Cotranscription and Intergenic Splicing of Human P2Y₁₁ and SSF1 Genes*

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The P2Y₁₁ receptor is an ATP receptor positively coupled to the cAMP and phosphoinositide pathways. Ssf1 is a Saccharomyces cerevisiae nuclear protein, which plays an important role in mating. The gene encoding the human orthologue of SSF1 is adjacent to the P2Y₁₁ gene on chromosome 19. During the screening of placenta cDNA libraries, we isolated a chimeric clone resulting from the intergenic splicing between the P2Y₁₁ and SSF1 genes. The fusion protein was stably expressed in CHO-K1 cells where it generated a cAMP response. According to both Western blotting and cAMP response, the expression of the fusion protein in the transfected cells was clearly lower than that of the P2Y₁₁ receptor. Both P2Y₁₁ and SSF1 probes detected a 5.6-kb messenger RNA with a similar pattern of intensity in each of 11 human tissues. The ubiquitous presence of chimeric transcripts and their up-regulation during granulocytic differentiation indicate that the transgenic splicing between the P2Y₁₁ and the SSF1 genes is a common and regulated phenomenon. There are very few examples of intergenic splicing in mammalian cells, and this is the first case involving a G-protein-coupled receptor.

P2Y₁₁ and SSF1 are adjacent genes located on chromosome 19 (1). The P2Y₁₁ receptor belongs to the P2Y family of G-protein-coupled nucleotide receptors (2); it is activated by ATP and positively coupled to the cAMP and the phosphoinositide pathways. It has been cloned from a human cDNA placenta library, but it is specifically expressed in the immune system (3). In particular, P2Y₁₁ messengers are present in HL-60 human promyelocytic leukemia cells and strongly up-regulated following exposure to various agents inducing their differentiation into neutrophil-like cells (4). Furthermore the induction of the granulocytic differentiation of HL-60 cells by ATP is mediated through the activation of P2Y₁₁ receptors (5, 6). On the other side, SSF1 is a Saccharomyces cerevisiae nuclear protein, which plays an important role in mating (7, 8, 9). SSF1 and its close homologue Ssf2 have been related to ppan, a gene involved in Drosophila larval growth (10). The cloning of the human orthologue of yeast Ssf1 was reported recently and the ubiquitous expression of human ssf1 mRNA is consistent with a general role in cell growth (11). Ssf1 (a suppressor of swi four) should not be confused with the homonymous Ssf1 (a second step splicing factor 1), an activity involved in the second step of pre-mRNA splicing in S. cerevisiae (11).

The presence of chimeric messengers resulting from intergenic splicing is not commonly observed in normal mammalian cells. It has been reported that cotranscription and intergenic splicing of human galactose-1-phosphate uridylyltransferase and interleukin-11 receptor α-chain genes generates a fusion transcript in normal cells (12). The intergenic splicing between the MDS1 and EVI1 genes has also been described (13). EVI1 is a protooncogene encoding a nuclear protein with several zinc finger domains, whereas MDS1 has been cloned as one of the partner genes of AML1 in the t(3;21)(q26;q22) translocation associated with myeloid leukemia. A third case reported in the literature is the intergenic splicing involving the murine Prnd and Prnp genes encoding the prion protein PrP and the PrP-like protein Doppel, respectively (14). It has been speculated that intergenic splicing would be a mechanism for generating new multidomain proteins and could therefore have major evolutionary implications.

During the screening of placenta cDNA libraries to isolate new P2Y receptors, we have isolated a cDNA clone encoding a SSF1-P2Y₁₁ chimeric transcript. We have then investigated the tissue expression of this fusion mRNA and the biological activity of the corresponding fusion protein.

EXPERIMENTAL PROCEDURES

Materials—Trypsin was from Flow Laboratories (Biotest, Switzerland). Culture media G418, fetal bovine serum, and restriction enzymes Taq and Platinum Pfx DNA polymerases were purchased from Life Technologies, Inc. [32P]ATP (800 Ci/mmol) was from Amersham Pharmacia Biotech. ATP, ATP-S (adenosine 5'-O-(3-thiotriphosphate)), benzoyl ATP (2'- and 3'-O-(4-benzoyl-benzoyl)adenosine 5'-triphosphate), ADP, UTP, UDP and all-trans-retinoic acid were obtained from Sigma. Ropilram was a gift from the Laboratoires Jacques Lopeix (Trappes, France). The human placenta cDNA library was kindly given by Prof. F. Chambon (Strasbourg, France). pEFI3 is an expression vector developed by Euroscreen (Brussels, Belgium). The human Multiple Tissue Northern (MTN) blots was from CLONTECH (Palo Alto, CA). HL-60

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cells were obtained from American Type Culture Collection (Manassas, VA). P2Y₁₁, C-terminal peptide (AAPKPFQGSRQELSGK) and bovine serum albumin-conjugated peptides (conjugation through an additional tyrosine) were from NeoSystems (Strasbourg, France).

