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Cloning of a Human Heptahelical Receptor Closely Related to the $P2Y_5$ Receptor[1]

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The 6H1 receptor cloned from activated chicken T cells was initially considered an orphan G-coupled receptor, but was later included in the P2Y family of receptors for purine and pyrimidine nucleotides on the basis of a significant amino acid identity and was renamed $P2Y_5$. Analysis of the expressed sequence tag database revealed the presence of a related sequence exhibiting 63% amino acid identity with this receptor. Starting from this partial sequence, we have isolated a complete clone and identified a 1113 base pair open reading frame encoding a new G-coupled receptor that we have called $P2Y_5$-like. This sequence exhibits 61% identity with the chicken $P2Y_5$ sequence and 30-33% with other P2Y subtypes. A construct encoding this $P2Y_5$-like receptor was transfected into COS-7, 1321N1, and CHO-K1 cells, and expression was documented by Northern blotting. None of the 40 nucleotides and nucleosides tested was able to elicit a response in any of four functional assays: inositol phosphate formation, stimulation or inhibition of cAMP formation, and extracellular acidification measured with a microphysiometer. These results suggest either that the natural ligand of the $P2Y_5$-like receptor is an uncommon nucleotide or alternatively that despite its structural similarity to the P2Y family it is not a nucleotide receptor.

G-protein coupled receptors mediate intracellular responses to a wide variety of extracellular ligands including peptides, lipids, nucleotides or ions. Among this superfamily, the nucleotidic receptors have been called P2Y. So far the cloning of 7 different subtypes has been reported: most of them are coupled to phospholipase C (PLC). They differ from each other by their sensitivity for various nucleotides and in particular by their relative affinity for adenine and uracil nucleotides (1). Whereas the $P2Y_1$ receptor (2,3) has a selectivity for adenine nucleotides, the $P2Y_3$ (4), $P2Y_4$ (5) and $P2Y_6$ (6,7) receptors have a preference for uracil over adenine nucleotides and the $P2Y_2$ receptor (8) is activated equipotently by ATP and UTP. Initially considered as an orphan receptor, the 6H1 receptor cloned from activated chicken T cells (9), was included in the P2Y family on the basis of sequence homology and radioligand binding studies and called $P2Y_5$ (10). The expression of that receptor was associated with the appearance of membrane binding sites for ATP and ADP, but its transduction mechanisms remain unknown. Its human ortholog was recently cloned (11). In this paper, we report the cloning and sequencing of a new human receptor closely related to the $P2Y_5$ receptor.

MATERIALS AND METHODS

Materials. Trypsin was from Flow laboratories, the culture media, reagents, foetal calf serum (FCS), restriction enzymes and Taq polymerase were purchased from Life Technologies, Inc. The radioactive products myo-D-2-[^{3}H]inositol (17.7 Ci/mmol) and [α-^{32}P]dATP (800 Ci/mmol) were supplied by Amersham. Dowex AG 1×8 (formate form) was from Bio-Rad Laboratories. ATP, dATP, ATPγS, BzATP, $\alpha\beta$meATP, ADP, ADPβS, $\alpha\beta$meADP, AMP, cyclic AMP, adenosine, adenine, UTP, dUTP, 5-BrUTP, UDP, UDP-glucose, UDP-galactose, UMP, uridine, TTP, dTTP, TMP, thymidine, ITP, IDP, inosine, CTP, CDP, CMP, cytosine, GTP, GDP, GMP, guanosine, AP_3A, AP_4A, AP_5A, AP_6A, NAD^+, PGE_1, carbachol and LiCl were obtained from Sigma. 2-MeSATP was from Research Biochemicals Inc. λDASHII library was from Stratagene. Rolipram was a gift from the Laboratoires Jacques Logeais (Trappes, France). Forskolin was purchased from Calbiochem. pBluescript SK^+ vector is from Stratagene. pcDNA3 is an expression vector developed by Invitrogen. Multiple human tissues Northern blots I and II were from Clontech. Rneasy kit from Qiagen was used for extraction of total RNA.

Cloning and sequencing. Specific primers were synthesized on the basis of EST sequence (accession number: H20663) and were

[1] Sequence data described in this article have been deposited with the GenBank Data Library under Accession No. AF005419.

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Abbreviations used: 2-MeSATP, 2-methylthio ATP; $\alpha\beta$meATP, α,β-methylene ATP; AP_XA, diadenosine polyphosphates; BzATP, 3′-O-(4-benzoyl)benzoyl ATP; NAD^+, nicotidamide adenine dinucleotide; PGE_1, protaglandine E_1; EST, expressed sequence tag.

