SHORT COMMUNICATION

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Cloning and characterization of the murine *P2XM* receptor gene

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Abstract We have isolated the murine counterpart of the human *P2XM* gene (*mP2XM*), a P2X purinoceptor that is expressed predominantly in skeletal muscle. The *mP2XM* gene, consisting of 12 exons that span 10kb of genomic DNA, encodes a 379-amino-acid product with 83% identity to the human homologue. Two potential transmembrane domains (M1 and M2) are present in the predicted product, and a segment resembling the H5 region of voltage-gated ion channels is completely conserved between human and mouse *P2XM* proteins. Predominant expression of *mP2XM* in murine skeletal muscle was confirmed by Northern-blot analysis, and a low level of expression was detectable in lung tissue, although human lung does not express *P2XM*.

Key words *P2XM* · Murine · Skeletal muscle · *p53* · *RP-2*

Introduction

As the *p53* gene is so frequently mutated in human tumors of various types, its tumor-suppressive function *p53*

continues to be the focus of many intensive studies (Hollstein et al. 1991). A variety of biological actions are attributed to *p53*, presumably as the result of transcriptional activation of downstream genes when *p53* binds to specific DNA motifs (Vogelstein and Kinzler 1992; Kastan et al. 1992; El-Deiry et al. 1993; Wu et al. 1993; Okamoto and Beach 1994; Buckbinder et al. 1995; Miyashita and Reed 1995; Furuhashi et al. 1996; Morris et al. 1996). For example, *p21/WAF1*, which is induced when DNA is damaged, inhibits cyclin-dependent kinases and thereby arrests the cell cycle (El-Deiry et al. 1993) and *GADD45*, which is associated with nucleotide excision repair (Kastan et al. 1992), and *BAX*, which promotes programmed cell death (apoptosis), are also transcriptionally activated by *p53* (Miyashita and Reed 1995). However, only a few such *p53*-inducible genes have been identified thus far, and the mechanisms by which *p53* maintains cellular control in normal tissues and/or suppresses tumorigenesis through downstream elements are still unclear.

Recently we isolated a novel human *p53*-inducible gene, *P2XM*, through an approach designed to facilitate cloning of potential *p53* targets by probing genomic DNA with *p53*-binding sequences (*p53*-tagged sites) (Urano et al. 1997). *P2XM* represents a new member of the P2X-receptor family of ATP-gated ion channels, and is expressed predominantly in skeletal muscle. In our report of that work we described the complete loss of expression, or alternative splices that removed the transmembrane domain of *P2XM*, in several cell lines derived from rhabdomyosarcomas or osteosarcomas (Urano et al. 1997). Those findings suggested that loss of normal function of *P2XM* may be associated with bone or soft tissue tumorigenesis when its ability to transduce signals is disrupted. However, no functional analysis of this gene or investigations into its association with tumor progression have yet been performed.

In this report we describe cloning of the murine counterpart of human *P2XM* and identification of its genomic structure, with the aim of facilitating the functional analysis of *P2XM* in a model species.

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Materials and methods

Screening of a murine cosmid library and sequencing analyses

We screened a cosmid library prepared from mouse genomic DNA using cDNA corresponding to human *P2XM* as a probe. Cosmid clones were isolated by colony hybridizations performed according to the method described previously (Urano et al. 1997), except that hybridization and washing were carried out at 50°C. One of the cosmids was digested with *Hind*III and *Bam*HI, and the restriction fragments were subcloned into pBluescript II SK(−) vector (Stratagene, La Jolla, CA, USA). Sequencing was performed with an Applied Biosystems model 373 DNA sequencer (Perkin Elmer Cetus, Norwalk, CT, USA) with a Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems, Forester, CA, USA). Exon-intron junctions were defined by comparing genomic DNA sequences to cDNA sequences.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA extracted from mouse skeletal muscle was reverse transcribed for single-strand cDNA using oligo(dT)₁₅ primer and Superscript II (BRL, Life Technologies, Rockville, MD, USA). PCR amplification was performed with Pfu polymerase (Clontech, Palo Alto, CA, USA), with an initial step of 94°C for 3 min followed by 35 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 2 min 30 s, on the GeneAmp PCR system 9600 (Perkin Elmer). Primer sequences used for amplifying the entire coding region were E1F2 (5'-CTGCGGCTAAATTTGCCTGCT-3') and E12R2 (5'-CCTTTGAATGAACCCAG GAGG-3').

