

Research article

Molecular cloning of the rat proteinase-activated receptor 4 (PAR4)

Willemijntje A Hoogerwerf*, Helen Lee Hellmich, Maria Adelaide Micci, John H Winston, Lei Zou and Pankaj J Pasricha

Address: Enteric Neuromuscular Disorders and Pain Laboratory, Division of Gastroenterology and Hepatology, University of Texas Medical Branch, Galveston, TX, USA

E-mail: Willemijntje A Hoogerwerf* - wahooger@utmb.edu; Helen Lee Hellmich - hhellmic@utmb.edu; Maria Micci - mmicci@utmb.edu; John H Winston - lzou@utmb.edu; Zou Lei - jhwinsto@utmb.edu; Pankaj J Pasricha - jpasrich@utmb.edu

*Corresponding author

Published: 18 February 2002

Received: 26 November 2001

BMC Molecular Biology 2002, **3**:2

Accepted: 18 February 2002

This article is available from: <http://www.biomedcentral.com/1471-2199/3/2>

© 2002 Hoogerwerf et al; licensee BioMed Central Ltd. Verbatim copying and redistribution of this article are permitted in any medium for any purpose, provided this notice is preserved along with the article's original URL.

Abstract

Background: The proteinase-activated receptor 4 (PAR4) is a G-protein-coupled receptor activated by proteases such as thrombin and trypsin. Although activation of PAR4 has been shown to modulate rat gastrointestinal motility, the rat PAR4 sequence was unknown until now. This study aimed to identify the rat PAR4 cDNA.

Results: The cDNA coding for the rat PAR4 homologue was cloned from the duodenum. Northern blots demonstrated a 3.0 kb transcript in the duodenum. Protein homology with mouse and human counterparts was 90% and 75% respectively. PAR4 is expressed predominantly in the esophagus, stomach, duodenum and the spleen. When expressed in COS cells, PAR4 is activated by trypsin (1 nM), thrombin (50 nM), mouse PAR4 specific peptide (500 μ M) and a putative rat PAR4 specific activating peptide (100 μ M), as measured by intracellular Ca^{2+} -changes.

Conclusions: We have identified and characterized cDNA encoding the rat PAR4 homologue. PAR4 is expressed predominantly in the upper gastrointestinal tract. It is activated by trypsin, thrombin and its newly identified rat PAR4 specific activating peptide.

Background

Proteinase-activated receptors (PARs) comprise a sub-family of G-protein coupled receptors, which are activated by serine proteases such as trypsin and thrombin. Cleavage of PARs unmasks an extracellular N-terminal domain that subsequently binds and activates the receptor, thus acting as a tethered ligand. Four related receptors have been identified to date [1–7]. Most recently, both human and mouse proteinase-activated receptor 4 (AR4) have been cloned [5,7]. Although best known for their role in the vascular and clotting systems, PARs have also been implicated as potential modulators of gastrointestinal motility [8–11] and recent studies have suggested a possible

