Primary structure and expression of a naturally truncated human P2X ATP receptor subunit from brain and immune system

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Abstract A novel member of the ionotropic ATP receptor gene family has been identified in human brain. This 422 amino acid long P2X receptor subunit has 62% sequence identity with rat $P2X_5$. Several characteristic motifs of ATP-gated channels are present in its primary structure, but this $P2X_5$-related subunit displays a single transmembrane domain. Heterologous expression of chimeric subunits containing the C-terminal domain of rat $P2X_5$ leads to the formation of desensitizing functional ATP-gated channels in *Xenopus* oocytes. The developmentally regulated mRNA, found in two splicing variant forms, is expressed at high levels in brain and immune system.

Key words: Transmitter-gated channel; Purinoceptor; Nucleotide; Lymphocyte; Cerebellum

1. Introduction

Mammalian P2X receptors belong to a multigene family of non-selective cation channels activated by extracellular ATP. The seven known members of the rat P2X family can be grouped into three functional categories according to their pharmacological profiles and to their properties of desensitization. $P2X_1$ [1] and $P2X_3$ [2,3] are highly sensitive to α,β-methylene-ATP (αβmATP) and desensitize rapidly; $P2X_2$ [4], $P2X_5$ [5,6] and $P2X_7$ [7] do not respond to αβmATP below 100 μM and do not desensitize, whereas $P2X_4$ [8–10] and $P2X_6$ [5] do not respond to αβmATP, are almost insensitive to co-applied non-competitive antagonists suramin and PPADS, and show moderate desensitization. Despite the fact that high levels of expression of P2X receptors are observed in many central and peripheral tissues, reinforcing the concept of an important role for ATP in intercellular communication [11], much less is known about their human counterparts. Excitatory ATP-gated channels play a specific role in sensory systems [12–14], therefore the development of subtype-specific P2X antagonists with analgesic properties should take into account functional differences between mammalian species. The reports on the expression of cloned human P2X orthologs of $P2X_1$ [15], $P2X_3$ [16], $P2X_4$ [17] and $P2X_7$ [18] emphasized both pharmacological and anatomical specificities that seriously undermine the relevance of our knowledge based on rodent systems for extrapolation to human fast purinergic transmission. In an effort to complete the genetic inventory of human ionotropic ATP receptors, we report here the identification, heterologous expression and anatomical distribution of a novel member of the human P2X gene family isolated from fetal brain.

2. Materials and methods

2.1. Molecular cloning and in vitro translation

Using the TBLASTN algorithm, virtual screening of the dbEST database [19] with the whole coding region of rat $P2X_4$ subunit led to the identification of human fetal brain EST sequences encoding a novel P2X gene (GenBank accession numbers T80104 and Z43811). The clone tagged by EST T80104 was sequenced on both strands and was shown to encode a short splicing variant of human P2X subunit related to rat $P2X_5$: $hP2X_{5R}$ (Fig. 1). A longer splicing variant of $hP2X_{5R}$ was detected in RT-PCR from human cerebellum mRNA with exact match primers. The EST clone was engineered to generate the long version of $hP2X_{5R}$ (Fig. 1), deposited in Genbank under accession number AF016709. This $hP2X_{5R}$ clone was transferred into the *Hin*dIII-*Not*I sites of eukaryotic vector pcDNA3 (Invitrogen) for CMV-driven heterologous expression in HEK-293 cells and *Xenopus* oocytes. Supercoiled $hP2X_{5R}$ plasmid was used for in vitro translation using the TnT system with T7 RNA polymerase (Promega) and [^{35}S]cysteine (ICN) according to the manufacturer's specifications.

2.2. Construction of epitope-tagged $hP2X_{5R}$ subunits and immunolocalization

$hP2X_{5R}$ subunit was epitope-tagged ($hP2X_{5R}$-FL) to facilitate the immunolocalization of the protein both in situ and in Western blot from transfected mammalian cells. The Flag octapeptide DYKDDDDK (IBI) was inserted by PCR in the C-terminal domain of $hP2X_{5R}$ using an antisense oligonucleotide primer designed for the replacement of its natural stop codon by an artificial in-frame *Xho*I site (TCACTCGAGCAACGTGCTCCTGTGGGGCT). Full-length mutant $hP2X_{5R}$ cDNA was amplified in PCR using *Pfu* polymerase (Stratagene), then ligated to an *Xho*I-*Xba*I cassette containing the Flag peptide followed by a stop codon [20] in the pcDNA3 vector.

