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Molecular cloning of a novel gene involved in serotonin receptor-mediated signal transduction in rat stomach

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Abstract **In *Xenopus* oocytes injected with small size mRNAs (500–700 b), obtained from rat stomach by fractionation, application of 10 μM 5-HT induced a substantial Ca^{2+}-activated Cl^- current ($I_{Cl\text{-}Ca}$). $I_{Cl\text{-}Ca}$ was not elicited by 5-HT in native oocytes. Consistent results from this assay in the oocyte expression system motivated cDNA cloning experiments. A novel cDNA (named rat stomach serotonin receptor-related cDNA: *RSS* cDNA) which encodes a small protein involved in specific 5-HT receptor-mediated $I_{Cl\text{-}Ca}$ activation was identified. The molecular weight of *RSS* protein in the reticulocyte lysate translation system (~10 kDa) is identical to that calculated from the amino acid sequence. Computer-aided analysis of the predicted protein does not show any obvious sequence homologies (<18%) to any other proteins including G protein-coupled receptors. Northern analysis revealed that *RSS* mRNA is ubiquitously expressed at varying levels in a number of different tissues. Furthermore, the binding of [^{3}H]spiperone, a 5-HT_2 receptor antagonist, was examined in CHO cells, which highly expressed *RSS* transcripts (named CHO-RSS). Specific binding of [^{3}H]spiperone was not clearly observed in native CHO but was detected in CHO-RSS. The dissociation constant was 10.3 nM in CHO-RSS. These results suggest that *RSS* protein may be a factor which facilitates 5-HT receptor expression or, alternatively, an enhancer of the affinity of native 5-HT receptor to 5-HT.**

Key words: 5-HT receptor; cDNA cloning; *Xenopus* oocyte; In vitro translation; Northern blotting; [^{3}H]Spiperone binding assay

1. Introduction

Serotonin (5-HT) is both a neurotransmitter [1] and an autacoid [2] which is involved in diverse psychological and physiological functions in the central and peripheral nervous systems and smooth muscle tissues [3]. 5-HT receptors are targets for many medications used in the treatment of psychiatric disorders and of problems related to intestinal and vascular smooth muscle tonus. 5-HT potently stimulates contractions of the isolated rat stomach fundus [4,5]. After many attempts to characterize pharmacologically the 5-HT receptor responsible for this effect [6–8], a novel receptor subtype, the 5-HT_{2B} receptor, was cloned from the rat stomach fundus in 1992 [9,10]. The pharmacological profile of 5-HT_{2B} is consistent with that of the receptor mediating contraction in the rat stomach fundus [11,12].

While making a survey of factors which can modulate 5-HT_{2B} receptor signal transduction, we found that *Xenopus* oocytes injected with fractionated mRNAs in a range of 500–700 b (much smaller than cDNA of 5-HT_{2B} receptor, 2020 bp) elicited substantial I_{Cl-Ca} in response to exogenously applied 5-HT. The present study was undertaken to identify this novel cDNA clone from a rat stomach cDNA library using *Xenopus* oocytes expression system. No such gene which may have a key role in 5-HT receptor-mediated signal transduction has been reported previously.

2. Materials and methods

2.1. Materials

Restriction endonucleases and other enzymes were purchased from Toyobo or NEB. [α-^{32}P]dATP (~3000 Ci/mmol), [α-^{32}P]dCTP (~3000 Ci/mmol), [γ-^{32}P]ATP (~3000 Ci/mmol) were from Amersham; [^{35}S]methionine (~1000 Ci/mmol) and [^{3}H]spiperone were from NEN. ZAP-cDNA synthesis kit, Gigapack II Gold Packaging extract, mCAP mRNA capping kit, and In vitro express translation kit were from Stratagene. CHO-K1 was obtained from Japanese Cancer Research Resources Bank (JCRB). Geneticin (G418) and D-MEM/F12 were from Gibco BRL. All other chemicals were obtained from Wako, Boehringer-Mannheim, Pharmacia, or Nippon Gene.

2.2. Isolation and fractionation of poly(A)$^+$ RNA from tissues

Total RNA was isolated from rat stomach by a modification of the method of Chirgwin et al. [13] and enriched for poly(A)-containing species by oligo(dT) chromatography [14]. Rat stomach poly(A)$^+$ RNA was fractionated on a linear sucrose gradient (10–30%, w/w) according to Sumikawa et al. [15]. Fractions of 400 μl each were collected and ethanol-precipitated twice and resuspended in 20 μl of DEPC-treated water.