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used in high stringency PCR using human genomic DNA as target, in order to amplify a receptor gene fragment. The conditions used were as follows: 93°C, 1 min.; 55°C, 2 min.; 72°C, 3 min.; 35 cycles. The obtained PCR product was subcloned in pBluescript SK^+ vector, sequenced by the Sanger dideoxy nucleotide chain termination method adapted for fluorescent primers and used as a probe to screen a human genomic library constructed in λDASHII. The hybridization was performed in 6× SSC (1× SSC: 0.15M NaCl, 0.015M sodium citrate) and 40% formamide at 42°C for 14 hours and the final washing conditions were 0.1× SSC, 0.1% sodium dodecyl sulphate (SDS) at 65°C. A λ phage DNA preparation was made from one of the purified clones. A restriction map and a Southern blotting analysis allowed us to subclone a 3.2-kb *SpeI* fragment into the pBluescript SK^+ vector. The complete receptor coding sequence was obtained on both strands after subcloning and sequencing of overlapping fragments in the same vector, using fluorescent primers and an automated DNA sequencer (Applied Biosystem 370A). Sequence analysis was carried out using DNASIS/PROSIS software (Hitachi) and the GCG software package (Gentic Computer Group, Madison, WI).

Cell culture and transfection. The open reading frame of the human $P2Y_5$-like receptor was inserted into the expression vector pcDNA3. COS-7 cells were transiently transfected with the recombinant pcDNA3-$P2Y_5$-like plasmid or the wild type pcDNA3 vector (as control) using the DMSO shock method (12). 1321N1 human astrocytoma and CHO-K1 cells were stably transfected with the same constructs using the phosphate calcium precipitation method (13). COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphothericin B, 1% sodium pyruvate. 1321N1 transfected cells were cultured in the same medium supplemented with 400 μg/ml G418. This cell line does not respond to extracellular nucleotides and was used by several investigators for the expression of P2Y receptors (5,7). CHO-K1 transfected cells were cultured in Ham's F12 medium with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphothericin B, 1% sodium pyruvate and 400 μg/ml G418. From the pool of transfected 1321N1 and CHO-K1 cells, individual clones were isolated by limiting dilution with the purpose of selecting clones with high expression of the receptor. The different clones were maintained in their respective medium containing 400 μg/ml G418.

Inositol phosphates measurements. Both transfected COS-7 and 1321N1 cells were used to investigate if the receptor is coupled to the PLC pathway. The cells were labelled for 24 h. with myo-D-2-[^{3}H]inositol (20 μCi for COS-7 cells in inositol-free complete DMEM and 5 μCi for 1321N1 cells in inositol-free DMEM containing 5% FCS, antibiotics, amphotericin, sodium pyruvate and G418). The cells were then washed twice with KRH buffer (124 mM NaCl, 5 mM KCl, 1.25 mM $MgSO_4$, 1.45 mM $CaCl_2$, 1.25 mM KH_2PO_4, 25 mM Hepes (pH 7.4) and 8 mM glucose) and incubated in the same medium for 30 min.; this was followed by a 20 min. incubation with KRH supplemented with LiCl 10 mM and the tested agonist. The incubation was stopped by the addition of an ice cold 3% perchloric acid solution. Dowex columns were used to isolate inositol phosphates (IP) as described (14). Total IP were measured in the case of COS-7 cells, while IP_3 was measured in the case of 1321N1 cells.

Cyclic AMP measurements. Stably transfected CHO-K1 cell lines were spread on Petri dishes (150.000 cells) and cultured in Ham's F12 medium containing 10% FCS, antibiotics, amphotericin, sodium pyruvate and G418. Cells are preincubated 30 min. in KRH buffer with rolipram (25 μM) and incubated 45 min. with the tested ligand with or without forskolin (1μM). The incubation was stopped by the addition of 1 ml HCl 0.1 M. After evaporation to dryness, the samples were resuspended into 400 μl of water and diluted as required. Cyclic AMP was quantified by radioimmunoassay after acetylation as previously described (15).

Microphysiometer measurements. The 1321N1 cell lines stably transfected by the recombinant $P2Y_5$-like-pcDNA3 construct or wild type vector were plated onto the membrane of Transwell cell capsules (Molecular Devices) at the density of 250,000 cells/capsule and cultured 2 days in complete DMEM medium. The capsules were placed into the sensor chamber of the microphysiometer (Cytosensor, Molecular Devices), and the cells were equilibrated for approximately 2 h. by perifusion of a fresh 1mM phosphate-buffered RPMI-1640 medium (pH 7.4) containing 0.2% BSA. Cells were then exposed to various nucleotides and nucleosides, added at 100 μM in the same medium, for 4 min. Acidification was measured at 1 min. intervals.

