (1). The major sites of 5-HT synthesis and storage are located in the periphery, in gut enterochromaffin cells and blood platelets, respectively. The large diversity of 5-HT functions is paralleled by the pharmacological complexity of 5-HT receptors. At least four classes of 5-HT receptors have been distinguished pharmacologically, reflecting the second messenger system to which the receptor is coupled. The family including 5-HT1 and 5-HT5 subtypes of receptors interacts negatively with adenyl cyclase, the 5-HT2B subfamily of receptors is coupled to the activation of the phospholipase C-β, the 5-HT3 receptor is a ligand-gated ion channel, and the family, including 5-HT4, 5-HT6, and 5-HT7 subtypes of receptors, activates adenylyl cyclase (2).

5-HT2 receptors mediate many of the central and peripheral physiological functions of 5-HT. Cardiovascular effects include constriction of blood vessels and shape change in platelets; central nervous system effects include neuronal sensitization to tactile stimuli and mediation of hallucinogenic effects of lysergic acid diethylamide and related phenylisopropylamine hallucinogens. The most characterized 5-HT2 receptor subtypes are the 5-HT2A (formerly 5-HT2) and the 5-HT2C (formerly 5-HT1C) both of which stimulate phospholipase C-β. Many investigators have observed that some peripheral 5-HT-like effects of 5-HT are mediated by “atypical” receptors (3). In the mouse, we cloned a new member (5-HT2B) of the 5-HT2 family which is mainly expressed in the cardiovascular system, gut, and developing brain (4). This mouse 5-HT2B receptor shares the highest degree of homology with the other 5-HT2B receptors cloned from rat libraries (5, 6) and, more recently, from human libraries (7–9) (for review, see Ref. 10) and from Drosophila (11).

5-HT, detected early in embryonic development (12), participates in craniofacial (13) and cardiovascular morphogenesis (14, 15) by unknown molecular mechanisms. We have investigated the growth factor properties mediated by the 5-HT2B receptor since (i) both Drosophila 5-HT2 and mouse 5-HT2B receptors are expressed during embryogenesis (11, 16), (ii) the mitogenic activity of 5-HT has been linked mainly to 5-HT2 receptor-dependent stimulation of phospholipase C-β/protein kinase C; when expressed at high density in NIH3T3 fibroblasts, both 5-HT2C and 5-HT2A have mitogenic effects induced by 5-HT (17, 18). In mammalian cells, the ability to activate the mitogen-activated protein (MAP) kinase cascade is a feature common to many extracellular stimuli, including growth factors, hormones, and neurotransmitters, leading to transcription factor phosphorylation and to cell division (19). The signaling pathways that activate the MAP kinase cascade use receptor tyrosine kinase or non-receptor tyrosine kinases. Other stimuli activate GTP-binding protein (G-protein)-coupled receptors generating second messengers or activating ion channels (20).

The present report concerns studies on the mitogenic and transforming activity of the mouse 5-HT2B receptor. This receptor mediates 5-HT stimulation of MAP kinase through the Ras pathway and can be classified as ligand-dependent proto-
oncogene since expression of the 5-HT2B receptor is necessary and sufficient to induce tumor formation in nude mice. In addition, expression of the 5-HT2B receptor is detected in vivo in spontaneous human and Mastomys natalensis carcinoid tumors (CT).

EXPERIMENTAL PROCEDURES

Materials—Ketanserin, ritanserin, and setoperone were kindly provided by J. anssen (Beere, Belgium). ICS 205-930 and MDL 72222 were gifts from Sandoz (Switzerland) and Merell-Dow (Strasbourg, France), respectively. Other neurochemicals were from RBI (Natick, MA). All other chemicals were reagent grade, purchased from commercial sources. The radioactive compound [3H](3S)-2-(2,5-dimethoxy-4-iodophenyl)-2-amino propane HCl ([3H]DOI, 81.4 TBq/mmol) was purchased from DuPont NEN. Dulbecco’s modified Eagle’s medium (DMEM) and fetal calf serum (FCS) were from Life Technologies, Inc.