2.3. Preparation of ^{32}P-labeled probe and Northern blotting

5-HT_{2B} receptor-specific 39-base oligonucleotides (5′-TTT CTG CTT ATG TGG TGC CCC TTT TTC ATT ACA AAC GTC-3′) were synthesized and were labeled on the 5′ ends with [γ-^{32}P]ATP using T4 polynucleotide kinase. Fractionated mRNAs from rat stomach were separated on a 1.0% agarose gel containing 2.2 M formaldehyde and 40 mM MOPS. Hybridization was carried out in 50% formaldehyde, 5×SSPE, 10×Denhardt's solution, 0.1% SDS, and 0.1 mg/ml denatured salmon sperm DNA at 42°C. After hybridization, each filter was washed three times in 5×SSC for 5 min at room temperature, and then autoradiographed with ~24 h exposure times and analyzed using a Fujix BAS2000 Bio-Imaging system.

2.4. cDNA library construction, screening, and DNA sequencing

A rat stomach oligo(dT)-primed cDNA library was constructed in the λ_{ZAP} vector. The library consisted of ~8×10^5 cDNA clones. Initially, the library was screened for full-length clones by in vitro transcription of five pools of 40 000 clones for each. The cDNA mixtures in each pool were transcribed to cRNA and injected into oocytes for electrophysiological assay of the response to 5-HT. A pool of cDNA which resulted in an active response to 5-HT was again divided into five fractions of 4000 clones for each. The purification of the cDNA clone, *RSS* cDNA was carried out by five repetitions of stepwise fractionation. As a result of the excision from the λ_{ZAP}, a clonal

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RSS cDNA was included in the plasmid pBluescript KS(−). Sequencing was performed by the dideoxynucleotide chain termination method [16] using the Taq DyeDeoxy Terminator Cycle Sequencing kit, with DNA sequencer 373A (Applied Biosystems).

2.5. RNA injection into oocytes and electrophysiology

Xenopus oocytes were isolated, collagenase-treated, and injected with poly(A)$^+$ RNA or synthesized cRNA using conventional methods [17]. Transcription was primed with the cap dinucleotide G(5′)ppp(5′)G and cRNA was injected at a concentration of 0.5–1.0 μg/μl. One or 2 days after the injection of cRNA, oocytes were voltage-clamped using two microelectrodes [18] in modified Barth's solution (MBS) containing 7.5 mM Tris-HCl (pH 7.6), 88 mM NaCl, 1.0 mM KCl, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$. Membrane currents were recorded using an amplifier (CEZ-1250, Nihonkoden) and a pen recorder (FBR-252A, Toadenpa). All experiments were performed at room temperature (23–26°C).

2.6. In vitro translation

Capped cRNAs were synthesized with T7 RNA polymerase as described above. The translation of the cRNAs was carried out using a reticulocyte lysate system (Stratagene) in the presence of [^{32}S]methionine. The reaction mixture was analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography for 12 h and measurement using a Fujix BAS2000 Bio-Imaging system.

2.7. Tissue distribution

Denatured total RNAs extracted from several tissues (10 μg for each) of the rat were separated by electrophoresis on a 1.0% agarose gel containing 2.2 M formaldehyde and 40 mM MOPS. Hybridization was carried out in 50% formaldehyde, 5×SSPE, 10×Denhardt's solution, 0.1% SDS, and 0.1 mg/ml denatured salmon sperm DNA at 42°C in fresh buffer to which a randomly primed, [α-^{32}P]dCTP-labeled probe (*Bam*HI/*Kpn*I digested fragment of *RSS* cDNA) was added. After hybridization, the filter was washed several times in 2×SSC for 30 min at room temperature. Filters were autoradiographed with 12–24 h exposure times and analyzed using a Fujix BAS2000 Bio-Imaging system.

2.8. Construction of eukaryotic expression vector

A mammalian expression vector, pRSV-X/pRSV-neo^r, was used for stable transfection in CHO-K1 cells. *RSS* cDNA was amplified by PCR using the following oligonucleotides as primers: 5′-CCG GAT CCA TGG CCC TGA TGC ATT CCA GT-3′ (underline: *Bam*HI site) and 5′-CCT CTA GAG GGC GAA TTG GGT ACC GGG CCC-3′ (underline: *Xba*I site). The PCR product included the whole coding region of *RSS* cDNA. Twenty-five cycles (94°C, 1 min; 55°C, 2 min; 72°C, 3 min) followed by 72°C for 5 min were done on a thermal cycler. The PCR products were extracted, double-digested with *Bam*HI and *Xba*I and ligated into pRSV-X/pRSV-neo^r. Recombinant vector with *RSS* cDNA (named pRSV-RSS/pRSV-neo^r) was identified by DNA sequencing.

2.9. Cell culture and transfections

CHO-K1 cells were maintained in Dulbecco's modified Eagle's medium (D-MEM/F-12) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and 2 mM glutamine, grown at 37°C in a humidified 5% CO_2 atmosphere, and passed at confluency. Cells were transfected with pRSV-RSS/pRSV-neo^r by calcium phosphate precipitation [19] and then selected in D-MEM/F-12 medium containing G418 (1.0 mg/ml). G418 resistance colonies were selected for high levels of *RSS* mRNA expression by Northern blot hybridization. Transfected cell lines, CHO-RSS, were maintained in supplemented D-MEM/F-12 medium with G418 and passaged confluently.