similar role for PAR4 [10]. However, to date, the rat PAR4 homologue has not been cloned and the tissue distribution of rat PAR4 in the gastrointestinal tract is unknown. In this report, we describe the cloning of a functional rat PAR4 cDNA. When expressed transiently in COS-1 cells, the rat PAR4 was activated by thrombin, trypsin, mouse PAR4 activating peptide and its own newly identified rat PAR4 tethered ligand. The rat PAR4 is mainly expressed in the rat esophagus, stomach, duodenum and the spleen.

```

1      ATGTGCTGGC  CGCTGCTGTA  TCCTCTGATG  CTCGGGTTC  GCATCAGCCC
51     GGC GGAATGC  CAGACGCCCA  GCATCTACGA  CGATGTAGAG  AGTACCAGGG
101    AAGGCCAAGA  AGCCTCTCTG  CGTCCCACAG  TAGAACTTAA  TGAGTCGAAG
151    TCCCCAGACA  AGCCTAATCC  ACGAGGCTTC  CCGGGCAAGC  CCTGTGCCAA
201    CAACAGTGAC  ACGCTGGAGC  TCCCAGCCAG  CTCTGAAGCA  CTGCTTCTGG
251    GGTGGGTCCC  CACGAGGCTG  GTACCTGCCA  TCTATGGGCT  AGTGGTGGTC
301    GTGGGGCTGC  CTGCCAATGG  GCTAGCGCTG  TGGGTGCTGG  CCACAAGGGT
351    CCCACGCTTG  CCATCCACCA  TTCTGCTCAT  GAACCTGGCA  GTGGCTGACT
401    TGCTACTGGC  CCTGGTGCTC  CCACCACGAC  TGGTTTATCA  CCTGCGTGCC
451    CAGCGCTGGC  CGTTTGGGGA  GGCTGCCTGC  CGGGTGGCCA  CAGCTGCCCT
501    CTATGGCCAC  ATGTATGGTT  CAGTGTGCT  GCTGGCCGCA  GTCAGCCTGG
551    ACAGATACCT  GGCCTGGTG  CATTCTTTGC  GGGCCCGGGC  ATTGCGTGGT
601    CAACGCCTCA  CCACCATACT  CTGCTTGGTG  GCCTGGCTCT  CTGCAGCCAC
651    TCTAGTCTTG  CCACTCACGT  TTCATCGGCA  GACCTTCCTA  TTGGCTGGCT
701    CCGATCGCAT  GCTGTGTCAT  GATGCGCTGC  CATTGGCGGA  GCAGACCTCC
751    CATTGGAGAC  CGGCCTTCAT  CTGCCTGGCT  GTCCTGGGCT  GCTTTGTGCC
801    ACTGCTGGCC  ATGGTCCTGT  GCTATGGGGC  CACCCTTCGT  GCACTGGCAG
851    CCAATGGTCA  GCGGTACAGC  CATGCAGTGA  GACTGACAGC  CCTGGTCCTG
901    TTCTCAGCAG  TGGCCGCTTT  CACACCTAGC  AATGTGCTTC  TGGTGCTGCA
951    CTATTCAAAC  CCAAGCCCTG  AGGCCTGGGG  CAATCTCTAT  GGAGCCTATG
1001   TTCCCAGCCT  GGCCTCAGC  ACCCTTAACA  GCTGCGTAGA  CCCTTTCATC
1051   TACTACTATG  TGTCCCACGA  GTTCAGGGAG  AAGGTACGGG  CTATGTTGTG
1101   TCGCCAGCTG  AAGGCCAGCA  GCTCCTCTCA  GGCCTCCAGG  GAGGCTGGAA
1151   GCCGAGGGAC  TGCCATTTGC  TCCTCTACAC  TTCTGTGACT  GTTAGCTGAG
1201   TTGGGAAAGG  GGACGTTTCAG  ACTCGACTGG  ATCTCCCCTT  AAAGTGTCCC
1251   TCCTGAACCC  TCAGGACATG  ACCTTATTTG  GATATGCACT  TGGCGCGACC
1301   TTCATTTAGT  GGAGCTGAGG  TCC

```

Figure 1
Nucleotide sequence of the rat PAR4 gene; CD: 1-1188.

Results

Cloning of rat PAR4

Primers, based upon the mouse PAR4 sequence, were designed to amplify the rat PAR4 coding region. We amplified a 1.1-kb fragment from rat duodenal RNA of which the nucleotide sequence shared 91 % and 82 % homology with mouse PAR4 and human PAR4 respectively. To establish the expression and corroborate the sequence of the PCR clone a rat duodenal cDNA library was screened. A partial clone was identified (0.7 kb), which contained part of the open reading frame (ORF) of the RT-PCR clone (nucleotides 596-1188) and a short segment of 3' untranslated region (nucleotides 1189-1323). To fill in the missing

5' end, 5' RACE was performed. This yielded a 0.6 kb fragment that represented the 5' end of the ORF (nucleotides 2-636). The ORF of the partial duodenal library clone and the 5' RACE fragments both shared 100% homology with the rat PAR4 PCR clone. Figure 1 shows the nucleotide sequence; figure 2 shows the deduced amino-acid sequence and its alignment with the human and mouse rat PAR4 homologue. The rat PAR4 nucleotide sequence is available in the GenBank under accession number AF310216.

Analysis of the coded amino-acid sequence revealed an overall 90% and 75% protein identity of the rat PAR4 sequence with the mouse and human PAR4, respectively.