Northern blot analysis. Four different blots were hybridized with a *HindIII-BamHI* 515 bp $P2Y_5$-like probe: two commercial blots (MTN I & II, Clontech) of human organs (2μg $polyA^+$ RNA/lane of heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon and blood leukocytes), a blot with 20 μg of total RNA from 7 different 1321N1 clones (n° 2, 3, 4, 5, 6, 7 and 8), a mix of 1321N1 cells transfected by the recombinant vector and 1321N1 control cells and a blot containing 20 μg of total RNA from 6 different CHO-K1 clones (n° 1, 2, 3, 4, 5, 6), a mix of CHO-K1 cells transfected by the recombinant vector and CHO control cells. The blots were prehybridized at least 6h at 42°C and hybridized with the [α-^{32}P]ATP labelled restriction fragment of the $P2Y_5$-like coding sequence as probe. According to the recommended protocol for the MTN I & II blots, the hybridization solution used contained 50% formamide and 2% SDS. The Northern membranes were washed for 40 min. at 42°C in a solution containing 2× SSC and 0.05% SDS and 30 min. at 55°C in a solution containing 0.1× SSC and 0.1% SDS. They were exposed to a X-ray film in the presence of an intensifying screen at −80°C for 12 days. 1321N1 and CHO-K1 Northern blots were hybridized in the same conditions but with 0.3% SDS instead of 2% and 10% dextran sulphate. These blots were exposed to the PhosphorImager SI (Molecular Dynamics) for 3 days.

RESULTS

Cloning and sequencing. Databases analysis revealed the existence of two partial human sequences, EST R91585 and H20663, related to the chicken $P2Y_5$ receptor. The EST R91585 sequence shares 75% amino acid identity and corresponds to its human ortholog. Recently, a full sequence has been published but this receptor was not pharmacologically characterized (11). The EST H20663 sequence has 63% identity, suggesting that it belongs to a distinct but closely related subtype. In order to obtain the complete sequence of that new P2Y subtype, a specific couple of primers was synthetized and used in PCR reactions on human genomic DNA, that allowed to amplify a 407 bp fragment. After restriction analysis and sequence checking in order to verify the amplified product, that fragment was used as a probe to screen at high stringency a human genomic DNA library built in λDASHII. DNA of a clone hybridizing strongly with the probe was amplified and purified; restriction and Southern blotting analysis allowed us to subclone a 3.2kb *SpeI* fragment in pBluescript SK+ vector. Several restriction fragments of the 3.2kb insert were subcloned into the pBluescript SK+ vector and sequenced using fluorescent primers methods and an automated DNA sequencer. The full sequence of a 1.4 kb fragment was obtained (FIG.1.) and an intronless open reading frame of 371 codons was identified. This sequence contains the LFLTCIS motive