Northern-blot analysis

A Northern blot containing poly(A) RNAs derived from normal mouse tissues (Clontech) was hybridized overnight at 42°C with DNA corresponding to the entire coding region of murine *P2XM* (*mP2XM*) that had been labeled by the random-primer method. The blot was washed twice with 0.1% standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) at 50°C for 10 min. and autoradiographed by exposure to X-ray film for 6 days at −80°C.

Results

Cloning of *mP2XM* coding region

In the course of screening a cosmid library constructed from murine genomic DNA, using human *P2XM* cDNA as the probe, we obtained 11 positive clones. We compared the partial sequences of these clones and identified three as

most closely matched with human *P2XM*. One of them, clone 1A, was confirmed to contain DNAs homologous to both ends of human *P2XM* (corresponding to exons 1 and 12) by Southern hybridization, and we analyzed it further by performing Southern hybridizations of restriction digests to identify fragments containing regions homologous to exon 1 or exon 12 of the human gene. A 5.8-kb *Hind*III fragment and a 3.0-kb *Bam*HI fragment, found to include exons 1 and 12, respectively, were subcloned into pBluescript II vector. Sequencing of these fragments confirmed that the 5.8-kb *Hind*III fragment contained DNA sequences homologous to exon 1 of human *P2XM*. Similarly, the 3.0-kb *Bam*HI fragment was confirmed to contain the 5' half of exon 12. We determined the DNA sequence of the 3' half of exon 12 using cosmid DNA as a template. From those sequence data we designed RT-PCR primers corresponding to exons 1 and 12; the 1.3-kb product, representing the entire coding region, was subcloned into pBluescript II and sequenced. The nucleotide sequence data reported in this paper have been submitted to the DNA Data Bank of Japan (DDBJ) and have been assigned the accession number AB010883.

Homology with human *P2XM*

The *mP2XM* cDNA and its predicted amino-acid sequences are shown in Fig. 1. This cDNA contains a 1137-bp open reading frame encoding 379 amino acids with a high degree of sequence homology to human *P2XM*. The murine product contains two potential transmembrane domains (M1 and M2) and also a segment that resembles the H5 region of voltage-gated K⁺ ion channels that is present in other members of the P2X family (Valera et al. 1994). A lesser degree of similarity exists between the human and mouse cDNAs in regions corresponding to exons 1 (75.0%) and 12 (61.5%), but greater than 80% identity at the nucleotide level can be seen in other exons. Overall identity between the predicted murine and human gene products was high, at 83%; in particular, the amino acid sequences in the H5 region were completely conserved (Fig. 2). However, the murine counterpart lacks 54 amino acids in the C-terminal cytoplasmic domain and contains two additional amino acids in the N-terminal region. Exon-intron junctions of *mP2XM* were determined by sequencing cosmid clone 1A (Table 1 and Fig. 3). The positions of junctional borders of each exon were exactly same as those in human *P2XM*, and the sizes of corresponding introns were also similar (Urano et al. 1997). The mouse gene consists of 12 exons spanning an approximately 10-kb genomic region (Fig. 3).

Expression profile of *mP2XM*

Northern-blot hybridization showed that a 2.4-kb transcript of *mP2XM* was expressed predominantly in murine skeletal muscle. However, although no transcript of *P2XM* has been detected in human lung, in the mouse gene a very low level of expression of its homolog does occur in lung tissue (Fig. 4).