Cloning and Sequencing—A human placenta cDNA library was screened with a cDNA fragment corresponding to the mature P2Y₁₁ receptor probe (GenBank Accession No. D14525) which had been inserted into the pBluescript SK±II vector. Hybridization conditions for screening were 6× SSC (1× SSC: 0.15 mM NaCl, 0.015 mM sodium citrate) and 40% formamide at 42 °C for 14 h, and the final washing conditions were 0.5× SSC, 0.1% SDS at 60 °C. One of the purified clones displayed an insert of 1.9 kb in length and was subsequently subcloned after acetylation as previously described (15).

Northern Blot Analysis—One blot containing 12 human mRNAs (MTN 12.1 μg of poly(A)⁺ RNA/lane; CLONTECH) was hybridized with specific probes corresponding to the P2Y₁₁ (1st exon) and SSF1 (exons 1–11) coding sequences. The blot was prehybridized 9 h at 42 °C in a 50% formamide, 2% SDS solution and hybridized for 18 h in the same solution supplemented with the α-²⁰P-labeled probe. The final washing conditions were 0.2× SSC and 0.1% SDS at 55 °C. The blot was exposed during 6 days and visualized as an autoradiograph or by using the PhosphorImager SI (Molecular Dynamics). A HL-60 blot hybridized previously with a P2Y₁₁ probe (4) was hybridized with a SSF1 probe. To realize this blot, total RNA was extracted using the Rneasy kit (Qiagen) from HL-60 cells unidentifiably or differentiated for various times with 1 μM retinoic acid.

Cell Culture and Transfection—We have amplified 14 specific PCR products encoding chimeric proteins starting at each of the ATG codons present in the SSF1 part of the chimeric cDNA using the Platinum² Pfx DNA polymerase (94 °C, 15 s; 66 °C, 30 s; 72 °C, 2 min for 30 cycles). The products corresponding to P2Y₁₁ or chimeric SSF1-P2Y₁₁ receptors were subcloned between the HinⅢ and dIII and sequenced using the BigDye Terminator cycle sequencing kit (Applied Biosystems, Warrington, Great Britain).

Reverse Transcription PCR Experiments—RNA was extracted from HL-60 cells with the Rneasy kit (Qiagen). The reverse-transcription was performed with 2 μg of total RNA using the Superscript kit (Life Technologies, Inc.). Specific primers located in the genomic sequence located upstream of the second exon of the P2Y₁₁ gene and a specific reverse primer located in the third transmembrane region of the P2Y₁₁ receptor were synthesized and used in reverse-transcription PCR experiments. The PCR amplification conditions with Taq DNA polymerase were 94 °C for 45 s, 50 °C for 30 s, and 72 °C for 1 min 30 s (35 cycles). The amplification products were subcloned in BlueScript SK± and sequenced using the BigDye Terminator cycle sequencing kit (Applied Biosystems, Warrington, Great Britain).

RESULTS
A human placenta cDNA library was screened at moderate stringency with a P2Y₁ probe (spanning transmembrane domains 3–7) to isolate novel P2Y receptors. Some clones encoded a novel P2Y receptor that has been characterized and named P2Y₁₁ receptor (3). One of the positive clones was clearly longer than the others (2500 base pairs (bp)). A 2385-bp open reading frame was identified in this clone (GenBank accession number: AJ300588). The first half of the corresponding protein displayed 40% amino acid identity with a S. cerevisiae protein involved in mating called Ssf1 (1), whereas the second half exactly matched the P2Y₁₁ sequence (3).