For transfection of $hP2X_{5R}$-FL subunits, HEK-293 cells (ATCC CRL1573) were grown in DMEM-10% heat-inactivated fetal bovine serum (Wisent, St Bruno, Quebec) containing penicillin and streptomycin. Freshly plated cells reaching 30–50% confluence were used for transient transfection using the calcium phosphate method on 90 mm dishes with 10 μg supercoiled plasmid/10^6 cells.

For immunofluorescence, transfected HEK-293 cells (48–72 h post-transfection time) were plated at 50–70% confluence in poly-lysine-coated chambers. Adherent cells were washed in PBS and fixed for 20 min at room temperature with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 8.0. After blocking non-specific sites with 2% normal goat serum, fixed cells were incubated with anti-Flag monoclonal antibody (mAb) M2 (1 μg/ml, IBI) for 1 h at room temperature in 0.05 M Tris-saline buffer pH 7.2 containing 0.2% Triton X-100, 5% normal goat serum and 5% dry milk powder. Bound primary antibodies were detected by immunofluorescence after 1 h incubation with Texas red-labeled goat anti-mouse (2 μg/ml) secondary antibodies (ImmunoResearch Labs).

For high resolution analysis of $hP2X_{5R}$-FL subcellular distribution, we used a Zeiss Laserscan Inverted 410 confocal microscope equipped with an argon-krypton laser set at 580 nm for Texas red. Serial images (512×512 pixels) acquired as single optical sections were averaged over 32 scans/frame and processed with the Zeiss CLMS software package.

For Western blots, transfected cells were lifted in Hanks' modified

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calcium-free medium with 20 mM EDTA, pelleted at low speed and homogenized in 10 volumes of 10 mM HEPES buffer pH 7.4 containing the protease inhibitors phenylmethylsulfonyl fluoride (0.2 mM) and benzamidine (1 mM). Lysates were pelleted at 14000×*g* for 5 min to remove cell debris before protein assay and crude membrane proteins in supernatants were solubilized with SDS-containing loading buffer. About 150 μg of protein/lane were run on 12% SDS-PAGE, then transferred to nitrocellulose. Immunoprobing was performed with mouse mAb M2 (1 μg/ml) followed by peroxidase-labeled secondary antibodies for visualization by enhanced chemiluminescence (Amersham).

2.3. *Functional expression of h-rP2X$_5$ chimera in Xenopus oocytes*

The domain of hP2X$_{5R}$ containing amino acids 1–318 was fused to the PCR-amplified domain corresponding to amino acids 318–455 of rat P2X5 using the natural *Bam*HI site of hP2X$_{5R}$ to generate the chimera h-rP2X$_5$. Oocytes surgically removed from mature *Xenopus laevis* frogs were treated for 2 h at room temperature with type II collagenase (Gibco-BRL) in Barth's solution under agitation. Selected stage IV–V oocytes were defolliculated manually before nuclear microinjection [21] of 10 ng cDNA of hP2X$_5$ or h-rP2X$_5$ chimera in pcDNA3 vector. After 2–4 days of expression at 19°C in Barth's solution containing 10 μg/ml gentamicin, oocytes were recorded in two-electrode voltage-clamp configuration using a OC-725B amplifier (Warner Inst.). Signals were acquired and digitized at 500 Hz using a Macintosh IIci equipped with an A/D card NB-MIO16XL (National Instruments) then traces were post-filtered at 100 Hz in Axograph (Axon Instruments). Agonists dissolved in Ringer's solution containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$ in 10 mM HEPES pH 7.4 at room temperature were applied on oocytes by perfusion in constant flow (10 ml/min).