	1					50
mouse	MCWP..LLYP	LVLGLSISLA	EGIQTPSIYD	DVESTRGSH	GPLGPTVELK	
rat	MCWP..LLYP	LMLGFSISPA	E.CQTPSIYD	DVESTREGQE	ASLRPTVELN	
human	.MWGRLLLWP	LVLGFSL..	GGTQTPSVYD	ESGSTGGGDD	ST..PSI...	
	51					100
mouse	EPKSSDKPNP	RGYPGKFCAN	DSDTLELPAS	SQALLLGWVS	TRLVPALYGL	
rat	ESKSPDKPNP	RGFPGKPCAN	NSDTLELPAS	SEALLLGWVP	TRLVPAIYGL	
humanLPAP	RGYPGQVCAN	DSDTLELPDS	SRALLLGWVP	TRLVPALYGL	
	101					150
mouse	VVAVGLPANG	LALWVLATRV	PRLPSTILLT	NLAVADSLLA	LVPPPRLAYH	
rat	VVVVGLPANG	LALWVLATRV	PRLPSTILLM	NLAVADLLLA	LVLPPRLVYH	
human	VLVVGLPANG	LALWVLATQA	PRLPSTMLLM	NLATADLLLA	LALPPRIAYH	
	151					200
mouse	LRGQRWPFGE	AACRVATAAL	YGHMYGSVLL	LAAVSLDRYL	ALVHPLRARA	
rat	LRGQRWPFGE	AACRVATAAL	YGHMYGSVLL	LAAVSLDRYL	ALVHSLRARA	
human	LRGQRWPFGE	AACRLATAAL	YGHMYGSVLL	LAAVSLDRYL	ALVHPLRARA	
	201					250
mouse	LRGQRLTTGL	CLVAWLSAAT	LALPLTLHRQ	TFRLAGSDRM	LCHDALPLTE	
rat	LRGQRLTTIL	CLVAWLSAAT	LVLPLTFHRQ	TFLLAGSDRM	LCHDALPLAE	
human	LRGRRALGL	CMAAWLMAAA	LALPLTLQRQ	TFRLARS DRV	LCHDALPLDA	
	251					300
mouse	QTSHWRPAFI	CLAVLGCFVP	LLAMGLCYGA	TLRALAANGQ	RYSHALRLTA	
rat	QTSHWRPAFI	CLAVLGCFVP	LLAMVLCYGA	TLRALAANGQ	RYSHAVRLTA	
human	QASHWQPAFT	CLALLGCFLP	LLAMLLCYGA	TLHTLAASGR	RYGHALRLTA	
	301					350
mouse	LVLFSAVASF	TPSNVLLVLH	YSNPSPEAWG	NLYGAYVPSL	ALSTLNSCVD	
rat	LVLFSAVA AF	TPSNVLLVLH	YSNPSPEAWG	NLYGAYVPSL	ALSTLNSCVD	
human	VVLASAVAFF	VPSNLLLLLH	YSDPSPSAWG	NLYGAYVPSL	ALSTLNSCVD	
	351					399
mouse	PFIYYYYVSHE	FREKVRA.ML	CRQPEASSSS	QASREAGSRG	TAICSSTLL	
rat	PFIYYYYVSHE	FREKVRA.ML	CRQLKASSSS	QASREAGSRG	TAICSSTLL	
human	PFIYYYYVSAE	FRDKVRAGLF	QRSPGDTVAS	KASAEGGSRG	MGTHSSLLQ	

Figure 2

Amino-acid sequence of rat PAR4 and alignment with mouse and human PAR4 (Genbank accession number AF080215, mouse, and AF080214, human). The putative rat PAR4 tethered ligand (GFPGKP) sequence has 3 amino-acids (GPG) in common with the human (GYPGQV) and 4 amino-acids (GPGK) with the mouse ligand (GYPGKF). Activating peptide sequences are printed bold.

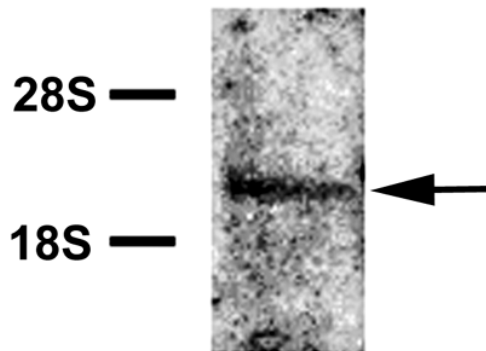


Figure 3
Northern blot of rat duodenal mRNA hybridized to a rat PAR4 probe. Left: ribosomal markers. The single hybridizing band corresponds to a mRNA of ≈ 3 kb.

Rat PAR4 shared 33% with rat PAR1 and 35% protein identity with rat PAR2. Identification of a protease cleavage site corresponding to the human PAR4 indicated that the putative rat PAR4 AP (GFPGKP) had 3 amino-acids (GPG) in common with the corresponding human PAR4 (GYPGQV) and 4 amino-acids (GPGK) in common with the mouse (GYPGKF) sequences [5,7].

Tissue distribution of rat PAR4

Northern blot analysis of poly(A)⁺ RNA from the duodenum detected a 3.0 kb band (Figure 3).

This corresponds in size to the predominant band previously described in human tissues. Expression in other tissues was measured by RPA (Figure 4).

Various amounts of total RNA were used based upon availability of the tissue, however analysis of the total amounts of PAR4 was expressed as the PAR4/actin ratio, thereby correcting for the different amounts of total RNA. Results showed that PAR4 is expressed at low levels in multiple gastrointestinal tissues including the esophagus, stomach, duodenum, jejunum and distal colon. It is also present in the spleen.