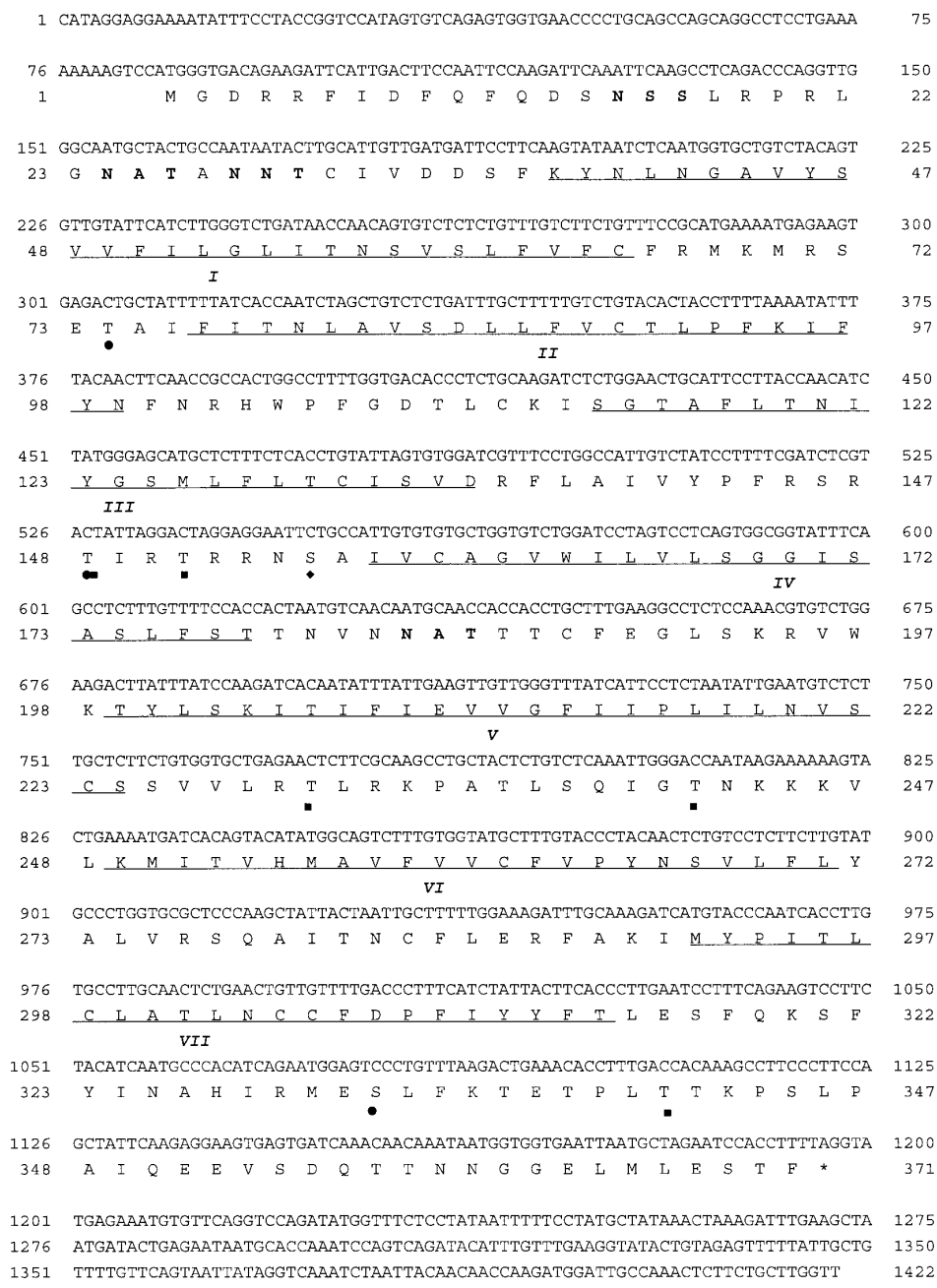

FIG. 1. Nucleotide and deduced amino acid sequence of the $P2Y_5$-like receptor. The putative membrane spanning domains are underlined and numbered **I** to **VII.** The four putative glycosylation sites are represented in boldface. The putative phosphorylation sites by protein kinase C, protein kinase A and calmodulin-dependent protein kinases are respectively indicated by black squares (■), black diamonds (♦) and black circles (●).

found in all P2Y subtypes cloned so far at the end of the third transmembrane segment (1). This complete sequence displays 61% identity with the chicken $P2Y_5$ receptor and about 30-33% with all the other receptors of the P2Y family, except the $P2Y_7$ receptor (25%) (16). The translated $P2Y_5$-like receptor sequence contains four potential N-linked glycosylation sites (residues N^{15}, N^{24}, N^{28} and N^{183}), five potential phosphorylation sites by proteine kinase C (T^{148}, T^{151}, T^{230}, T^{242} and T^{341}), three potential phosphorylation sites by calmodulin-dependent protein kinases (T^{74}, T^{148} and S^{332}) and a potential phosphorylation site by protein kinase A (S^{155}). None of the four positively charged amino acids (H^{262}, R^{265}, K^{289} and R^{292}) reported to play a crucial role in the $P2Y_2$ receptor activation by ATP and UTP (17) is conserved in the $P2Y_5$-like or in the $P2Y_5$ sequences.