2.4. *RT-PCR detection of hP2X$_{5R}$ mRNA from human cerebellum and lymphocytes*

Total RNA from post-mortem adult human cerebellar cortex and adult lymphocytes were isolated using Trizol reagent (Gibco-BRL). Then 5 μg of each RNA was subjected to random-primed reverse-transcription with Superscript (Gibco-BRL). Around 100 ng of RT-cDNA was used as template for PCR with the following primers: forward TTCGACTACAAGACCGAGAAGT (P1, see Fig. 1B) and

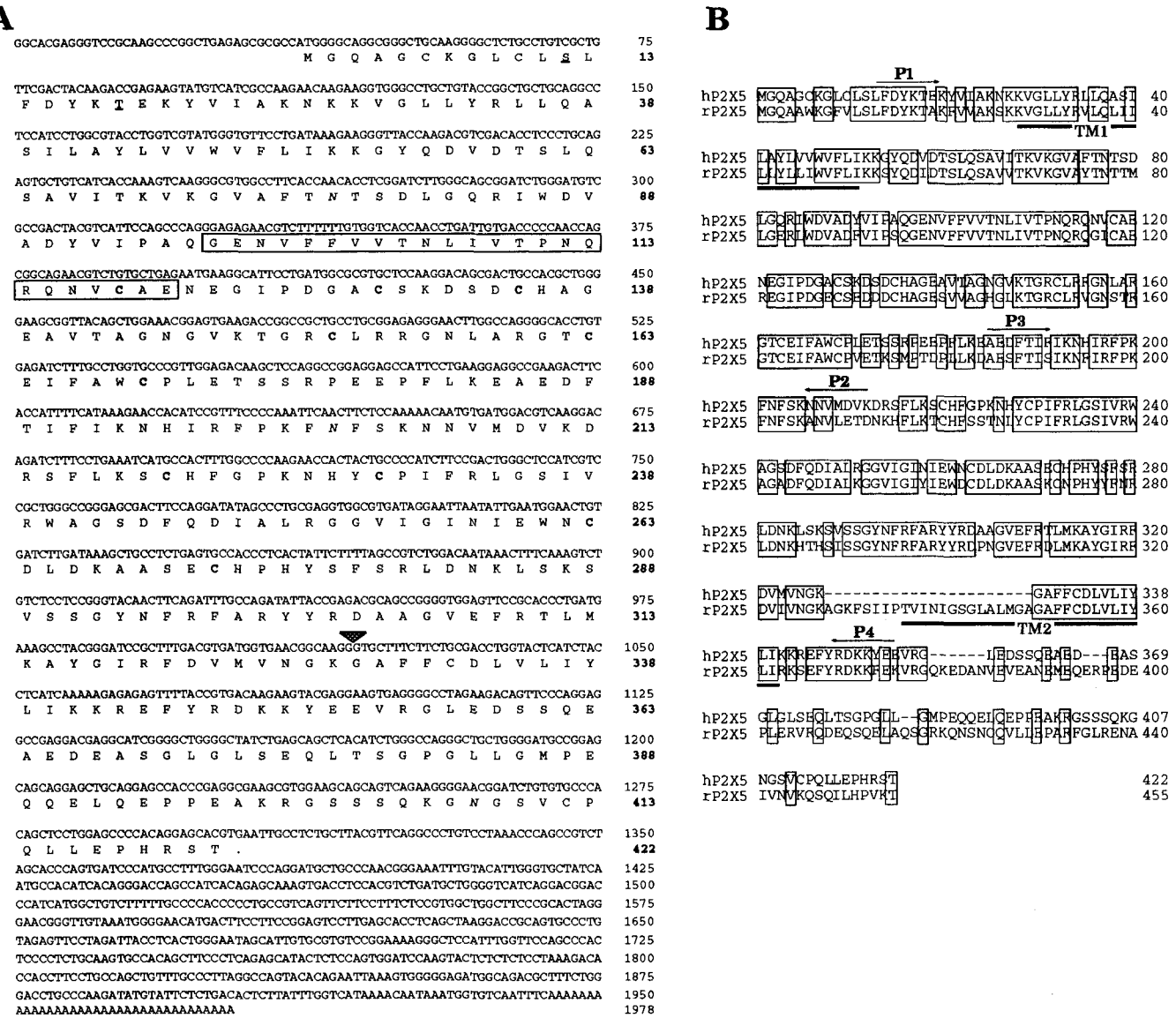

Fig. 1. Molecular cloning of a novel member of the human P2X ATP gene family: hP2X$_{5R}$. A: Nucleotide sequence and ORF domain. Potential intracellular phosphorylation sites are indicated, as well as cysteine residues (bold) and *N*-glycosylation sites (italics) in the putative extracellular domain of the subunit. The spliced domain of 24 amino acids (Gly^{97}–Glu^{120}), absent in the short variant from brain, is boxed. The putative splicing site of missing TM2 sequence is indicated by the arrowhead. B: Alignment of human and rat P2X$_5$ amino acid sequences and positions of PCR primers based on hP2X$_{5R}$ primary structure.

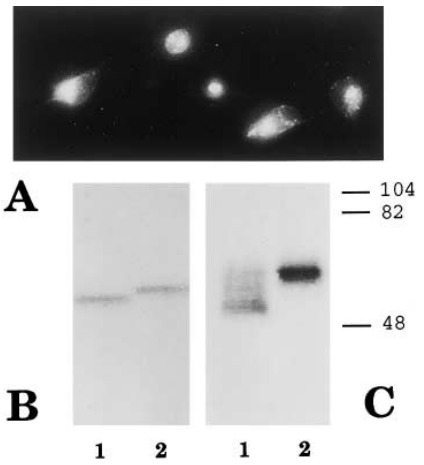

Fig. 2. Heterologous expression and immunodetection of $hP2X_{5R}$ subunits. A: In vitro translation of recombinant wild-type (1) and Flag-tagged $hP2X_{5R}$ (2). B: Detection in Western blot of epitope-tagged $hP2X_{5R}$ (1) and rat $P2X_4$ (2) from transfected HEK-293 cells.

reverse CTTGACGTCCTTCACATTGT (P2) to amplify the region corresponding to nucleotides 75–671 around the splicing event, as well as forward GAGGCCGAAGACTTCACCAT (P3) and reverse CCTCGTACTTCTTGTCACGG (P4) to detect the expression of a complete second transmembrane domain (TM2). Non-RT samples amplified in the same conditions provided the negative controls. Integrity and representativity of RT-cDNA were checked by amplification of human $P2X_7$ subunit message [18] and human $P2X_4$ message [17] with appropriate primers in lymphocytes and in cerebellum, respectively (data not shown).