All antibodies are from Santa Cruz Biotechnology (dilution 1:1000) and include a rabbit polyclonal antibody against v-Ha-Ras (259) which also recognizes Ki- and N-Ras p21, rabbit antisera against the amino-terminal sequence of mouse Gαs/11 (E17), rabbit polyclonal antibody against the carboxyl terminus of MAP kinase-p44ERK-1 (C16) which also reacts with p42, rabbit antisera against the amino-terminal sequence of bovine Gαi2 (A16), and rabbit polyclonal antibody against the carboxyl terminus of mouse Gαi2 (T20) which also reacts with Gαi2,-2,-3,4.

Protection and Stabilization of the 5-HT2B Mason Receptor—For this study, the previously described LMS (21) clone is used. It expresses 24 ± 8 fmol of 5-HT2B receptor/mg of protein, as measured by [125I]DOI binding experiments. Parental LMTK- and transfected LMS cells were grown in DMEM containing 10% 5-HT-depleted FCS prepared as described.

Determination of GTase Activities—Whole cell GTase activity was measured on cell extracts prepared after incubation in the presence of 1 μM 5-HT as described in Refs. 21 and 22 by monitoring the release of [32P]Pi from [32P]ATP. Membranes were prepared as described in Ref. 21, incubated for 2 min at 20°C, mixed with 0.15 M NaCl, 0.05 M Tris (pH 7.40), containing 5% (w/v) Norit-A charcoal, and the supernatant was taken for scintillation counting. Reactions were performed in quadruplicate with blanks containing no added membranes. [32P]Pi released from [γ-32P]GTP, in the absence of membranes represented 0.7 to 2% of the added [γ-32P]GTP. GTase activity is expressed as femtograms of GTP hydrolyzed per mg of protein per min.

Data Analysis and Statistics—Binding data were analyzed using the iterative nonlinear fitting software LIGAND 3.0 (23). This allows calculation of dissociation constants in saturation experiments (Kd). Data points were tested (F-test) to fit a single- or two-site model with or without an additional parameter regarded as nonspecific binding. The statistical analysis on small groups were performed by nonparametric tests (24). The chosen significance criterion was p < 0.05. All values are given as arithmetic means ± S.E. of the indicated number of experiments.

Cellular Division Assay—Cells were plated at an initial density of 104 cells/35-mm dish, in DMEM containing 10% 5-HT-free FCS. After 24 h, the medium was replaced with DMEM containing 0.6% 5-HT-free FCS. In the presence or absence of serotoninergic drugs as indicated. The medium was changed every 2 days. After 5 and 8 days, cells were detached from dishes with trypsin (1:250) and counted twice.

Focus Formation Assay—Cells were grown in 25-cm² flasks with 10% FCS until confluency. After incubation for 2 or 3 weeks in medium containing 3% 5-HT-free FCS with or without 1 μM 5-HT or 0.1 μM DOI and/or 1 μM ritanserin, foci were scored. The medium was changed every 2 days. In some instances, foci were isolated and expanded in DMEM containing 10% 5-HT-free FCS and the initial inducer (1 μM 5-HT or 0.1 μM DOI).

Tumor Formation in Nude Mice—Cells were nonenzymatically removed from culture dishes, washed with PBS, and injected subcutaneously into nude mice (Charles River). At two locations, 2-10 × 10⁶ cells were injected around each site. Tumor cell lines were established by plating dissociated cells in DMEM containing 10% FCS (25).

Ras Activation Assay—Activation of Ras was determined by analyzing the ratio of GTP- versus GDP-bound immunoprecipitated Ras from control and stimulated cells (26). Briefly, cells were incubated with 30 μCi/ml [32P]GTP for 6 h and then stimulated. Cell lysates were prepared, Ras was immunoprecipitated, and the bound [32P]-GTP and [32P]-GDP was resolved using PEI-cellulose thin-layer chromatography. Quantitation of radio labeled GDP and GTP was accomplished using an automated Lab 2832 radioactivity scanner (Berthold, Wilbad, Germany). Control refers to immunoprecipitations performed in the absence of primary anti-Ras antibody.