2.10. [^{3}H]Spiperone binding assay

For saturation binding studies, CHO-RSS cells were washed twice with phosphate-buffered saline (PBS), scraped off and centrifuged at 2000×*g* for 10 min at 4°C. Cells were suspended in binding buffer (50 mM Tris-HCl, pH 7.4, 4 mM $CaCl_2$). This procedure was repeated for three washes in total. Binding assays in intact cells were performed in triplicate. Volumes of 100 μl of cell suspension (approximately 1.5×10^6 cells) and 100 μl of binding buffer containing 0 or 400 μM spiperone for total or non-specific binding, respectively, were added to 200 μl of binding buffer which contained [^{3}H]spiperone at various final concentrations (0.1–20 nM). The mixture was incubated at 37°C for 1 h and then rapidly filtered over Whatmann GF/B filters that had been presoaked in 0.5% polyethyleneimine and pre-cooled with ice-cold binding buffer. The amount of [^{3}H]spiperone trapped on the filters was determined by liquid scintillation counting. Actual free [^{3}H]spiperone concentrations were determined by sampling the supernatant of parallel saturation experiments in which bound radioactivity had been separated by centrifugation. Each experiment was carried out in triplicate and analyzed with a non-linear least-squares curve-fitting procedure.

3. Results

3.1. Response to 5-HT in Xenopus oocytes by expression

Rat stomach mRNA was size-fractionated on a sucrose gradient, and fractions 1–10 were used in pairs for electrophysiological analysis and Northern hybridization analyses. The upper panel of Fig. 1A shows an ethidium bromide-stained agarose gel with lanes containing mRNA from each fraction. The mRNA in each fraction was injected into oocytes and the electrical responses to 10 μM 5-HT was examined under voltage clamp. Fig. 1B shows a Northern blot of the fractionated mRNA in a separate gel with a probe of 39-mer oligonucleotides which is specific for 5-HT_{2B} mRNA. The mRNA of about 2000 b in the second fraction was hybridized. Although some contamination was also observed in lanes 3–5, the 5-HT_{2B} mRNA is mainly included in fraction 2.

Fig. 1C shows inward currents elicited by application of 10 μM 5-HT to an oocyte injected with mRNA in fraction 7 (500–700 b). The 5-HT-induced current at holding potential of −60 mV was mainly Ca^{2+}-activated Cl^- current (I_{Cl-Ca}) since 50 nM niflumic acid or 100 μM 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) blocked this current. No or undetectably small responses to 5-HT were observed in native oocytes of the same origin (not shown, $n>20$). I_{Cl-Ca} in response to application of 5-HT was also observed in oocytes injected with mRNA in fraction 2 but not in those injected with mRNA in fractions 4, 5, 6, 8, 9, and 10. When 10 μM ACh was applied to the oocytes injected with mRNA in fraction 7, electrical responses were small and not different from those obtained in native oocytes (not shown, $n=8$). These data strongly suggest that a small mRNA of rat stomach is responsible for the expression of a novel protein involved in 5-HT receptor-mediated signal transduction in *Xenopus* oocytes.

3.2. Isolation of cDNA encoding 5-HT receptor related protein

A cDNA expression system that permits identification of functional cDNA clones in the absence of protein sequence information was designed based upon the cloning method originally developed by Masu et al. [20]. First, a cDNA library was constructed from total mRNA of the rat stomach. Appropriate numbers of the λcDNA clones were plated after in vitro packaging. The clonal λcDNA mixture was then extracted and cleaved with a restriction enzyme, *Not*I. The resultant λcDNA mixture was transcribed in vitro by specific T7 RNA polymerase in the presence of the capping nucleotide. The mRNA mixture synthesized was injected into oocytes, which were then tested for the expression of the response to 5-HT by measuring I_{Cl-Ca}. The target cDNA clone, *RSS* cDNA, was purified by stepwise fractionation of those cDNA mixtures, on the basis of the in vitro cRNA synthesis and electrophysiological assay in oocytes. The screening was