Pharmacological characterization assays: IP measurements. The open reading frame was amplified by PCR reaction on human genomic DNA using a couple of specific primers. The PCR product was checked for the absence of mutation and inserted into the pcDNA3 expression vector. We transiently transfected COS-7 cells by the recombinant or wild-type pcDNA3 vector. The transfected cells were tested for their response to ATP, $\alpha\beta$meATP, 2-MeSATP, ADP, $\alpha\beta$meADP, AMP, adenosine, UTP, UDP, UMP, uridine, CTP, CDP, GTP, GDP, ITP, IDP, TTP, AP_3A, AP_4A, AP_5A and AP_6A, at a concentration of 100 μM. In control cells, transfected with pcDNA3 alone, we observed a weak increase over the resting level when ATP, ADP, UTP, UDP, AP_4A and ITP were added, which is due to the presence of endogenously expressed $P2Y_2$ receptors in the COS-7 cells (12). Identical responses were observed in cells transfected with the recombinant construct (data not shown). Different nucleotides at a concentration of 100 μM were added on a mix of 1321N1 human astrocytoma cells in order to stimulate the formation of IP_3: ATP, dATP, 2-MeSATP, $\alpha\beta$meATP, BzATP, ADP, AMP, UTP, 5-BrUTP, UDP, dUDP, UDP-glucose, UDP-galactose, AP_3A, AP_4A, AP_5A, AP_6A, TTP, ITP, GTP, CTP and carbachol (500 μM, as positive control) were tested. None of these nucleotides induced a detectable response; while carbachol produced a 6-fold increase. Five different 1321N1 clones (n° 2, 3, 4, 6 and 9) were also tested, following addition of ATP, dATP, $\alpha\beta$-meATP, ADP, UTP (100 μM) and carbachol (500 μM) as positive control. The IP_3 formation was not increased by the nucleotides in any of the five clones, including the clone 3 which has the highest level of receptor expression according to Northern blot analysis (see below) and which was extensively tested (FIG. 2A.).

Pharmacological characterization assays: Cyclic AMP measurements. CHO-K1 cells were also transfected by the recombinant ($P2Y_5$-like-pcDNA3) or the wild-type vector. Cyclic AMP was measured following addition of different nucleotides (ATP, ADP, UTP, UDP, ITP, TTP, GTP and CTP) at a concentration of 100 μM with or without forskolin (1 μM). None of these nucleotides did either stimulate or inhibit the formation of cyclic AMP (FIG. 2B.).

Pharmacological characterization assays: Acidification rate measurements with the microphysiometer. The lack of modulation of PLC and adenylate cyclase pathways in cells transfected by the $P2Y_5$-like receptor may be due to the fact that this receptor is coupled to another signalling cascade. Therefore, we used the microphysiometer: this device allows to detect changes in extracellular acidification rate reflecting cell activation in response to a receptor agonist, whatever transduction mechanism is coupled to that receptor (18). This device has been successfully used to characterize several orphan receptors such as the chemokine receptors (19). We perfused 1321N1 cells with ATP, ATPγS, dATP, $\alpha\beta$meATP, 2-MeSATP, BzATP, ADP, $\alpha\beta$-meADP, AMP, cyclic AMP, adenosine, adenine, UTP, dUTP, 5-BrUTP, UDP, dUDP, UDP-glucose, UDP-galactose, UMP, uridine, ITP, IDP, inosine, TTP, dTTP, TMP, thymidine, GTP, GDP, GMP, guanosine, CTP, CDP, CMP, cytosine, AP_3A, AP_4A, AP_5A, AP_6A, NAD^+, PGE_1 and carbachol. All the molecules were tested at a concentration of 100 μM, except carbachol (500μM). Only PGE_1 and carbachol, the two positive controls, increased strongly the acidification rate in both $P2Y_5$-like transfected and control cell lines. As another positive control, 1321N1 cells stably expressing the human $P2Y_6$ receptor (7) were perfused by a solution containing 100 μM of UDP (the most potent agonist of that receptor) and a strong increase of the extracellular medium acidification rate was also observed (FIG. 3.).