Assay of MAP Kinase Activity—Cells (4 × 10⁶ per 100-mm plate) were incubated for 20 h in DMEM without serum but with 0.1% bovine serum albumin to achieve quiescence. Cells were then collected and MAP kinase activity was measured essentially as described (27). Cells were rinsed three times and harvested by scraping in ice-cold phosphate-buffered saline. Cell lysis was accomplished in 50 mM β-glycero phosphosphate (pH 7.20), 100 mM sodium vanadate, 2 mM MgCl₂, 1 mM EGTA, 0.5% Triton X-100, 10 μg/ml leupeptin, 0.02 unit/ml aprotinin, and 1 mM dithiothreitol. Cell lysate supernatants were collected after microcentrifugation for 10 min at 4°C (removing insoluble cell components), normalized for protein using the bicinchoninic acid assay, and 0.5 ml of each sample was loaded onto Mono Q columns of a fast protein liquid chromatography system equilibrated in 50 mM β-glycero phosphosphate (pH 7.20), 100 mM sodium vanadate, 1 mM EGTA, and 1 mM dithiothreitol. Proteins were eluted with a linear gradient of NaCl (0–0.4 M) and collected in 1-ml fractions. Twenty μl of each fraction were added to a reaction buffer containing 1 μCi of [32P]ATP, 20 μM ATP, and 1.5 mg/ml myelin basic protein (MBP) (Sigma) for 20 min at 30°C, essentially as described (28).

5-HT2B Antiserum—Mouse 5-HT2B receptor carboxyl terminus synthetic peptide was used for rabbit immunization according to Fisher et al. (29). The peptide has the following sequence, CSTQGSSIIIILLDTLLL-TENDGDKAEQVSYV1, and has no homology with other known 5-HT receptors. Antibodies were affinity-purified and tested as described (30). The specificity was assayed by immunostaining of the cells transfected by 5-HT receptor cDNA (5-HT1A, -1B, -2A, -2B, -2C) and by Western blotting of their protein extracts (final concentration 0.2–2 μg/ml). The 5-HT2B-transfected cells show immunoreactivity which is abolished by preincubation of the serum with an excess of the immobilized receptor extract revealing protein band of the right size on Western blot. In addition, this serum recognizes the mouse, Mastomys, and human 5-HT2B receptor protein.

RESULTS

GTase Activity—We have previously shown (21) that the mouse 5-HT2B receptor, stably expressed in fibroblast LMTK- cells (L5), induces GTase activity and inositol 1,4,5-trisphosphate production when stimulated by agonist, whereas the parental LMTK- cells are unresponsive. This activity is blocked by antibodies against Gαq/11, but not by pertussis or cholera toxins or by anti-Gαi or anti-Gαs antibodies, indicating that it is mediated by the G-protein, Gαq/11, but not by Gε or Gγ (21). A more detailed examination of the GTase activity in LMS cells induced by 5-HT reveals a first activation at 1.5 min followed by a second activation peaking at 5 min, whereas the LMTK- cells are unresponsive (Fig. 1A). Therefore, we investigated the possibility of other coupling mechanisms for the 5-HT2B receptor. Interestingly, the early phase of GTase activation (at 1.5 min after 5-HT addition) can almost completely be blocked by incubation in the presence of anti-Gαq/11, antibody or γγ2 subunit antibodies, without affecting the LMTK- cells (Fig. 1C). In contrast, at 5 min after 5-HT addition, more than 95% of the GTase activation is resistant to treatment by anti-Gαq antibody, but about 35% of the activity is sensitive to treatment by anti-β1,-4 or γγ2 subunit antibodies and by Ras antibodies, without affecting the LMTK- cells (Fig. 1D). This suggests that the small G-protein Ras participates, at least partially, at the late phase of GTase activation.