We have then obtained the complete cDNA and genomic sequences of human SSF1, which is split into 12 exons, and have shown that its mRNA is expressed in all human tissues tested (1). We have also shown that the P2Y₁₁ and SSF1 genes are contiguous on chromosome 19p31 (Fig. 1). The genomic organization of the SSF1 gene has been previously discussed (1). Existence of the fusion cDNA is due to a transgenic splicing removing the genomic sequence included between the first third of the last exon of the SSF1 gene and the second exon of the P2Y₁₁ gene (Fig. 1A). This splicing occurs in the absence of a consensus splicing donor site (residue 426 of the SSF1 protein) (Fig. 1B). The last 47 amino acids of the SSF1 protein (residues 427 to 473) are present in the fusion SSF1-P2Y₁₁ protein. From these observations, it was clear that in fact the first three amino acids, MDR, of the P2Y₁₁ sequence, which we have previously published (3), were coming from the SSF1 sequence. In the placenta cDNA library, we had first obtained a partial clone of the SSF1-P2Y₁₁ fusion protein in which these three amino acids appeared to be the beginning of the P2Y₁₁ cDNA sequence (3). After we obtained the complete SSF1-P2Y₁₁ fusion protein sequence and the genomic organization of the SSF1 gene, we performed PCR experiments to identify the true first exon of the P2Y₁₁ gene to clarify its genomic organization. We identified the first exon of the P2Y₁₁ receptor by reverse-transcription PCR experiments using primers located upstream of potential ATG starting sites in the genomic sequence included between the last exon of the SSF1 gene and the second exon of the P2Y₁₁ gene.

Antibody Production—An anti-P2Y₁₁ polyclonal antibody was generated in rabbits using a synthetic peptide located at the extremity of the C-terminal part of the human P2Y₁₁ receptor (AAPKPFQGSRQELSGK). Rabbits were injected subcutaneously with 2 μg of peptide emulsified in 10 mg/ml bovine serum albumin, concentrated, and dialyzed on a filter microsep 30-kDa membrane (Northborough, MA). Concentrated antibody was stored in 50% glycerol at −80 °C.

Western Blot Analysis—At confluency, CHO-K1-transfected cells were washed by PBS (pH, 7.3) (157 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·2H₂O, with 1.4 mM KH₂PO₄) and scrapped, and the pellets were solubilized in Laemmli buffer (10% w/v glycerol, 5% (w/v) mercaptoethanol, 2.3% (w/v) SDS, 62.5 mM Tris-HCl (pH 6.8). The protein concentration was determined using the method of Minamide and Bamburg (16). The same amount of proteins for each condition was electrophoresed in a 7.5% SDS-polyacrylamide gel electrophoresis. Proteins were then transferred overnight at 60 V and 4 °C onto a nitrocellulose membrane using 20 mM Tris, 154 mM glycine, 20% (v/v) methanol as a transfer buffer. Immunodetection was achieved using the enhanced chemiluminescence Western blotting detection system (ECL, Amersham Pharmacia Biotech) using a biotinylated-secondary rabbit antibody (1/50000). The anti-P2Y₁₁ polyclonal antibody was used at a 1/200-dilution.

1 The abbreviations used are: kb, kilobase(s); PCR, polymerase chain reaction; CHO, Chinese hamster ovary; kb, kilobase(s); bp, base pair(s).

2 The sequences corresponding to P2Y₁₁ or chimeric SSF1-P2Y₁₁ receptors were synthesized and used in reverse-transcription PCR experiments. The PCR amplification conditions with Taq DNA polymerase (94 °C, 15 s; 50 °C, 30 s; 72 °C, 2 min for 30 cycles). The amplification products were subcloned in BlueScript SK± and sequenced using the BigDye Terminator cycle sequencing kit (Applied Biosystems, Warrington, Great Britain).

3 The sequences corresponding to P2Y₁₁ or chimeric SSF1-P2Y₁₁ receptors were synthesized and used in reverse-transcription PCR experiments. The PCR amplification conditions with Taq DNA polymerase (94 °C, 15 s; 50 °C, 30 s; 72 °C, 2 min for 30 cycles). The amplification products were subcloned in BlueScript SK± and sequenced using the BigDye Terminator cycle sequencing kit (Applied Biosystems, Warrington, Great Britain).

4 The reverse primer corresponding to P2Y₁₁ and chimeric SSF1-P2Y₁₁ receptors was subcloned between the HinⅢ and dIII and sequenced using the BigDye Terminator cycle sequencing kit (Applied Biosystems, Warrington, Great Britain).

5 The reverse primer corresponding to P2Y₁₁ and chimeric SSF1-P2Y₁₁ receptors was subcloned between the HinⅢ and dIII and sequenced using the BigDye Terminator cycle sequencing kit (Applied Biosystems, Warrington, Great Britain).
P2Y11 receptor is expressed (4). One of the PCR products sequenced has allowed us to identify the first exon of the P2Y11 gene, which is located 1.9 kb upstream of the second one. This 445-bp product was obtained using the reverse primer and the following 5'–primer containing an EcoRI restriction site (in italic) 5'–TCCGGAATTCAGACACAGGCTGAGGA-3'. The exon encodes the six first amino acids of the P2Y11 receptor, MAANVS (Fig. 1B). An in-phase stop codon (in bold and underlined in the 5'–primer) is located 33 bp upstream of the starting codon, which is in a Kozak consensus. In conclusion, the sequence MAANVSK is the true beginning of the non-chimeric P2Y11 receptor, whereas MDRGAK represents the junction between the SSF1 and P2Y11 gene products (Fig. 1B). The correct non-chimeric P2Y11 sequence has been submitted to GenBankTM/EMBL (accession number: AJ298334).