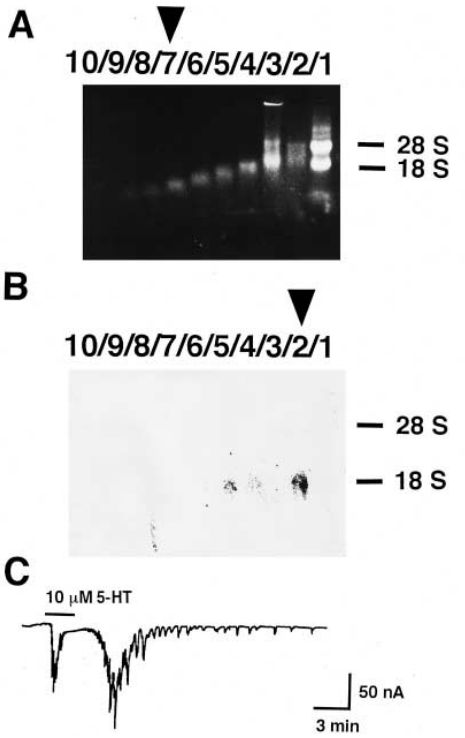


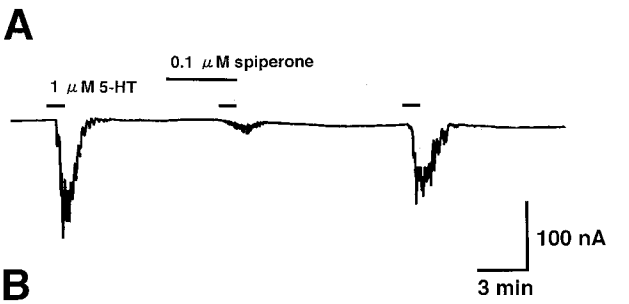
Fig. 1. Fractionated mRNAs by sucrose gradient and hybridization of the fractions with a specific probe for rat 5-HT_{2B} receptor. A: Ethidium bromide staining pattern, demonstrating the size fractionation of the RNA. The arrow indicates the low molecular weight fraction which is capable of expressing the Cl^- current response to 5-HT in injected oocytes. B: Northern blot of the fractions with an oligonucleotide probe specific for the cRNA encoding 5-HT_{2B} receptor. The arrow indicates the positively hybridized fraction. The numbers of lanes in both panels indicate the fraction numbers and correspond to each other in the two panels. Note that expression occurs when mRNAs in fraction 7 was injected (A) whereas positive hybridization is observed in fraction 2 (B). C: Oscillatory inward currents were elicited by a 3 min application of 10 μl 5-HT to an oocyte injected with mRNA (in fraction 7) of the rat stomach. The holding potential was −60 mV.

started from a λcDNA mixture comprising 4×10^5 cDNA clones. A reproducible response to 5-HT was observed in oocytes injected with the cRNA derived from the cDNA mixture. The peak amplitude of I_{Cl-Ca} induced by 5-HT in each screening step increased gradually; ~40 000 clones: 28.4 ± 5.5 nA, $n=6$; ~4000 clones: 30.2 ± 1.3 nA, $n=6$; ~400 clones: 64.0 ± 9.5 nA, $n=6$; 40 clones: 84.0 ± 13.9 nA, $n=6$; 6 clones: 106.3 ± 20.8 nA, $n=6$. Finally, a single clone (λ_{ZAP}) that was capable of inducing an electrophysiological response to 5-HT (147.0 ± 26.7 nA, $n=12$) was obtained.

3.3. *Nucleotide sequence of RSS cDNA and deduced amino acid structure*

The *RSS* cDNA was sequenced by the chain termination method using the Taq DyeDeoxy Terminator Cycle Sequencing kit, with DNA sequencer 373A (Applied Biosystems). The cDNA insert (448 bp) from this λ_{ZAP} phage was subcloned with the flanking T7 and T3 promoters by rescue excision to generate the plasmid pBluescript-RSS. To search for *RSS*-related cDNAs which may have longer nucleotide sequence than *RSS* cDNA, we performed the plaque hybridization analysis of the radiolabeled *RSS* cDNA probe in the rat stomach cDNA library. However, all positive clones (~20 clones) had the same inserts with *RSS* cDNA in the nucleotide length (~450 bp). Therefore, we confirmed that *RSS* cDNA obtained from electrophysiological assay in oocytes had an approximately complete nucleotide sequence. Pharmacological analysis of the response to 5-HT in *RSS* cRNA injected oocytes revealed that the responses had properties similar to those of the 5-HT_2 subtypes, since superfusion of oocytes with 0.1 μM spiperone markedly reduced the current evoked by 1 μM 5-HT in injected oocytes ($n=6$) (>80% inhibition) (Fig. 2A). In oocytes injected with total mRNA of the rat stomach, repetitive application of 1 μM 5-HT led to a marked decrease in the amplitude of the response. Foguet et al. [9] have reported that such desensitization to 5-HT is induced in oocytes expressing the 5-HT_{2B} receptor. However, the I_{Cl-Ca} response to 1 μM 5-HT in oocytes injected with *RSS* cRNA can be characterized by a lack of desensitization (not shown). The I_{Cl-Ca} response to 10 μM ACh was not changed in either native or cRNA-injected oocytes ($n>20$) (not shown). Therefore, the expressed protein specifically induced or facilitated the signal transduction triggered by 5-HT in oocytes. To confirm the existence of *RSS* mRNA in fraction 7 (Fig. 1B), Northern blot analysis was performed using ^{32}P-labeled *RSS* cDNA probe and the same nitrocellulose filter in Fig. 1B. In fraction 7 alone, hybridized mRNA was detected as a single band (not shown).