In several signaling paradigms thought to involve the function of the Ras protein, stimulation with growth factors leads to the rapid accumulation of Ras protein in its GTP-bound (i.e. active) state. The immunoprecipitation by anti-Ras antibodies of extracts of LMS5 cells indicates that stimulation by 5-HT, transiently and specifically, increases the amount of the GTP-bound Ras complex (Fig. 1B). This activity is not seen in the
parental cell line nor in presence of the antagonist ritanserin. Interestingly, the LMS cell basal level of Ras GTPase activity is more than three times higher than that of LMTK- cells. Compared to basal levels of LMS cells, the increase in GTP-bound Ras induced by 5-HT is nearly 4-fold at 5 min, 78.2% ± 4.3 (n = 4) of which is sensitive to βγ antibodies (not shown). By 30 min after agonist addition, GTP-bound Ras returns to basal levels.

5-HT Action on Cell Division—The activation of Ras, known to stimulate cell division, has been investigated further by measuring phosphorylation by MAP kinase of MBP. 5-HT stimulation of LMS cells induces MAP kinase activation (Fig. 2A) which is not seen in the presence of ritanserin. At 10 min, this 5-HT stimulation, not observed in LMTK- cells, is almost completely blocked by ritanserin, a 5-HT2 antagonist, or by incubation in the presence of antibodies against the Ras, β1, 2, 4, and γ2, or Gα11 subunit (Fig. 2B).

Since stimulation of MAP kinases can lead to cell division (31), we investigated further the effect of 5-HT on the rate of LMS cell division. This analysis indicates that 5-HT acts through the 5-HT2B receptor on the steady state number of cells: both 5-HT2 agonists 5-HT (1 μM) and DOI (0.1 μM) stimulate cell growth of the LMS clone (but not of the LMTK- cells). These effects can be inhibited by incubation of agonists together with 1 μM ritanserin (Fig. 3A). Interestingly, in the absence of agonist and in 5-HT-free serum, the LMS cells have a cell division rate that is 2-fold greater than the LMTK- cells, and this rate is reduced by the antagonist ritanserin alone (Fig. 3A).

Long-term formation and tumor induction in nude mice—The long-term effect of the 5-HT2B receptor on the growth properties of the LMS cells was also investigated: LMS cells were grown to confluence, and the loss of contact inhibition was scored by counting the number of foci formed after 2 weeks or longer. The control cells (nontransfected LMTK- cells) never generated foci in 3% 5-HT-free FCS, with or without addition of 5-HT, DOI, or ritanserin. In contrast, in the absence of 5-HT2B agonists in the medium, foci occurred in LMS cells, although at a frequency 10 to 25 times lower than that observed in agonist-treated cells (Fig. 3B and Table I). To determine whether the 5-HT2B receptor-expressing cells were transformed, tumorigenicity was assessed by subcutaneous injection into nude mice. No tumors were observed upon injection of control LMTK- cells. In contrast, tumors appeared in all LMS-injected mice within 4 weeks. Similar results were observed when 105 foci-derived cells from agonist-stimulated or from nonstimulated LMS cells were injected separately into nude mice. Tumors were observed in every case, but tumors formed by foci-derived cells appeared earlier (2–3 weeks) and never spontaneously regressed.

We then investigated receptor expression in tumors by examining the presence of 5-HT2B-specific binding on tumor-derived cell lines. Interestingly, 2 weeks of treatment of nude mice-bearing tumors with the antagonist ritanserin induces a significant reduction in tumor size, suggesting the presence of the 5-HT2B-like receptors on these tumors (Table I). DOI bind-
ing under nonsaturating conditions (2 nM [125I]DOI) indicates a relative value of receptor densities. We, therefore, examined the persistence of the 5-HT2B receptor expression by plating the tumor-derived cells and estimating the receptor density from the DOI binding values. All tumor-derived cell lines expressed a significantly (p < 0.01, KS test) greater density of 5-HT2B receptors than the parental LM5 cell line (Table I). This suggests that the growth of these tumors is associated with selective expansion of cells which express the highest number of 5-HT2B receptors (Table I). Furthermore, all cell lines derived from tumors retain the ability to form foci, with a rough correlation between the number of foci and the density of 5-HT2B receptors (Table I). To test whether the formation of foci requires activation of 5-HT2B receptors, tumor-derived cell lines were plated on a lawn of LMTK2 cells in the presence or absence of agonists, ritanserin, or mesulergine for 2 weeks. As for the parental foci, the ability of the tumor-derived cell lines to form foci appears almost (95%) completely blocked by agonists (Table I). Thus, foci formation by these tumor-derived cells remain ligand-dependent.