2.5. Distribution of $hP2X_{5R}$ transcripts in human tissues

Known amounts of mRNA blots corresponding to equivalent transcription levels of housekeeping genes from various fetal and adult human tissues (Masterblot, Clontech) were probed with full-length random-primed [^{32}P]$hP2X_{5R}$ cDNA at high stringency (final elution at 65°C in 0.3×SSC buffer). Hybridization signals were quantitated using a Storm phosphorimager and the ImageQuant application (Molecular Dynamics) before subtraction of background and normalization of transcription levels.

3. Results and discussion

The longest reading frame of $hP2X_{5R}$ cDNA encodes a protein of 422 amino acids that displays 62% sequence identity with rat $P2X_5$ and several unique motifs conserved in all P2X receptor subunits (Fig. 1). In the intracellular N-terminal domain, $hP2X_{5R}$ displays an highly conserved threonine residue (Thr^{18}), potentially susceptible to phosphorylation by protein kinase C. In addition, a serine residue (Ser^{12}) is found in a consensus site for phosphorylation by casein kinase II. In the putative extracellular domain of $hP2X_{5R}$, all 10 cysteine, all proline, 91% of glycine and 75% of lysine residues are present at homologous positions in all P2X subunits, indicating their critical roles in the folding and function of this sensor region. We checked the real position of the predicted stop codon by in vitro translation as well as Western blot (Fig. 2). The recombinant subunit and its epitope-tagged mutant migrate at a M_r of 49 ± 2 kDa and 51 ± 2 kDa, respectively, in agreement with the calculated MW of 47 kDa for wild-type $hP2X_{5R}$ (Fig. 2B). At least one of the 3 *N*-linked glycosylation consensus sites present in the extracellular domain of $hP2X_{5R}$ is used, according to the difference of M_r (up to 11 kDa) between in vitro translated $hP2X_{5R}$ and $hP2X_{5R}$ expressed in transfected HEK-293 cells (Fig. 2B,C). The multiple bands observed in Western blot from transfected cells could be due to the co-expression of several glycosylated forms of $hP2X_{5R}$ (Fig. 2B).