The deduced amino acid sequence which was assigned from

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  1  ATG GCC CTG ATG CAT TTC CAG TTT ACG TTC AAA CAG TTC GAA CAG
  1  M   A   L   M   H   F   Q   F   T   F   K   Q   F   E   Q
 46  CGT AAA AGC ATC CGC AGT ACC GCC CGC AAA GCC CGC GAT GAC TTT
 16  R   K   S   I   R   S   T   A   R   K   A   R   D   D   F
 91  GTC GTT GTA CAG ACG GCG GAC CTT TTT CAC GTT GCC TTT CAT TAC
 31  V   V   V   Q   T   A   D   L   F   H   V   A   F   H   Y
136  GGT ATT GCC CAA CGT GGC CTG ACC ATC ACC AGC GAT GAC CAC ATG
 46  G   I   A   Q   R   G   L   T   I   T   S   D   D   H   M
181  GCC GTT ACG GCG TAC GCT TAC TAT AGT TGT CAC GAG CTG ACC CCT
 61  A   V   T   A   Y   A   Y   Y   S   C   H   E   L   T   P
226  TGG TTA CGA ATA CAG AGT ACA AAC CCC GTA CAA AAG TAC GGG GCA
 76  W   L   R   I   Q   S   T   N   P   V   Q   K   Y   G   A
271  TAA
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Fig. 2. A: Electrophysiological assay of a response to 5-HT using *Xenopus* oocytes injected with *RSS* cRNA. Oscillatory inward currents were elicited by the application of 1 μM 5-HT to an oocyte injected with *RSS* cRNA for 1 min. The holding potential was −60 mV. Changing over to superfusate containing 1 μM 5-HT and/or 0.1 μM spiperone is indicated by bars above the trace. B: cDNA sequence and predicted amino acid sequence of the gene involved in 5-HT receptor-mediated signal transduction in the rat stomach. Nucleotides are numbered in the 5′-3′ direction, beginning with the first residue of the ATG triplet encoding the initiative methionine. The number of the first nucleotide residue of each line is given on the left. The deduced amino acid sequence code (in one-letter code) is shown below the nucleotide sequence. Amino acid residues are numbered beginning with the initiative methionine. Numbers of the first residues in each line are given on the left. The termination codon TAA at the end of open reading frame is marked by asterisks. The sequence has been deposited in the DDBJ/EMBL/GenBank databases and assigned accession number D89965.

the longest open reading frame of the *RSS* cDNA sequence is shown in Fig. 2B. This polypeptide consists of 90 amino acid residues and has a relative molecular weight of 10454 Da. The protein encoded by *RSS* transcripts was synthesized in vitro from the *RSS* cRNA using a reticulocyte lysate translation system. SDS polyacrylamide gel electrophoresis analysis [21] of the synthesized protein yielded a molecular weight of ~10000 Da (Fig. 3), which is consistent with the value calculated from the amino acid sequence. Thus, we concluded that the protein sequence encoded by the cDNA is composed of 90 amino acids. Computer-aided analysis of the predicted protein did not show any obvious sequence similarity to any proteins registered in the National Biomedical Research Foundation/European Molecular Biology Laboratory (NBRF/EMBL) database or to any other known G protein-coupled receptor protein including 5-HT receptors (similarity < 18%). The hydropathy plot (Hopp and Woods algorithm) of the deduced amino acid sequence shows that hydrophobic domains are abundant in the amino-termini (Fig. 4A), and that basic residues were abundant in the amino-termini (Fig. 4B).

3.4. Tissue distribution of RSS mRNA

The tissue distribution of *RSS* mRNA was analyzed by Northern blot experiments using randomly ^{32}P-labeled *RSS* cDNA (Fig. 5). Total RNA was obtained from a number of different tissues from young rat: brain, heart, intestine, kidney, liver, lung, skeletal muscle (soleus), spleen and stomach. Hybridized mRNA was detected as a single band with the molecular sizes of ~550 bp. *RSS* mRNA was observed in all tissues examined, suggesting that this transcript is expressed ubiquitously. Similarly, we performed Northern blot analysis using mRNAs obtained from native oocytes under the same condition. However, mRNAs obtained from native oocytes did not hybridize to the *RSS* cDNA probes (not shown), indicating that practically *RSS* mRNAs are not contained in native oocyte mRNAs.