In Vivo Localization of 5-HT2B Receptor in Tumors—The observation that 5-HT2B receptor expression is tumorigenic for LMTK2 cells raises the question of its physiological relevance. Since the 5-HT2B receptor is expressed in several peripheral tissues, we looked at its expression in peripheral tissue-derived tumors. Most of the human tumors derived from the enterochromaffin cell, carcinoid tumors (CT), have the property of synthesizing high amounts of 5-HT (32). An animal model of spontaneously appearing CT has been described in the aged M. natalensis (muridae) bearing spontaneous, malignant CT, originally described in Ref. 33 and homotransplanted to syngenic animals by Hosoda et al. (34). Antibodies, raised against the carboxyl-terminal portion of the mouse 5-HT2B receptor, which
are specific for 5-HT2B receptors, were used on tissue sections from human CT and Mastomys E-line CT (mainly 5-HT-secreting (25, 35)). Positive cells are organized in neuroendocrine-like structures (Fig. 4, A and B) in all examined human and Mastomys CT samples, whereas, in LM5-derived nude mice tumor, 5-HT2B receptors are present in fibrosarcoma-like structures (Fig. 4D). Therefore, in vivo, the 5-HT2B receptor is also expressed in spontaneously occurring malignant tumors.

5-HT Stimulates Ras Activity in Mastomys CT—After plating dissociated Mastomys CT cells, we investigated their response to 5-HT stimulation. As shown on Fig. 5, immunoprecipitation of tumor cell extracts with anti-Ras antibodies indicates that 5-HT induces an activation of the Ras-GTPase activity in a manner similar to that observed in LM5 cells, stimulation which is blocked by the antagonist ritanserin. However, the level of Ras stimulation by 5-HT is only 2.2 times the basal level which is high, probably due to endogenous Ras activity in these tumor cells (Fig. 5).

**DISCUSSION**

The aim of this study is to characterize further biochemical signals transduced by 5-HT in 5-HT2B receptor transfected cells (LM5). The early GTPase activation involves Gαq, β1,4, and γ subunits (1.5 min) (Fig. 1, A-D). The late phase of GTPase activation (5 min) is resistant to anti-Gαq antibodies and, therefore, implicates secondary activation of other G-proteins; 35% of this late GTPase is sensitive to anti-Ras and to anti-βγ antibodies (Fig. 1, A, C, and D). In addition, 5-HT2B agonists stimulate the accumulation of the GTP-bound form of Ras with similar kinetics peaking at 5 min after 5-HT stimulation (Fig. 1B). These results suggest that Ras is involved in the secondary phase of GTPase activation and that the βγ subunit is mediating this effect. The amount of GTP-bound Ras is stimulated 4-fold by 5-HT, and this is blocked in the presence of ritanserin (Fig. 1B). However, the Ras-GTPase activity determined by immunoprecipitation of Ras (Figs. 1B and 5) represents only 18% of the maximal total cellular GTPase activity (Fig. 1A), which in turn corresponds to approximately half of the GTPase activity blocked by anti-Ras antibodies (Fig. 1D). This indicates that other processes, that are inhibited by anti-Ras antibodies, may also participate in the 5-HT2B-dependent GTPase stimulation. Agonist stimulation of heterotrimeric G-proteins involves dissociation of the Gαβγ subunit from the receptor (36). The βγ subunit, subsequently released from Gαq (37, 38), stimulates the Ras-GTPase activity, raising, at least in part, the late GTPase activity. The Ras stimulation by the βγ subunit remains to be dissected out. Candidates to mediate this stimulation are proteins containing plekstrin homology domain since βγ subunits have affinity for such domains at the carboxyl terminus of β-adrenergic kinase (39). This includes the GTPase activating proteins, whose noncatalytic domain has been reported to interfere with transformation induced by G-protein-coupled receptors (40, 41) or other protein kinases (see Ref. 42 for review).