The integrity of the protein was also checked by detection of heterologously expressed epitope-tagged $hP2X_{5R}$ mutants using in situ immunofluorescence (Fig. 2AFig. 3). Analysis of the subcellular localization of $hP2X_{5R}$-FL in HEK-293 cells using confocal microscopy indicated a correct targeting of the subunit to the plasma membrane (Fig. 3), quantitatively similar to the surface distribution of the functional P2X receptors rat $P2X_1$-FL and rat $P2X_4$-FL [14]. When expressed in *Xenopus* oocytes, homomeric $hP2X_5$ channels do not respond to extracellular ATP applied at concentrations up to 1 mM. However, the chimera h-r$P2X_5$, made of the N-terminal domain of $hP2X_5$ linked to the C-terminal domain of rat $P2X_5$, revealed the formation of cationic ATP-gated ion channels (Fig. 4). These channels run down rapidly and do not recover fully from desensitization after extensive washing of agonist lasting several minutes in constant perfusion (Fig. 4). There-

Fig. 3. Subcellular distribution of $hP2X_{5R}$ subunits expressed in HEK-293 cells using confocal microcope immunofluorescence. Arrows indicate the localization of $hP2X_{5R}$ subunits on the plasma membrane.

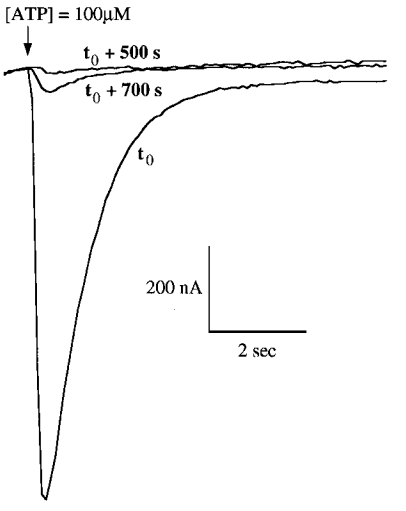

Fig. 4. Functional expression of the chimera h-r$P2X_5$ in *Xenopus* oocytes. Fast and desensitizing inward currents evoked by 100 μM extracellular ATP were recorded after 2–4 days of expression in two-electrode voltage-clamp configuration (holding potential $v_h = -100$ mV). Repeated applications of agonist at time to +500 s or to +700 s showed profound desensitization of the chimeric ATP-gated channels.

fore, despite the high sequence similarity with non-desensitizing wild-type rat $P2X_5$ (62%), this chimeric channel displays a unique functional property not found in any other known homomeric or heteromeric subtypes. These data suggest that the kinetics of desensitization of P2X channels is dependent on subtle rules of complementarity between the N-terminal and the C-terminal halves of the subunits, as has been shown for $P2X_1$ and $P2X_2$ [23].

We observed the expression of a splicing variant identified in fetal and adult brain by RT-PCR (Fig. 5), differing from the long form of $hP2X_{5R}$ by the absence of a cassette of 24 amino acids, corresponding to the domain Gly^{97}–Glu^{120} (Fig. 1). This cassette contains one of the 10 extracellular cysteines, so the expression of this short variant is due either to some inaccuracy in the intron/exon splicing mechanisms or to some functional regulation. Interestingly, the region including amino acids Ala^{328}–Ala^{349} in rat $P2X_5$, corresponding to the pre-TM2 region and the outer mouth of TM2 in P2X receptor subunits [22], is consistently absent in $hP2X_{5R}$ mRNA from brain and from lymphocytes, despite the high sensitivity of the RT-PCR method used and the high levels of expression in both tissues (Fig. 6). Furthermore, attempts to detect a variant of $hP2X_{5R}$ with a complete TM2 failed in tissues where homologous rat $P2X_5$ has been localized by in situ hybridization [5], like spinal cord and trigeminal sensory ganglia (data not shown).