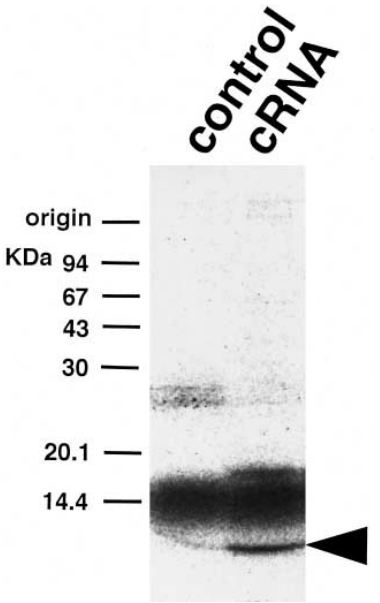

Fig. 3. SDS-PAGE mobility of in vitro translation product of the cRNA derived from *RSS* cDNA. The in vitro translation product of the cRNA was prepared with the reticulocyte lysate translation kit in the presence of [^{32}S]methionine at 30°C for 1 h. The labeled protein sample was reduced and subjected to electrophoresis on a 15% SDS-polyacrylamide gel (lane: cRNA). The control translation mix was treated exactly as the *RSS* translation procedure except that mRNA was not added (lane: control). Apparent molecular mass was visualized by staining with Coomassie brilliant blue and determined from the migration of protein standards resolved on the same gel. The migration of protein standards of 94, 67, 43, 30, 20.1, 14.4 kDa is shown on the left. The band at ~12 kDa was produced non-specifically by the lysate in the absence of any exogenous RNA. The arrow indicates a translation product of ~10 kDa from the cRNA. Autoradiograms were scanned using a Fujix BAS2000 Bio-Imaging system.

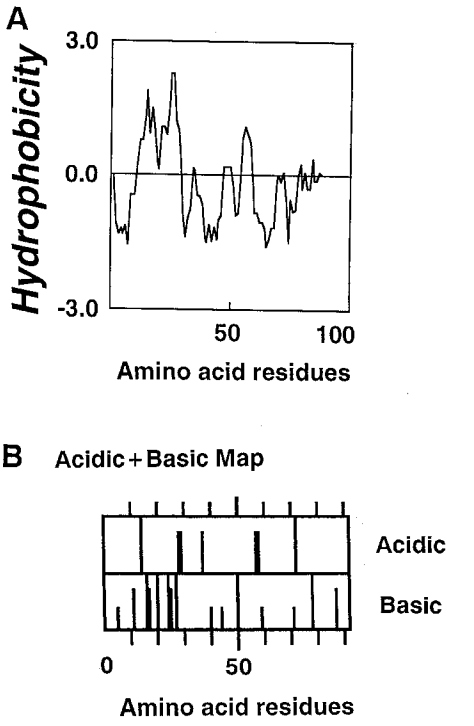

Fig. 4. Hydropathy plot and acidic+basic map of the protein sequence of *RSS*. A: Hydrophobicity values were calculated according to the Hopp and Woods algorithm and were plotted along the amino acid residue numbers. B: The acidic amino acid residues (Asp and Glu) and basic amino acid residues (His, Lys, and Arg) are indicated by upward and downward vertical lines, respectively. In acidic amino acid residues, short and long bars indicate Asp and Glu, respectively. In basic amino acid residues, short, intermediate, and long bars indicate His, Lys, and Arg, respectively.

3.5. Cloning of the CHO cells expressing RSS mRNA

The fragment of *RSS* cDNA was amplified by polymerase chain reaction (PCR) using the specific primers and subcloned in the mammalian expression vector pRSV-X/pRSV-neo^r, named pRSV-RSS/pRSV-neo^r. Neomycin-resistant gene was used as a selective marker gene that allowed *RSS* mRNA-expressing cell populations to grow in a medium containing G418 (1 mg/ml), which is an analog of neomycin. Approximately 50 clonal cells expressing *RSS* mRNA at various levels were isolated by this selection method and the clonal CHO cell stably expressing it at the highest level (named CHO-RSS) was screened by Northern blot hybridization. In Fig. 6A, total cellular RNAs isolated from CHO-K1, CHO cells transfected permanently with vector DNA alone (CHO-V), and CHO-RSS were analyzed by Northern blot hybridization with a DNA probe corresponding to randomly ^{32}P-labeled *Bam*HI/*Kpn*I fragment of *RSS* cDNA. Only CHO-RSS cells expressed

~2.3 kb mRNA which includes the SV40 origin of replication, an enhancer, a promoter, and SV40 polyadenylation signal of mammalian expression vector, pRSV-RSS/pRSV-neo^r (Fig. 6A).

3.6. [^{3}H]Spiperone binding assay

To determine changes in the expression level of 5-HT receptor in CHO cells, the binding of spiperone (a 5-HT$_2$-type receptor antagonist) to 5-HT receptor protein was measured. CHO-V and CHO-RSS were subjected to saturation binding analysis using [^{3}H]spiperone as a ligand in a concentration range of 0.1–20 nM. Parallel incubations in the presence of 100 μM spiperone were conducted to determine non-specific binding as a function of [^{3}H]spiperone concentration. Substantial specific binding of [^{3}H]spiperone was found in CHO-RSS but not in CHO-V. Similar results were obtained in three separate experiments. Scatchard plot analysis of the specific binding in CHO-RSS was performed by linear regression. The results indicate a single binding site of spiperone (not shown) and the binding parameters are $K_d = 10.25$ nM and $B_{max} = 8.2 \times 10^{-20}$ mol/cell (Fig. 6B).