In LM5 cells, 5-HT triggers the activation of p42mapk/p44mapk (ERK2/ERK1) MAP kinase cascade (Fig. 2A). This "extracellular signal-regulated kinase" pathway is classically used by
activity was determined by analyzing the amount of GTP and GDP bound to immunoprecipitated Ras from control and stimulated cells as in Fig. 1B. Stimulation of LMTK − (white boxes), of LM5 (gray boxes), or of Mastomys dissociated CT cells (black boxes) alone (○) or in the presence of 10 nM 5-HT (□) or of 10 nM 5-HT plus 1 nM ritanserin (x) have been performed 4 times. The results, after 5 min of stimulation (peak of Fig. 1B), are expressed as femtomoles of GTP bound per mg of protein per min. The LMTK − cells are not responsive to 5-HT in LM5 cells as in Fig. 1B, the amount of GTP-bound Ras is stimulated 4 times by 5-HT and blocked by ritanserin, whereas in the Mastomys tumor cells the amount of GTP-bound Ras is stimulated 2.2 times and is also blocked by ritanserin. We notice that the level of active Ras in the Mastomys tumor cells is nearly as high as that of stimulated LM5 cells, indicating that Ras is already activated in these tumor cells.

growth factor stimulation of intracellular tyrosine kinase activity (43). This cascade leads to cell division (31), differentiation, and/or transformation and can be potentiated by stimulation of Ga αq (44). Ligands for multiple G-protein coupled receptors have been shown to stimulate tyrosine kinase activity (42, 45–47), and angiotensin II AT1 receptor recently has been reported to stimulate directly the Jak/STAT pathway in rat aortic smooth muscle (48). The contribution of the 5-HT2B receptor to tyrosine kinase activation remains to be investigated. However, MAP kinase activity has been reported to be stimulated by Ga αq, a pertussis toxin-sensitive G-protein (47, 49), and by Ga αq (50–52) or inhibited by Ga αq (see Ref. 53 for review). Furthermore, 90% of the 5-HT-induced MAP kinase activation in LM5 cells is specifically blocked by antibodies against either Ga αq-β γ γ subunit or Ras (Fig. 2B), also suggesting that the dissociation of the Ga αq and β γ γ as well as Ras activation are involved in this stimulation. In contrast to other receptor systems (52, 54–57), the contribution of the phospholipase C-β stimulation to MAP kinase activation seems only minor. Nevertheless, our results add 5-HT to the 5-HT2B receptors to the growing list of receptors, including α1-adrenergic, bombesin, m1–5 acetylcholine muscarinic, prostaglandin, and thrombin, which activate Ras by Ga αq (58–60) or MAP kinases by Ga αq and β γ γ subunit (51, 52).

The stimulation of the 5-HT2B receptor not only triggers the MAP kinase cascade, but also activates the LM5 cell division rate (Fig. 3A). This is confirmed by the enhancement of [3H]thymidine incorporation in response to agonists (data not shown). Therefore, these data demonstrate that 5-HT has a mitogenic effect on LM5 cells. This mitogenic effect probably results from MAP kinase stimulation via Ras, Ga αq, and β γ γ, as already shown in other systems by use of the dominant negative form of Ras (58) or of constitutively active Ga αq (44, 61). In addition to short-term effects, MAP kinase activation may lead to long-term effects, including cell transformation and/or differentiation. LM5 cells grown to confluence are forming foci, an effect which is stimulated by agonist and inhibited by antagonist (Fig. 3B). Surprisingly, the potency of antagonist is greater with regard to the ability to inhibit foci formation than with the rate of cell division (Fig. 3, A and B). This difference may be related to different responses in different physiological states (exponential growth versus quiescent) and probably corresponds to short-term effects versus long-term effects involving different effectors of the receptor. In addition, all nude mice-derived tumor cells express more 5-HT2B binding sites than LM5 cells. This indicates that a threshold level of receptor expression (1 pmol/mg of protein) is probably necessary for transformation. However, these events remain 5-HT and 5-HT2B receptor-dependent (Table I). Although the contribution of Ras stimulation to the long-term alterations is probable, since activated Ras is known to transform NIH3T3 fibroblasts (62) and since expression of a dominant negative form of Ras inhibits the muscarinic m5 receptor-dependent transformation (60), additional transduction mechanisms are probably involved. Nevertheless, expression of the 5-HT2B receptor triggers both the 5-HT-mediated mitogenicity and the transformed phenotype of the LMTK − cells, indicating that the 5-HT2B receptor behaves as a ligand-dependent proto-oncogene for the LMTK − cell line.