We have then investigated whether the fusion transcript could be translated into a functionally active chimeric receptor. Because there are 14 potential starting codons in the first half of the clone corresponding to the SSF1 part and potential extracellular region of this chimeric receptor, we have amplified 14 specific PCR products encoding chimeric proteins starting at each of these ATG codons. The sequences of these products have been checked after insertion in the pEFIN3 expression vector and transfected into CHO-K1 cell lines.

In the transfected cells, the construction inducing the greatest functional response corresponded to the P2Y11 receptor alone, previously characterized following stable expression in CHO-K1 cells (3, 6). However each transfected chimeric construction led to a significant but much lower cAMP response to ATP (100 μM), even the construction corresponding to the full-length SSF1-P2Y11 fusion protein (Fig. 2). The basal level was considerably lower in the cell lines transfected with the chimeric transcript as compared with the P2Y11-transfected cells, suggesting a constitutive activity of the P2Y11 receptor. No ATP response was observed with CHO-K1 cells transfected with the pEFIN3 empty vector, whereas the forskolin-induced cAMP accumulation was comparable in all the transfected cell lines (Fig. 2). The cAMP data obtained for the intermediate constructions were similar to those obtained for the full chimeric receptor (data not shown). Northern blotting revealed comparable amounts of messengers for the different constructions in the various transfected CHO-K1 cell lines (data not shown).

Other adenine nucleotides, known to produce a strong activation of the P2Y11 receptor, were tested on cells transfected with the chimeric SSF1-P2Y11 construction. ATP,S and benzoyl ATP behaved as full agonists of the chimeric receptor and were apparently more potent than ATP (Fig. 2) as observed.
previously for the recombinant P2Y11 receptor (6). No effect of ADP, UTP, or UDP was observed (data not shown).

We have used a polyclonal antibody generated in the rabbit against a peptide located at the extremity of the C terminus of the P2Y11 receptor (AAPKPSEPQSRELSQ). This antibody was used on CHO-K1 cells transfected with P2Y11, full-length-SSF1-P2Y11, and with the empty vector (Fig. 3). In P2Y11-transfected cells, three strong bands were clearly detected around 45 kDa. A weak single band was detected in SSF1-P2Y11-transfected cells around 90 kDa (Fig. 3). These bands were not detected in the presence of 2 μg/ml of the corresponding peptide (data not shown). No band was detected in CHO-K1 cells transfected with pEFIN3 vector alone in the absence (Fig. 3) or the presence of the corresponding peptide (data not shown). No band was detected in CHO-K1 cells transfected with either SSF1-P2Y11 or P2Y11 or was loaded with the empty vector. Immunodetection was achieved using the enhanced chemiluminescence Western blotting detection system (ECL, Amersham). The anti-P2Y11 antibody was used at 1/200 dilution. The detected proteins are indicated by black arrows.

Northern blotting experiments were performed with specific probes of the SSF1 and P2Y11 genes (Fig. 4, A and B) on a blot containing mRNA from 11 human tissues and blood leukocytes. With an SSF1 probe, two prominent messengers (1.7 and 5.6 kb) were revealed in each tissue. Additional weaker bands were also detected (2.6 and 3.5 kb) (Fig. 4A). As shown previously, a P2Y11 probe hybridized to a 2-kb mRNA in human spleen (3) and liver (Fig. 4B). However, a second band was present in each tissue (Fig. 4B) and had a size indistinguishable from that revealed by the SSF1 probe at 5.6 kb. It seems that 1.7-, 2.6-, and 3.5-kb messengers were only detected with a SSF1 probe and correspond to SSF1 messengers (Fig. 4A), whereas 2-kb messengers shown in panel B correspond to P2Y11 messengers. The 5.6-kb band detected with the two probes corresponds apparently to a chimeric SSF1-P2Y11 messenger. This 5.6-kb unique band was also detected with a probe corresponding to the chimeric transcript and not with other unrelated probes (data not shown). A 5.6-kb messenger detected with a P2Y11 probe in the HL-60 cells (4) was also revealed with an SSF1 probe with a similar pattern (Fig. 4C) and corresponded apparently to the chimeric SSF1-P2Y11 messenger. This messenger was up-regulated during granulocytic differentiation of HL-60 cells by retinoic acid (Fig. 4C).