To assess the anatomical distribution, we measured the relative levels of transcription of the $hP2X_{5R}$ gene in quantitative mRNA dot blot. Hybridization signals from a variety of fetal and adult tissues showed widespread but selective distribution of central and peripheral $hP2X_{5R}$ transcripts (Fig. 6). The two systems primarily involved in the processing of extracellular information, i.e. the central nervous system and the immune system, show the highest levels of $hP2X_{5R}$ expression. We measured a dramatic developmental down-regulation of $hP2X_{5R}$ mRNA in the thymus from the fetal to the adult stage (Fig. 6) that could be related to the massive apoptosis of immature thymocytes occurring during negative selection [24]. $P2X_1$ mRNA has been found to be upregulated during the apoptosis of immature thymocytes [25] so the distribution of $hP2X_5$ argues strongly for an important role of P2X receptors in clonal deletion of immune cells. With only one transmembrane region, the structure of $hP2X_{5R}$ is reminiscent of tyrosine kinase receptors [26]. We speculate that $hP2X_{5R}$ could be activated by the binding of an unidentified extracellular ligand or could be involved in the regulation of channel subunit interactions both in the brain and in the immune system.

The rat $P2X_5$ is the neuronal subunit with the most limited

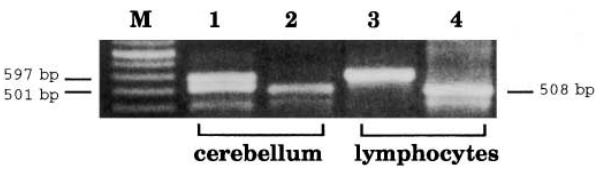

Fig. 5. The $hP2X_{5R}$ transcripts are expressed in two alternatively spliced forms in human brain as detected in RT-PCR amplification (1, primers P1+P2). The full-length form is predominantly found in lymphocytes (3, primers P1+P2). Both $hP2X_{5R}$ mRNA populations from brain (2, primers P3+P4) and lymphocytes (4, primers P3+P4) encode subunits with a single transmembrane domain.

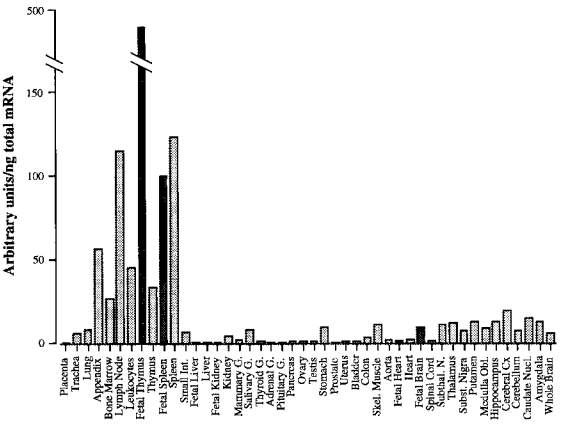

Fig. 6. Relative levels of expression of central and peripheral $hP2X_{5R}$ mRNA in human fetal (shaded bars) and adult tissues using hybridization of a full-length cDNA probe on poly(A)$^+$ RNA blot.

distribution, so one striking difference between rat and human P2X receptor genes is the high expression of $hP2X_{5R}$ in the brain and in peripheral tissues. The developmental regulation of levels of transcription and the tissue specificity observed in mRNA splicing do not seem compatible with the expression of a processed pseudogene [27]. However, it is possible that undetected splicing variants of $hP2X_{5R}$ with two transmembrane domains would form functional human $P2X_5$ channels in homomeric form or assemble in heteromeric complexes in specific cell types. In any case, as has been described in other classes of multimeric integral proteins, i.e. the neurotrophin receptors [28], the steroid receptors [29] and voltage-gated K channels [30], the regulatory effect of a dominant negative truncated $hP2X_{5R}$ subunit on human heteromeric ATP-gated channels remains to be investigated.

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