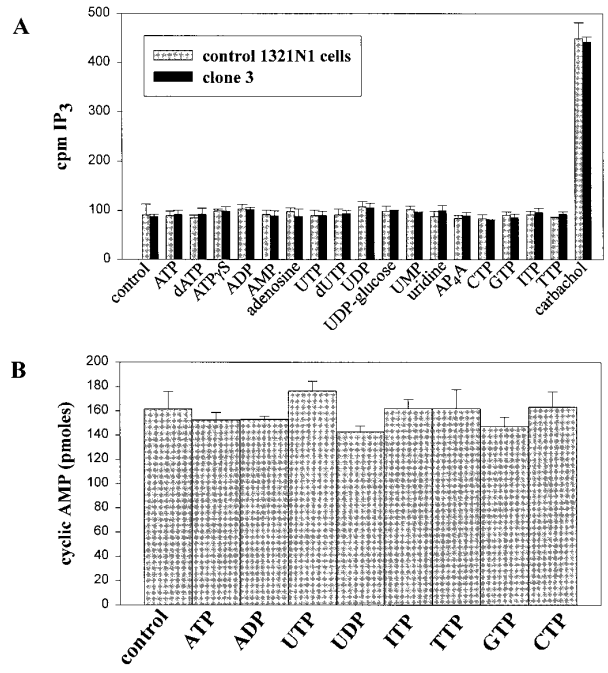

FIG. 2. (A) Measurement of IP_3 accumulation in 1321N1 cells expressing the $P2Y_5$-like receptor. Clone 3 from 1321N1 cells transfected with the $P2Y_5$-like coding sequence inserted in the pcDNA3 vector and control cells were incubated with different nucleotides, nucleosides (100 μM) or carbachol (500 μM) for 20 min. in presence of LiCl 10 mM. (B) Measurement of cAMP in CHO-K1 cells stably expressing the $P2Y_5$-like receptor. The cells were incubated 45 min. in the presence of forskolin (1 μM) with or without several nucleotides (100 μM). The data represent the means ±SD of triplicate experimental points and are representative of two independent experiments.

Northern blot analysis. Two blots containing RNA from 16 different human tissues (MTN I & II) were hybridized with a *HindIII-BamHI* restriction fragment of the $P2Y_5$-like receptor coding sequence used as a probe. No signal was observed (data not shown). Northern blots of 1321N1 and CHO-K1 cell lines transfected