4. Discussion

4.1. Cloning of RSS cDNA and functional expression

In the present study, cDNA encoding a novel protein related to 5-HT receptor has been cloned from a cDNA library of rat stomach. The fractionated mRNA containing *RSS* mRNA in Fig. 1 (fraction 7) had a molecular size of approximately 600 b. Correspondingly, the hybridized mRNA by the *RSS* probe was detected as a single band with a molecular size of ~550 b (Fig. 5). The cloned *RSS* cDNA, however, did not contain a poly(A)$^+$ tail and had an open reading frame of 270 bp. Expression of the protein by the injection of *RSS* cRNA in oocytes resulted in the induction of response to 5-HT. The response to ACh was not affected by the preinjection of *RSS* cRNA in oocytes. *RSS* protein is, therefore, an extraordinarily small molecule which may have a significant cellular function in 5-HT receptor-related signal transduction. The possibility that the protein is functional as an oligomer was not examined in the present study and could not be ruled out. Northern hybridization analysis showed that the *RSS* mRNA is widely distributed in various tissues of the rat.

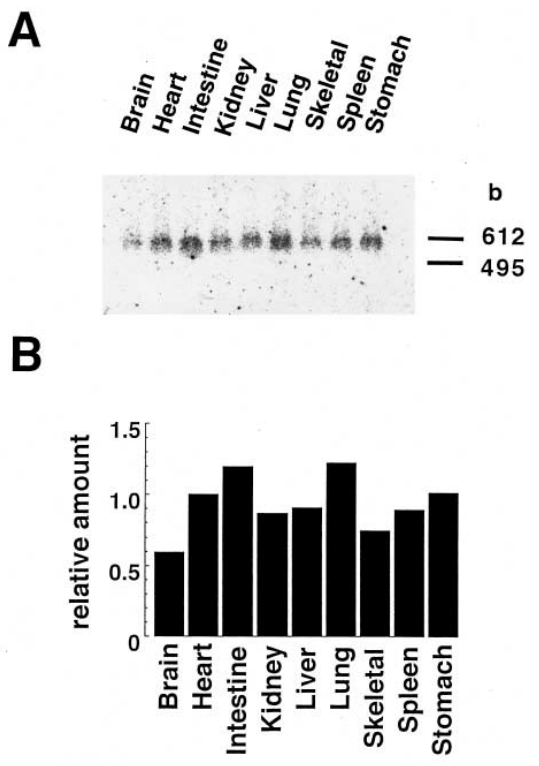

Fig. 5. Northern hybridization analyses of total RNAs isolated from various tissues of rat. 10 μg aliquots of total RNAs were isolated from the following tissues: Br: brain; He: heart; In: intestine; Ki: kidney; Li: liver; Lu: lung; Sk: skeletal muscle; Sp: spleen; St: stomach. Northern blot analysis was conducted using ^{32}P-labeled probes for the *Bam*HI/*Kpn*I fragment of *RSS* cDNA. The molecular marker is marker V (ΦX174/*Hinc*II digest) (Nippon Gene). Autoradiograms were analyzed using a Fujix BAS2000 Bio-Imaging system.