1	TT GCC TGC TGA CTC ATG CAG TTG CAG CCA GCA GGA ACT GGG AAC ATG GCT TCT GCA GCC GCA GCA GCA CTC GTG AGC TGG GGG TTT CTG	89
	M A S A A A A A L V S W G F L	15
90	GAT TAC AAG ACG GAG AAA TAT GTG CTA ACC AGG AAC TGT CGG GTG GGC GTC TCC CAG AGG CTG CTG CAG CTG GCA GTG GTA GTC TAC GTG	179
16	D Y K T E K Y V L T R N C R V G V S Q R L L Q L A V V V Y V	45
	M1	
180	ATA GGG TGG GCC CTC TTG GCC AAA AAA GGC TAC CAG GAA CGG GAC TTG GCC CCT CAG ACT TCT GTC ATC ACC AAA CTC AAG GGG GTT TCT	269
46	I G W A L L A K K G Y Q E R D L A P Q T S V I T K L K G V S	75
270	GTA ACC CAG GTT AAG GAG CTG GAG AAC CGG CTG TGG GAC GTG GCT GAC TTT GTG AAG CCA TCA CAG GGA GAG AAC GTG TTC TTC CTG GTA	359
76	V T Q V K E L E N R L W D V A D F V K P S Q G E N V F F L V	105
360	ACC AAC TTC CTC GTG ACA CCA GCT CAA GTC CAG GGC AGA TGT CCA GAG CAT CCT TCC GTT CCT CTG GCT AAC TGC TGG GCT GAT GAA GAC	449
106	T N F L V T P A Q V Q G R C P E H P S V P L A N C W A D E D	135
450	TGC CCC GAA GGG GAG ACG GGG ACA TAC AGC CAT GGC ATA AAA ACT GGT CAG TGT GTG GTG TTC AAC GGA ACC CAC AGG ACC TGT GAG ATC	539
136	C P E G E T G T Y S H G I K T G Q C V V F N G T H R T C E I	165
540	TGG AGC TGG TGC CCA GTG GAG AGT GGT GCT GTG CCC AGG AAG CCC CTG CTA GCT CAG GCC AAG AAC TTC ACA CTC TTC ATC AAA AAT ACT	629
166	W S W C P V E S G A V P R K P L L A Q A K N F T L F I K N T	195
630	GTA ACC TTC AGC AAG TTC AAC TTC TCC AGA TCC AAT GCC TTA TTA ACC TGG GAC AAC ACA TAT TTC AAG CAT TGT CTC TAT GAT CCA CTC	719
196	V T F S K F N F S R S N A L L T W D N T Y F K H C L Y D P L	225
720	TCC AGT CCG TAC TGC CCA GTG TTC CGC ATC GGG GAC CTT GTG GCC ATG GCT GGA GGG GAC TTT GAG GAC CTG GCC TTG CTG GGT GGT GCT	809
226	S S P Y C P V F R I G D L V A M A G G D F E D L A L L G G A	255
810	GTG GGC ATC ACC ATT CAC TGG GAT TGC AAC CTG GAC ACG AAA GGC TCT GAC TGC TGT CCC CAG TAC TCC TTC CAG CTG CAG CAG AAA GGG	899
256	V G I T I H W D C N L D T K G S D C C P Q Y S F Q L Q Q K G	285
900	TAC AAC TTC AGG ACA GCC AAT CAC TGG TGG GCA GCC TCA GGC GTG GAG ACC CGG AGC CTG CTC AAG CTC TAT GGA ATC CGT TTT GAC ATT	989
286	Y N F R T A N H W W A A S G V E T R S L L K L Y G I R F D I	315
	H5	
990	CTT GTC ACT GGG CAG GCA GGG AAG TTC GCG CTC ATC CCT ACG GCC ATC ACA GTG GGC ACA GGA GCG GCT TGG CTG GGC ATG GTC ACC TTC	1079
316	L V T G Q A G K F A L I P T A I T V G T G A A W L G M V T F	345
	M2	
1080	CTC TGT GAC CTG CTG CTA CTG TAT GTG GAT AGA GAG GCC GGT TTC TAC TGG AGA ACC AAA TAT GAA GAG GCA AGA GCC CCA AAG ACG ACT	1169
346	L C D L L L L Y V D R E A G F Y W R T K Y E E A R A P K T T	375
1170	ACC AAC AGT TCA TAG ACC GAG CCA GCC TTC TCA CCC GCT AAC CCT GTT ACC CAA GTG TCC TAG GAG TGG TTT GCA CCT GCC CCA TGT CTG	1259
376	T N S S *	379
1260	CTG CTG CTG GGA TCC AGG CAT CCA CGC ACC GCA CCG CAC CGA TGG CCT GTC TGG ATG GCT GCC ACC CAC TGG CCT ATT CAT TCC TGA GGC	1349
1350	TTA CAC CCT CTC CCT GCT GGT TAA AGT TTA GGG CTT GGG AAG GAA GAG GGT TCT GGC TTG GGA AAA TGG AGG ACA AAG CTT CAG CGG GAC	1439
1440	TAG AGG GGA GTT GGG ATA ATT CCG ACA GAC TCC GAA TGC AGA TTC	1484