Interestingly, the unstimulated LM5 cells have a higher cell division rate than the parental LMTK − cells. This basal activity is partially reduced in the presence of the antagonist ritanserin (Fig. 3A) (inverse agonist) and probably corresponds to an intrinsic activity of the receptor which has also been shown for the 5-HT2C receptor (63). Similarly, in unstimulated LM5 cells, foci spontaneously appear and the basal level of Ras activity is elevated suggesting intrinsic activity of the 5-HT2B receptor. Therefore, intrinsic activity and a high level of expression may be two important parameters for tumor development. Tumor regression in nude mice induced by ritanserin treatment may open lines of investigation to develop new types of therapeutic agents.

In several aspects, the oncogenic properties of the 5-HT2B receptor seems to differ from those described for the other 5-HT2 receptors: very high levels of 5-HT2A-2C receptor expression are required in NIH3T3 cells to induce foci formation (>10 pmol of receptor/mg of protein), cells derived from nude mice tumors induced by these foci are 5-HT independent (17, 18), and expression of the 5-HT2C receptor does not transform the CCL39 cell line (64). Several factors may be responsible for these differences. (i) Use of 5-HT-depleted serum (<1 nM 5-HT) in this report may be important for the full activity of the receptor. (ii) The different cell lines (LMTK −, NIH3T3, or CCL39) may express different levels of G-proteins (65) or different combinations of G-protein subunits (66), some of which may be crucial for transformation; the γ γ subunit has also been implicated in G αq-mediated Ras activation (51). (iii) Despite common coupling to phospholipase C-β, the ability to stimulate Ras has not been reported for the 5-HT2A or 5-HT2C receptors. (iv) The carboxyl-terminal region of the third intracellular loop, close to the sixth transmembrane domain, contains important amino acids involved in coupling to different G αq subtypes of G-protein (67) and is not conserved among 5-HT2 receptors. This region controls the G αq-dependent mitogenicity of the m1–5 muscarinic receptors (65) and, when mutated, leads to constitutive activation of the Ga αq by the α1-adrenergic receptor (68).

Furthermore, 5-HT which is present early in embryonic development (12) participates in craniofacial (13) and cardiovascular morphogenesis (14, 15). Therefore, some 5-HT trophic functions during embryogenesis may be controlled by the mitogenic and transforming properties of 5-HT2B receptor. Its embryonic expression starts earlier than the 5-HT2A or 5-HT2C receptors (69) and is located in heart primordia and in neural fold before neural tube closure (4, 16). In addition, the
5-HT2B receptor, which is expressed by immortalized teratoma-derived cells 1C11 before complete serotonergic cAMP-induced differentiation\(^3\) (21), seems to have an autocrine function.\(^2\)

Finally, the 5-HT2B receptor is expressed in tumors from both human and M. natalensis species (Fig. 4, B and C). The Mastomys CT expresses a 5-HT2B receptor very similar to the Mastomys species (Fig. 4, B and C). The Mastomys CT expresses a 5-HT2B receptor very similar to the Mastomys 5-HT2B receptor, which is expressed by immortalized teratoma-derived cells 1C11 before complete serotonergic cAMP-induced differentiation\(^3\) (21), seems to have an autocrine function.\(^2\)

Therefore, this report makes, for the first time, a parallel between induced tumorigenicity by expression of the 5-HT2B receptor in non-transformed fibroblasts and its expression by spontaneous tumors, which are both coupled to Ras activation (Fig. 5). However, the participation of the 5-HT2B receptor in enterochromaffin cell malignant transformation remains to be proven.

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