Functional studies

COS-1 and COS-7 cells were transiently transfected with a pcDNA3.1+ expression vector containing the rat PAR4 1.1 kb amplicon. Cells were treated with trypsin, thrombin, mouse PAR4 AP (GYPGKF) or rat PAR4 AP (GFPGKP) and changes in intracellular Ca²⁺ were measured. The reversed sequence of the mouse PAR4 AP (FKGPYG) was used as a control peptide, as we did not have the rat PAR4 control

peptide available. In addition, untransfected COS cells were used as controls.

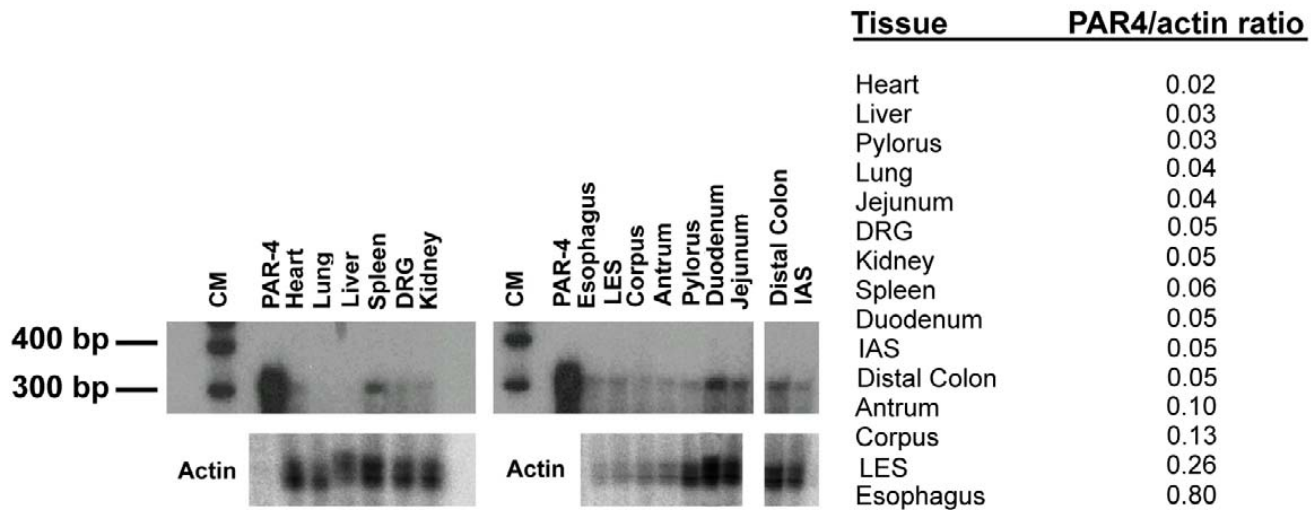
Transfected cells responded to mouse PAR4 AP 500 μ M, trypsin 1 nM, thrombin 50 nM and the putative rat PAR4 AP 100 μ M (Figure 5). Control COS cells showed no response to any agonists tested (data not shown) and transfected cells failed to respond to the mouse control peptide. After incubation with mouse PAR4 AP, the cells did respond to a second incubation with the mouse PAR4 AP 500 μ M, although the magnitude of the response was less. Rat PAR4 AP caused an increase in intracellular Ca²⁺ at 100 μ M. Subsequent stimulation of the receptor with trypsin did not demonstrate an increase in intracellular Ca²⁺. When these agonists were applied in reversed order, the same observations were made: the receptor was activated by trypsin but subsequently did not respond to stimulation with rat PAR4 AP.

Discussion

We have cloned a rat PAR cDNA from the rat duodenum. Based on its sequence homology with the mouse and human PAR4 and its functional characteristics we conclude that the amplicon represents the rat PAR4 homologue. Although our cloning strategy involved PCR techniques in both the initial PCR cloning of the rat PAR4 and in filling in the partial cDNA library clone with 5' RACE, we feel confident that the sequence of the rat receptor as reported here, is accurate. In both cases, we used the most accurate proofreading polymerase known, Pfu-Turbo, and in essence, we independently derived two PCR clones from our cloning experiments and found that their sequence was identical. In addition, the rat PAR4 is fully functional in a transient transfection assay, thus corroborating the accuracy of the sequence.

Synthetic peptides that mimic the tethered ligands of the PAR receptors have been used as specific agonists to study the function of these receptors. Site-directed mutagenesis studies by Xu *et al.*, who simultaneously and independently cloned the human PAR4 receptor, suggested that the Arg-47/Gly-48 cleavage site is critical for subsequent receptor activation by thrombin and trypsin [5]. The rat PAR4 receptor contains such a cleavage site at position Arg-58/Gly-59. Indeed, both thrombin and trypsin function as a ligand for the rat PAR4 receptor.