**DISCUSSION**

Intergenic splicing is extremely uncommon in mammalian cells with only three cases reported in the literature: MDS1 and EVII (13), galactose-1-phosphate uridytransferase and interleukin-11 receptor α-chain (12), and Prnd and Prnp (14). In this paper we have reported a new case of fusion mRNA resulting from in-frame intergenic splicing between the human SSF1 and P2Y11 genes; this is the first case involving a G-protein-coupled receptor. It is interesting to note that the transgenic splicing between these two unrelated genes leads to the addition of a potential ATP binding site present in SSF1 (GVGEGK, residues 289 to 294) to the sequence of a purinergic receptor. However, apparently this has no major effect on the responsiveness to nucleotides.

We have clarified the genomic organization of the P2Y11 gene. An exon encoding the first six residues of the non-chimeric P2Y11 receptor has been identified 1.9 kb upstream of the second exon which encodes the seven transmembrane regions of the receptor. This first exon contains the starting codon and encodes a potential N-glycosylation site (MAANVS). This first exon is not present in the chimeric messenger. The first three residues of the previously published P2Y11 sequence, MDR, (3) were thus a consequence of the intergenic splicing and belong in fact to the SSF1 sequence.

Theoretically the fusion transcript encodes a receptor with a very large extracellular domain displaying no peptide signal sequence. Because binding assays using radiolabeled nucleotides are not a valid method to quantitate P2Y receptors (17–19), we have performed functional assays to determine whether the transfection of the chimeric cDNA could lead to a biochemical response to ATP in the transfected cells. Indeed cAMP assays showed that CHO-K1 cells transfected with a SSF1-P2Y11 construction exhibited a cAMP response to nucleotides qualitatively similar to that observed in cells expressing the P2Y11 receptor alone (6). However, both the basal cAMP level (reflecting possible constitutive activity) and the maximum accumulation of cAMP in response to ATP were much lower in cells expressing the fusion protein than in P2Y11-expressing cells. The pharmacological data could be correlated with the level of expression of the fusion protein, which seems clearly lower than that of the P2Y11 receptor. Whereas three strong bands, probably corresponding to different degrees of glycosy-
lation, were observed in cells expressing the P2Y11 receptor, a weak 90-kDa band corresponding to the expected molecular mass of the fusion protein was detected in cells transfected with the SSF1-P2Y11 construction. Although the level of mRNA detected was comparable between cells transfected with the chimeric receptor and the P2Y11 receptor, we can speculate that the fusion protein is less translated or less stable than the P2Y11 receptor. It appears that the ATP response observed in cells expressing the fusion protein is not due to its cleavage at the fusion site because no lower band corresponding to the P2Y11 receptor was detected.

Both P2Y11 and SSF1 probes detected the same 5.6-kb messenger with a similar pattern of intensity in each tissue. The detection of the chimeric transcript in all the tested tissues was surprising as was its up-regulation in HL-60 cells in response to an agent inducing granulocytic differentiation. It indicates that the cotranscription and transgenic splicing between the P2Y11 and the SSF1 genes is a frequent, ubiquitous, and regulated phenomenon. However its functional significance remains unclear. It is important to emphasize that we have obtained cAMP data for the chimeric receptor in a system of overexpression of the recombinant fusion protein, but it is unclear whether the chimeric transcript observed in all the tested tissues is translated into fusion protein in vivo. Indeed in most cells ATP is unable to increase cAMP. Alternatively, the production of SSF1-P2Y11 fusion mRNAs could be a way to down-regulate the expression of active P2Y11 receptors by misleading transcription, or the fusion protein might have another function which remains to be determined.

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FIG. 4. Northern blot analysis of SSF1 and P2Y11 messenger expression. Each lane of the MTN blot (A and B) and the HL-60 blot (C) contains, respectively, 2 μg of poly(A)+ RNA and 15 μg of total RNA. Hybridization with the SSF1 (A and C) or P2Y11 (B) probes was performed as described under “Experimental Procedures.” The HL-60 cells were differentiated during various times (h, hour(s); d, day(s)) with 1 μM retinoic acid (RA). CONT, control (non-differentiated HL-60 cells). The pictures were obtained from an autoradiography (A) and from a PhosphorImager SI (B and C). The transcripts are indicated by black arrows.
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