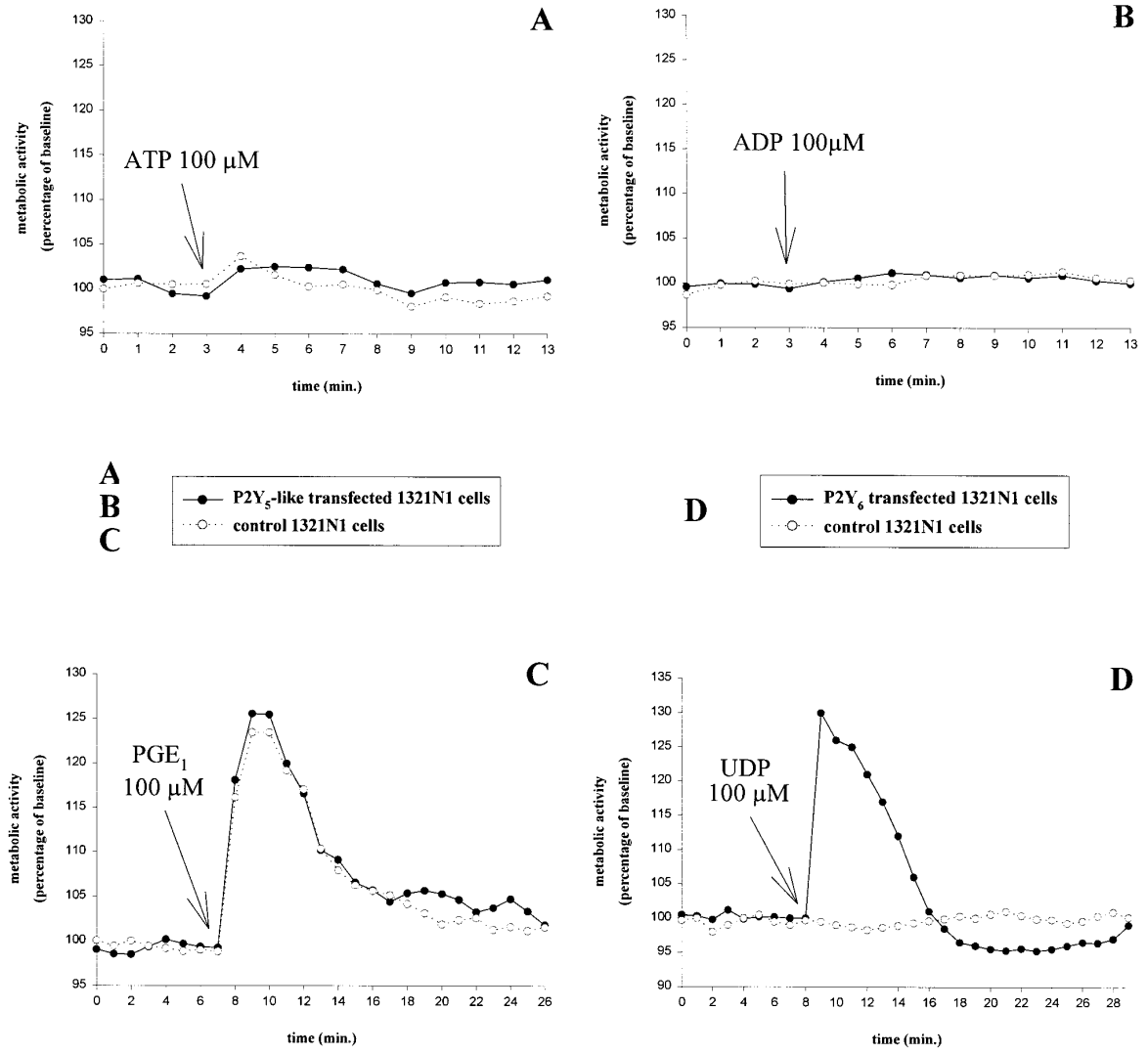

FIG. 3. Monitoring of the metabolic activity of control and $P2Y_5$-like or $P2Y_6$ receptor expressing 1321N1 cells with a microphysiometer. Results are expressed as a percentage of the baseline. Abscissa indicates time expressed in min. Following its addition (indicated with an arrow), the ligand is left for 4 min. in the presence of the cells before being washing out. A, B, C: metabolic activity of $P2Y_5$-like transfected and control 1321N1 cells following addition of ATP (A), ADP (B) and PGE_1 at 100μM. (D) Metabolic activity of $P2Y_6$ transfected and control 1321N1 cells following the addition of UDP (100 μM).

with the $P2Y_5$-like coding sequence were also hybridized with the $P2Y_5$-like probe. The 1321N1 blot revealed a 2 kb messenger for clones 2, 3, 6, 7 and for the cell mix; the clone 3 signal was the strongest (FIG. 4.). With the CHO-K1 blot, a signal was obtained for all the clones and was especially strong with the cell mix (FIG. 4.).

DISCUSSION

Initially considered as an orphan member of the G-coupled receptors superfamily, the 6H1 receptor cloned from activated chicken T lymphocytes (9) was later included in the P2Y family and called $P2Y_5$ on the basis of 2 arguments (10). On one hand the 6H1 sequence exhibits a limited but significant amino acid identity (around 30%) with the various P2Y receptors. On the other hand, the expression of this receptor in COS-7 cells induced the appearance of binding sites for ATP and ADP, which were detected using [^{35}S]dATPαS as radioligand. Databases analysis revealed the existence of 2 EST human sequences related to the $P2Y_5$ receptor. The R91585 sequence shares 75% identity with the chicken $P2Y_5$ receptor: a complete clone has now been isolated and its full coding sequence shares 70% amino acid identity with the chicken $P2Y_5$ sequence, indicating that it constitutes its human ortholog (11). The H20663 sequence displays 63% identity with the chicken $P2Y_5$ receptor, suggesting that it belongs to a distinct but closely related subtype. We have now iso-

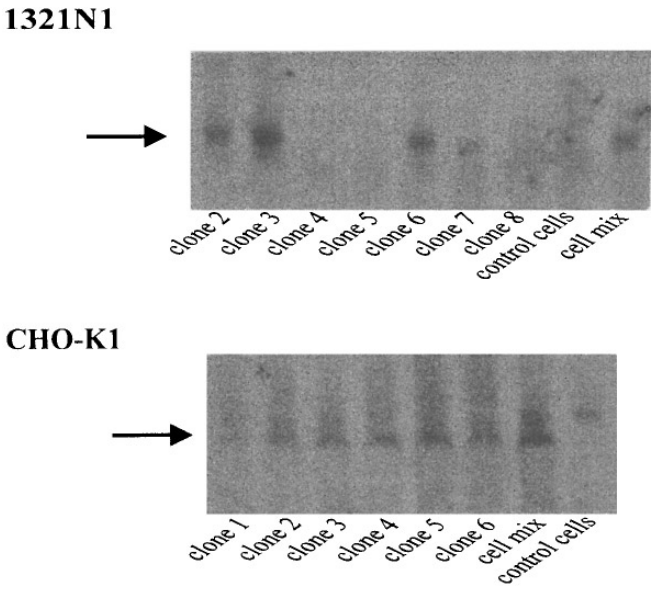

FIG. 4. Northern blot analysis of the $P2Y_5$-like receptor expression in 1321N1 and CHO-K1 cell lines. Each lane of the two blots contains 20 μg of total RNA. The first blot (1321N1) contains RNA of 7 different clones, control cells and $P2Y_5$-like transfected cell mix. The second blot (CHO-K1) contains RNA of 6 different clones, $P2Y_5$-like transfected cell mix and control cells. Hybridization with the $P2Y_5$-like probe was performed as described under Materials and Methods and followed by exposition to PhosphoImager for three days.