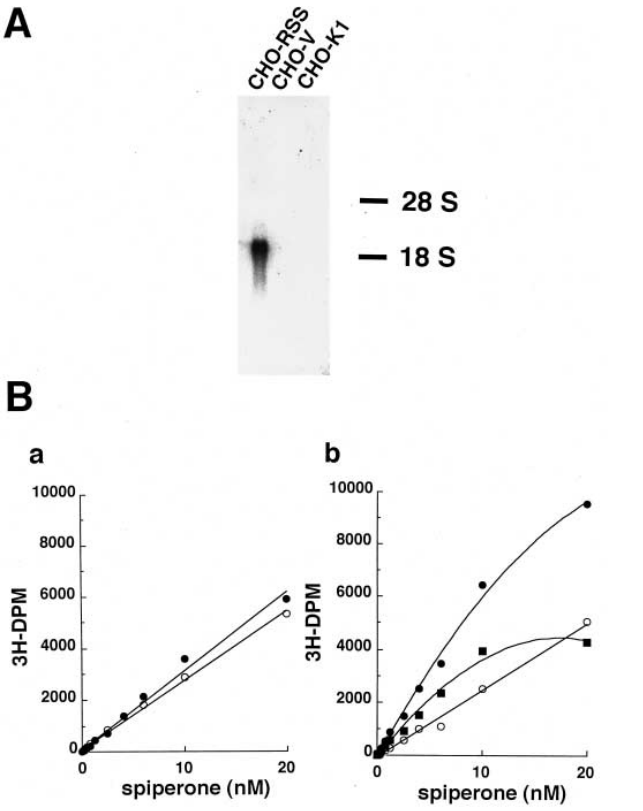

Fig. 6. A: Northern blot hybridization analyses of *RSS* mRNA in CHO cells. Total RNAs were isolated as described in Section 2 from nontransfected cells (CHO-K1), cells transfected with the vector DNA alone (CHO-V), and cells transfected with the *RSS* cDNA (CHO-RSS). Aliquots of total RNAs (10 μg) were loaded into adjacent lanes of a formaldehyde-agarose gel, fractionated by electrophoresis for 3 h with 4 V/cm, and then transferred to a nitrocellulose filter. The filter membrane was hybridized with a ^{32}P-labeled DNA probe corresponding to *RSS* cDNA. The filter was autoradiographed and analyzed using a Fujix BAS2000 Bio-Imaging system. Migration of 28S and 18S rRNA is shown on the right. B: Radioligand binding analyses in CHO-V (a) and CHO-RSS (b). [^{3}H]spiperone binding to 1.5×10^6 cells was expressed as dpm and plotted against the corresponding concentration of [^{3}H]spiperone in the binding solution. Total binding and non-specific binding were obtained in the absence and in the presence of a large excess of unlabelled spiperone, respectively. Data shown are the result of a single experiment performed in triplicate. Open and closed circles indicate non-specific and total binding, respectively. Closed squares indicate specific binding.

Although the co-existence of 5-HT receptors was not demonstrated, transcripts of the 5-HT_{2B} receptor gene have been detected by Northern blot, RT-PCR or immunohistochemical experiments in the various peripheries: aorta, liver, lung, heart, kidney and placenta in the human, and stomach, intestine, pulmonary smooth muscle, heart, kidney in the mouse [22–24]. The amino acid sequence of the *RSS* protein does not show any obvious similarity with known proteins, making it difficult to determine its exact cellular function in 5-HT receptor-related signal transduction.

4.2. Putative cellular function of RSS protein

One possibility is that *RSS* protein may be a factor regulating the transcription or translation of the 5-HT receptor itself. As shown in Fig. 4, basic residues of *RSS* protein may form a DNA binding site, however signal peptides which are required to transfer into nuclei [25] are not present in *RSS* protein. Although the electrical response to 5-HT was undetectably small in most native oocytes, there may be some 5-HT receptors coupling with PLC. The possibility that the expression of 5-HT receptors is enhanced in oocytes by injection of *RSS* cRNA was not examined in the present study. It is, however, shown that the specific binding of [^{3}H]spiperone is observed in CHO-RSS which permanently express *RSS* mRNA at high level, but not found in CHO-V. Although the amount of B_{max} in CHO-RSS was not large, the results may suggest that the expression of 5-HT receptors, which is undetectably low in CHO-K1 and CHO-V, was enhanced in CHO-RSS.

An alternative explanation for the marked enhancement of the response to 5-HT after *RSS* cRNA injection in oocytes is that the *RSS* protein may be a component or a cofactor involved in the pathway of signal transduction triggered by this G protein-coupled 5-HT receptor activation. *RSS* protein may increase the binding affinity of the receptor to 5-HT and/or sensitize one of the steps of the signal transduction which activates PLC. Actually, there are several reports suggesting a requirement for cofactors for membrane insertion and post-translational activation of G protein-coupled receptors [26–28]. Moreover, some proteins which markedly affect G protein activity or receptor-G protein coupling have been identified. For example, GAP-43 is a 43 kDa protein, which facilitates responses to ACh and 5-HT [29] and can affect G-protein activity or receptor-G protein coupling in the cellular milieu. Surprisingly, endogenous small peptides which consist of 2–4 amino acids and specifically reduce 5-HT binding to 5-$HT_{1B/1D}$ receptors have been found in mammalian brain [30]. There is no homology between the amino acid sequence of the most effective small peptide (LSAL) and that of *RSS* protein.

It is likely that the activity of one or more proteins in the signal transduction pathway may be regulated by phosphorylation/dephosphorylation reactions. Phosphorylation of receptor proteins by receptor-specific kinases is a common mechanism for autoregulation of various types of receptors (β-receptor [31]; muscarinic receptor [32]; rhodopsin [33]). The *RSS* protein could be a cofactor for a 5-HT receptor-specific kinase, and may progressively up-regulate the affinity of the receptor to 5-HT. However, a 5-HT receptor-specific kinase has not been identified yet.

4.3. Conclusion

Although the function of the *RSS* protein has not been completely revealed in the present study, the fact that such a small protein regulates transcription or translation of 5-HT receptor or the receptor affinity to 5-HT provides new insights into the molecular regulation of 5-HT receptor and related signal transductions. It will be interesting to examine whether this type of specific factors is involved in other receptor activation and following signal transduction cascades.

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