The putative rat PAR4 tethered ligand contains a phenylalanine in the second position, as do the human PAR1 and PAR3. This phenylalanine in the second position of the tethered ligand of the human PAR1 and PAR3 receptor is critical for function of this domain and we speculate that it is of equal importance in the rat PAR4 tethered ligand. By contrast, in the mouse PAR4, it is a tyrosine in the second position of the tethered ligand that is critical [7]. Fu-

**Figure 4**

A Detection of PAR4 mRNA in rat tissues by RPA. Tissue sources are given at the top and the size of the protected fragment is given on the left. (CM, central marker; LES, lower esophageal sphincter; IAS, internal anal sphincter; DRG, dorsal root ganglia). B The PAR4/actin ratio suggests that PAR4 is predominantly expressed in the esophagus, LES, and the stomach.

ture studies, requiring the construction of different receptor-related peptides using site-directed mutagenesis, are needed to confirm the role of the various amino-acids in conferring activity to the PAR4 ligand.

Although the mouse PAR4 tethered ligand (GYPGKF) differs from the putative rat PAR4 AP sequence (GFPGKP), it was nonetheless able to activate the rat PAR4 transfected COS cells. This may represent the ability of mouse PAR4 activating peptide to serve as a partial agonist, causing enough of an alteration in the conformation of the receptor to initiate transduction of a signal across the plasma membrane. In keeping with this theory, *in vitro* studies using rat tissue have previously reported functional responses to mouse PAR4 activating peptide [10,11].

When transfected COS cells were challenged with either rat PAR4 AP or trypsin, activation occurred as demonstrated by an increase in intracellular Ca^{2+} . However, subsequent stimulation with rat PAR4 AP or trypsin did not evoke a biological response suggesting that desensitization had occurred. It is conceivable that activation of the receptor prevents subsequent stimulation by a second agonist, analogous to studies on PAR2 mechanisms of desensitization [12]. By contrast, mouse PAR4 AP did not completely abolish a response to a second exposure to mouse PAR4 AP, although the magnitude of the response was less. This may be due to a weaker agonistic effect, reflecting differences in its sequence.

Rat PAR4 message is found mainly in the esophagus, stomach, the duodenum and the spleen. The potential effect of PAR4 activation in the upper gastrointestinal tract and a hematopoietic organ like the spleen is an interesting topic for further study. Recently Andrade-Gordon *et al* partially cloned guinea-pig PAR4 and identified the guinea-pig activating peptide SFGQA [14]. Guinea pig PAR4 appears to be involved in platelet aggregation. However, the exact physiological and pathophysiological roles of PARs in the gastrointestinal tract remains to be fully defined. Several studies have suggested a role for PARs in the modulation of gastrointestinal motility [8–10] and based on previous literature using cross-species experiments (mouse AP on rat GI tissue), a role for PAR4 has also been proposed in gastric contractility [11] and in the relaxation of esophageal muscularis mucosae [10]. The identification of the rat PAR4 homologue, its tethered ligand and its tissue distribution will now allow the testing of the specific PAR4 AP in various well established models of rat motility or other aspects of gastrointestinal function such as epithelial ion transport, as has been suggested by some studies [15].

In summary, we identified a rat PAR4 cDNA sequence from a rat duodenal cDNA library. A putative protease cleavage site (Arg-58/Gly-59) was identified. The rat PAR4 clone mobilized calcium in transiently transfected COS cells in response to thrombin, trypsin, mouse PAR4 AP and its newly identified rat PAR4 AP. The highest levels of