lated a complete clone encoding this subtype, that we have called $P2Y_5$-like, and determined its full coding sequence. The human $P2Y_5$-like receptor exhibits 61% identity with the chicken $P2Y_5$ receptor, and 30-33% with the other P2Y subtypes, except the $P2Y_7$ subtype with which the identity is only 25%. Another argument to include this new receptor in the P2Y family is the presence of a LFLTCIS motive which is found in all the P2Y receptors cloned so far (1). A plot of structural relatedness (FIG. 5.) indicates that the $P2Y_5$ and $P2Y_5$-like receptors constitute a distinct subgroup close to the P2Y family. Northern blot analysis failed to reveal $P2Y_5$-like messenger in the 16 human organs represented on MTN I & II blots, which have been used previously to determine the tissue expression of $P2Y_1$ (12) and $P2Y_6$ (7) receptors. Functional expression of the $P2Y_5$-like receptor was accomplished in 3 distinct systems: transient expression in COS-7 cells, stable expression in 1321N1 and CHO-K1 cells. Expression was documented by Northern blotting and was particularly striking in some clones. Four types of cellular responses were measured: stimulation of inositol phosphates formation, stimulation or inhibition of cAMP accumulation and extracellular acidification using the microphysiometer. This last method has the advantage to detect cell activation independently of a particular signalling cascade (18). None of the 40 nucleosides and nucleotides tested had an effect on any of these parameters. In particular, the cell clones having the highest level of expression according to Northern blotting were completely unresponsive. A functional response to nucleotides or nucleosides has not been described so far in cells expressing the recombinant $P2Y_5$ receptor. The only evidence that the $P2Y_5$ receptor is functionally a purinergic receptor derives from radioligand binding studies. However the pitfalls of binding studies of P_2 receptors relying on labelled nucleotides have been underscored in several studies (20, 21) and we have not performed similar studies following the expression of the $P2Y_5$-like subtype. One interpretation of these results might be that the $P2Y_5$-like receptor is not a receptor for nucleotides. This is consistent with the low, though significant, percentage of amino acid identity with other P2Y subtypes, which is not much larger than that of other non-nucleotide receptors, such as the thrombin receptor and the proteinase activated receptor 2 (PAR2). Also, mutagenesis experiments on the $P2Y_2$ receptor have demonstrated the crucial role of four positively charged amino acids in transmembrane segments **VI** and **VII** in the activation of this receptor by ATP and UTP. These residues are at least partially conserved in all P2Y subtypes cloned so far, while none is conserved either in the $P2Y_5$ or in the $P2Y_5$-like sequences. Alternatively the natural ligand of the $P2Y_5$-

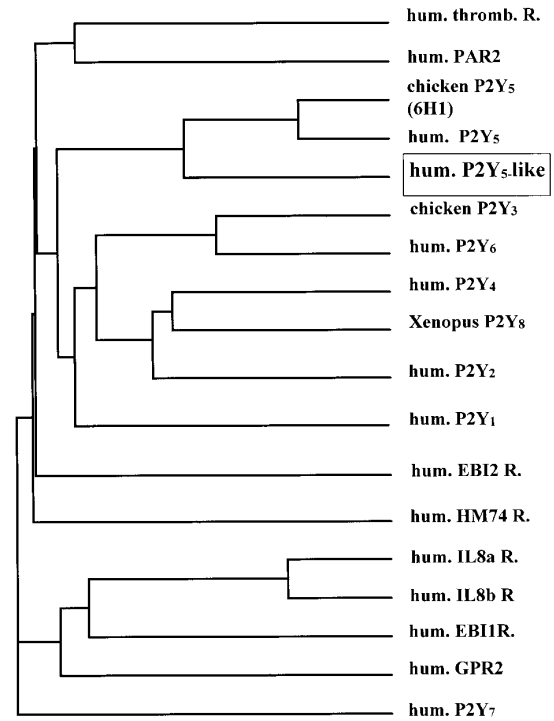

FIG. 5. Representation of structural similarities of the human $P2Y_5$-like receptor within the G-coupled receptors family. The plot was obtained using the multiple sequence alignment program Pileup of the GCG package. For each receptor, the program takes into account the total coding sequence.

like receptor might be an uncommon nucleotide that was not included in the repertoire of compounds that we have tested. In conclusion, we have cloned a new human G-coupled receptor, closely related to the $P2Y_5$ receptor. These two receptors constitute a distinct subgroup in the G-coupled receptors family and are structurally closer to the P2Y family than to any other known family. However, the true identity of their natural ligand(s) remains unknown.

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