Fig. 1 Nucleotide sequence of the entire coding region of the murine *P2XM* (*mP2XM*), receptor gene and predicted amino acid sequence. The 379 amino acids encoded by this gene include two potential trans-

membrane domains (M1 and M2) and a segment resembling the H5 region of voltage-gated ion channels (shown by underlines)

Discussion

Human *P2XM*, a novel member of the P2X family of receptors that open cationic channels by binding ATP, was cloned through an approach designed to isolate *p53*-inducible genes (Urano et al. 1997). This gene, which encoded a 431-amino-acid protein in 12 exons, was mapped to human chromosome 22q11. In the present study, we isolated the murine homolog of *P2XM* and determined its complete genomic structure, including exon-intron boundaries. The products of the human and mouse genes both contain two transmembrane domains and an H5 region characteristic of voltage-gated K⁺ channels. Also conserved among these and all P2X receptors reported so far are N-glycosylation consensus

sites and ten cysteine residues, which may be components of disulfide bridges in the extracellular domain (Soto et al. 1996a; Urano et al. 1997). Homology searches in the public database using the FASTA program (DNA Information and Stock Center, National Inst. of Agrobiological Resources, Tsukuba, Japan) revealed that *mP2XM* was 94% identical in nucleotide sequence and 93% identical in amino acid sequence with rat *P2X6*, another member of the P2X family (Collo et al. 1996; Soto et al. 1996a). Despite this high similarity, however, the expression pattern is different; *P2X6* is expressed widely in the central nervous system and peripheral tissues of the rat, but *mP2XM* is expressed predominantly in skeletal muscle.

Other P2X receptors are expressed in brain or peripheral nerve; *P2X4*, like *P2X6*, is widely expressed in the central

Fig. 3 Genomic structure of the *mP2XM* gene. Exons are numbered and shown as boxes according to their relative sizes

P2X6, for example, is slowly desensitized to ATP and is insensitive to suramin and PPADS (Collo et al. 1996). The differently expressed P2X subgroups are thought to conform to either homomultimeric or heteromultimeric ion channels that mediate such functions as cellular proliferation or differentiation; control of these processes is thought to be strictly regulated at the transcriptional level. Although the high degree of amino acid conservation between *P2XM* and *P2X6* suggests that, phenotypically, their functions are similar, their transcriptional regulation is clearly different. It is likely, therefore, that *mP2XM* is the murine counterpart of human *P2XM* rather than of rat *P2X6*, on the basis of its expression pattern. The similarities in genomic structure and expression profile of the *P2XM* genes in human and mouse suggest that their promoter or enhancer regions are also conserved and under the same transcriptional regulation.

As human *P2XM* contains a domain typical of ATP-gated ion channels (Urano et al. 1997), its similarity to *RP-2*, a gene that is induced when thymocytes are undergoing apoptosis (Owens et al. 1991; Valera et al. 1994; Urano et al. 1997), suggests that it may be associated with programmed cell death mediated by ATP or synaptic transmission. Therefore, since its expression is regulated by *p53*, *P2XM* may play a role in the apoptosis of skeletal muscle; specifically, in the maintenance of a normal apoptotic pathway. Loss of *P2XM* function could, presumably, lead to the formation or progression of tumors in bone and soft tissue (Urano et al. 1997).

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Fig. 4 Northern-blot analysis of *mP2XM* expression. Lanes contain poly(A) RNA derived from the murine tissue indicated

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