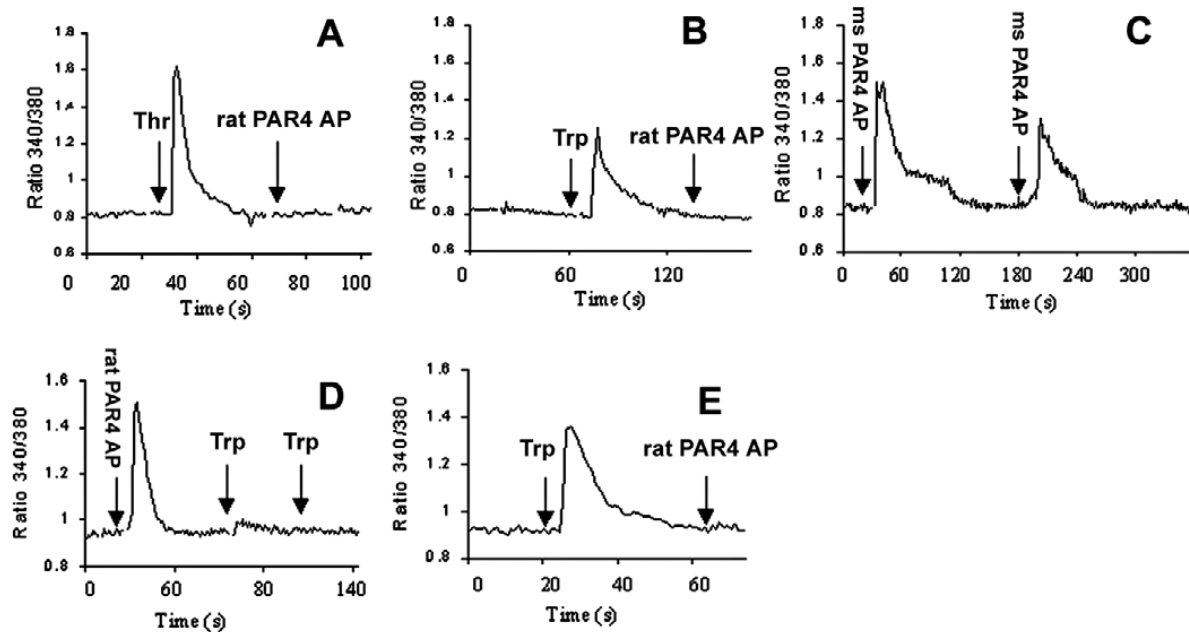


Figure 5

PAR4 mediated Ca^{2+} mobilization in transfected COS cells. (A) Rat thrombin 50 nM (1st arrow) followed by rat PAR4 AP 100 μM (2nd arrow), (B) Trypsin 1 nM (1st arrow) followed by rat PAR4 AP 100 μM (2nd arrow), (C) Mouse PAR4 AP 500 μM followed by repeat mouse PAR4 AP 500 μM , (D) Rat PAR4 AP 100 μM (1st arrow) followed by trypsin 1 nM (2nd and 3rd arrow), (E) Trypsin 1 nM (1st arrow) followed by rat PAR4 AP 100 μM (2nd arrow). Thr, thrombin; trp, trypsin; ms PAR4 AP, mouse PAR4 activating peptide.

expression of this receptor were observed in the esophagus and the stomach.

Methods

RT-PCR cloning of rat PAR4

Total RNA was made from the duodenum of normal male Sprague-Dawley rats, using the UltraspecTM RNA isolation system (Biotex Laboratories, Inc., Houston, TX) and treated with DNase I. Using the ProSTAR Ultra HF RT-PCR System (Stratagene, La Jolla, CA), RT-PCR was performed with mouse PAR4 specific primers (Forward primer 5'-ATG TGC TGG CCG CTG CTG TA-3' and reverse primer 5'-CAG TCA CAG AAG TGT AGA GG-3', Genbank accession number AF080215) which were designed to amplify the entire coding sequence. PCR conditions were as follows: initial denaturation 95°C for 1 min (1 cycle), 95°C for 1 min, 56°C for 1 min, 68°C for 15 min (40 cycles), 68°C final extension for 10 minutes. The PCR products were run on a low melt agarose gel, excised and purified with the PCR prep kit (Promega, Madison, WI) and blunt-

end ligated into a plasmid vector, pGEM 5Z(f)+ (Promega, Madison, WI). The PCR clone was completely sequenced using an automated ABI sequencer and its identity as a rat PAR4 was established by alignment of its sequence with the mouse and human PAR4 clones. To confirm the sequence of the rat PAR4 amplicon, a rat duodenal cDNA library was screened.

DNA library screening

Approximately 1×10^6 recombinant phage of a rat duodenal cDNA library constructed with the ZAP Express cDNA synthesis kit (Stratagene, La Jolla, CA) were screened using standard filter hybridization techniques with a ³²P-labeled random primer (Prime-it II kit; Stratagene, La Jolla, CA) rat PAR4 PCR amplicon as a probe. The filter was washed at moderate stringency (20 min at 37°C in 2× SSC, 0.1% SDS and for 20 min at 55°C in 0.5× SSC, 0.1% SDS) and subsequently exposed to Biomax film with an intensifying screen overnight at -80°C. A single cDNA clone was plaque-purified after two more rounds of screening and

subjected to *in vivo* excision as per manufacturer's protocol. The rat PAR4 cDNA was sequenced completely and found to be missing 595 bases at the 5' end.

5' RACE

Rapid amplification of cDNA ends (RACE) technique was used to complete the rat PAR4 cDNA sequence (Marathon RACE kit, Clontech, Palo Alto, CA). Double stranded rat duodenal cDNA was ligated to universal adapter primer and used as a template in 5' RACE reactions. The adapter-ligated cDNA was used in a PCR reaction with a sense primer corresponding to the adapter primer sequence and a PAR4 specific antisense primer (nucleotides 698–718 of the rat PAR4 sequence). RACE PCR conditions were similar as described under RT-PCR and also performed using a proofreading DNA polymerase (Pfu-Turbo-Stratagene La Jolla, CA). Fragments of the expected size were run on a low melt agarose gel, purified as described previously and blunt-end ligated into a plasmid vector, pGEM 5Z(f)+ (Promega, Madison, WI). The identity of the PCR product was confirmed by sequence analysis using an automated ABI sequencer.

Northern blot analysis

mRNA was purified using the mRNA Poly(A)Pure Purification Kit (Ambion, Austin, TX). Two μ g of duodenal mRNA was run on a 1.0 % formaldehyde-agarose gel, transferred to a nylon membrane and UV-crosslinked. The Northern blot was pre-hybridized and hybridized in ExpressHyb (Clontech, Palo Alto, CA) at 68°C with the ³²P-labeled random primer rat PAR4 PCR-amplified DNA clone. The membrane was washed at high stringency (20 min at 37°C in 2× SSC, 0.1% SDS, 20 min at 65°C in 0.1 × SSX, 0.1% SDS and exposed to Biomax film with an intensifying screen overnight at -80°C.

RNase Protection assay

The levels of rat PAR4 transcripts in a variety of rat tissues were analyzed using a ribonuclease protection assay (RPA). The RPA template DNA was amplified by PCR incorporating a T7 promoter sequence before the antisense primer (5'CTCGGTAATACGACTCACTATAGGGATGGCAAGCGTGGCACCCTTGTG-3'). The antisense riboprobe was labeled with alpha-[³²P] uridine triphosphate (UTP) according to manufacturer's protocol (Maxiscript, Ambion Inc., Austin, TX). Various amounts of total RNA were precipitated and dissolved in 8 μ l of hybridization buffer. One microliter each of rat PAR4 (80,000 counts/ μ l) and (β -actin (10,000 counts/ μ l) riboprobes were added to the hybridization mixture. Samples were heated to 90°C for 5 min and then hybridized at 56°C for 16 hours. After hybridization and RNase treatment, protected fragments were resolved on a 6.0 % sequencing gel. Radioactive bands were quantified with Cyclone Storage Phosor

Imaging System (Packard, Meriden, CT). PAR4 ratios were presented against the actin band for each tissue.

Rat PAR4 functional studies

Two oligonucleotide primers were designed to amplify the entire rat PAR4 coding region from rat duodenal RNA. The 5' primer contained a *Bam* HI site and a Kozak sequence (5'-GCTGGTACCGGATCCGCCATGTGCTGGCCGCTGCTGATCCTCTGATG-3') and the antisense primer contained a *Xba*1 site (5'-GCCGTCTAGAGCGCCGCATCACAGAAGTGTAGAGGAGCAAATGGCAGT-3'). The amplified PAR4 cDNA was cleaved with the restriction enzyme *Bam* HI and *Xba*1, directionally cloned into the expression vector pcDNA3.1+ (Invitrogen, Carlsbad, CA) and its sequence verified. For the transfection, COS-7 cells and COS-1 cells were grown in DMEM with 10% fetal bovine serum (FBS) supplemented with penicillin/streptomycin. Cells were plated 1 day before transfection. PAR4 DNA (1 μ g) was cotransfected with green fluorescent protein (GFP) according to manufacturer's protocol (FuGene™ 6 Transfection Reagent; Roche, Indianapolis, IN) and the cells were incubated for 36–48 hours. The cells were then loaded with the calcium indicator fura-2 and [Ca²⁺]_i-mobilization was measured using the 340/380 ratio. Specific PAR4 agonists were used including trypsin (Sigma, St. Louis, MO), rat thrombin (Sigma, St. Louis, MO) and rat and mouse PAR4 AP. (rat PAR4, GFPGKP-NH₂; mouse PAR4, GYPGKF-NH₂; reversed mouse PAR4, FKGYPG-NH₂). All peptides were synthesized at the UTMB Core Protein Facility using standard solid phase synthesis procedures).

Abbreviations

PAR, proteinase-activated receptor; RPA, ribonuclease protection assay; AP, activating peptide; ORF, open reading frame

Acknowledgement

We thank Brenda Kenworthy for excellent technical assistance in the preparation of this manuscript.

References

1. Vu TK, Hung DT, Wheaton VI, Coughlin SR: **Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation.** *Cell*, 1991, **64**:1057-1068
2. Nystedt S, Emilsson K, Wahlestedt C, Sundelin J: **Molecular cloning of a potential proteinase activated receptor.** *Proc. Natl. Acad. Sci.*, 1994, **91**:9208-9212
3. Nystedt S, Emilsson K, Larsson AK, Strömbeck B, Sundelin J: **Molecular cloning and functional expression of the gene encoding the human proteinase-activated receptor 2.** *Eur. J. Biochem.*, 1995, **232**:84-89
4. Saifeddine M, Al-Ani B, Cheng C-H, Wang L, Hollenberg MD: **Rat proteinase-activated receptor-2 (PAR-2): cDNA sequence and activity of receptor-derived peptides in gastric and vascular tissue.** *Br. J. Pharmacol.*, 1996, **118**:521-530
5. Xu W, Andersen H, Whitmore TE, Presnell SR, Yee DP, Ching A, Gilbert T, Davie EW, Foster DC: **Cloning and characterization of human protease-activated receptor 4.** *Proc. Natl. Acad. Sci.*, 1998, **95**:6642-6646

6. Ishihara H, Connolly AJ, Zeng D, Kahn ML, Zheng YW, Tommons C, Tram T, Coughlin SR: **Protease-activated receptor 3 is a second thrombin receptor in humans.** *Nature*, 1997, **386**:502-506
7. Kahn ML, Zheng YW, Huang W, Bigornia V, Zen D, Moff S, Farese JR RV, Tam C, Coughlin SR: **A dual thrombin receptor system for platelet activation.** *Nature*, 1998, **394**:690-694
8. Cocks TM, Sozzi V, Moffatt JD, Selemidis S: **Protease-activated receptors mediate Apamin-sensitive relaxation of mouse and guinea pig gastrointestinal smooth muscle.** *Gastroenterology* 1999, **116**:586-592
9. Kawabata A, Kuroda R, Nishikawa H, Kawai K: **Modulation by protease-activated receptors of the rat duodenal motility in vitro: possible mechanisms underlying the evoked contraction and relaxation.** *Br. J. Pharmacol.*, 1999, **128**:865-872
10. Kawabata A, Kuroda R, Kuroki N, Nishikawa H, Kawai K: **Dual modulation by thrombin of the motility of rat oesophageal muscularis mucosae via two distinct protease-activated receptors (PARs): a novel role for PAR-4 as opposed to PAR-1.** *Br. J. Pharmacol.*, 2000, **131**:578-584
11. Hollenberg MD, Saifeddine M, Al-Ani B, Gui Y: **Proteinase-activated receptor 4 (PAR4): action of PAR-4 activating peptides in vascular and gastric tissue and lack of cross-reactivity with PAR-1 and PAR-2.** *Can J. Physiol.*, 1999, **77**:459-464
12. Bohm SK, Khitin LM, Grady EF, Aponte G, Payan DG, Bunnett NW: **Mechanisms of desensitization and resensitization of proteinase-activated receptor-2.** *J. Biol. Chem.*, 1996, **271**:22003-22016
13. Déry O, Corvera CU, Steinhoff M, Bunnett NW: **Proteinase-activated receptors: novel mechanisms of signaling by serine proteases.** *Am. J. Physiol.*, 1998, **274**:C1429-C1452
14. Andrade-Gordon P, Derian CK, Maryanoff BE, Zhang HC, Addo MF, Cheung W, Damiano BP, D'Andrea MR, Darrow AL, de Garavilla L, Eckardt AJ, Giardino EC, Haertlein BJ, McComsey DF: **Administration of a potent antagonist of protease-activated receptor-1 (PAR-1) attenuates vascular restenosis following balloon angioplasty in rats.** *J. Pharmacol. Exp. Ther.*, 2001, **298**:34-42
15. Vergnolle N, MacNaughton WK, Al-Ani B, Saifeddine M, Wallace JL, Hollenberg MD: **Proteinase-activated receptor 2 (PAR-2)-activating peptides: Identification of a receptor distinct from PAR-2 that regulates intestinal transport.** *Proc. Natl. Acad. Sci.*, 1998, **95**:7766-7771

Publish with **BioMed Central** and every scientist can read your work free of charge

"BioMedcentral will be the most significant development for disseminating the results of biomedical research in our lifetime."

Paul Nurse, Director-General, Imperial Cancer Research Fund

Publish with **BMC** and your research papers will be:

- available free of charge to the entire biomedical community
- peer reviewed and published immediately upon acceptance
- cited in PubMed and archived on PubMed Central
- yours - you keep the copyright

Submit your manuscript here:

<http://www.biomedcentral.com/manuscript/>



BioMedcentral.com

editorial@biomedcentral.com