

Molecular cloning, expression and potential functions of the human proteinase-activated receptor-2

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We used PCR to amplify proteinase activated receptor-2 (PAR-2) from human kidney cDNA. The open reading frame comprised 1191 bp and encoded a protein of 397 residues with 83% identity with mouse PAR-2. In KNRK cells (a line of Kirsten murine sarcoma virus-transformed rat kidney epithelial cells) transfected with this cDNA, trypsin and activating peptide (AP) corresponding to the tethered ligand exposed by trypsin cleavage (SLIGKV-NH₂) induced a prompt increase in cytosolic calcium ion concentration ([Ca²⁺]_i). Human PAR-2 (hPAR-2) resided both on the plasma membrane and in the Golgi apparatus. hPAR-2 mRNA was highly expressed in human pancreas, kidney,

colon, liver and small intestine, and by A549 lung and SW480 colon adenocarcinoma cells. Hybridization *in situ* revealed high expression in intestinal epithelial cells throughout the gut. Trypsin and AP stimulated an increase in [Ca²⁺]_i in a rat intestinal epithelial cell line (hBRIE 380) and stimulated amylase secretion in isolated pancreatic acini. In A549 cells, which also responded to trypsin and AP with mobilization of cytosolic Ca²⁺, AP inhibited colony formation. Thus PAR-2 may serve as a trypsin sensor in the gut. Its expression by cells and tissues not normally exposed to pancreatic trypsin suggests that other proteases could serve as physiological activators.

INTRODUCTION

Most cell-surface receptors are activated by binding to a soluble extracellular ligand, such as a hormone or neurotransmitter. Recently a new family of G-protein coupled, seven transmembrane domain receptors has been described that are activated by proteolytic cleavage [1]. The thrombin receptor is the first member of this family [2,3]. Thrombin cleaves this receptor within the extracellular N-terminus at LDPR↓SFLI, forming an N-terminal tethered ligand that activates the receptor. The second member of this family is PAR-2, recently cloned from a mouse genomic library [4,5]. Trypsin cleaves the N-terminus of PAR-2 at SKGR↓SLIG, forming a tethered ligand. Peptides corresponding to the tethered ligand sequences of the thrombin receptor and PAR-2 are full agonists and thus can be used to investigate receptor functions [2–5].

Only mouse PAR-2 has been cloned (although during the review of this manuscript the sequence of human PAR-2 was published [6]). Thus structural similarities between different species have not been studied. Moreover, the sites of expression and biological actions of PAR-2 are unknown. The aims of this study were to (1) clone human PAR-2; (2) determine levels of expression in human tissues and cell lines; (3) examine cellular localization and signal transduction; and (4) define potential functions of PAR-2.

EXPERIMENTAL

Cloning and expression of human PAR-2

Degenerate oligonucleotides to conserved sequences in transmembrane domain II (5'-CCNGCNGTATQAYATG-3') and extracellular loop II (5'-CRTGRCANGTNGTWATRTT-3') of

mouse PAR-2 and human thrombin receptor were used to amplify human kidney cDNA (Clontech, Palo Alto, CA, U.S.A.). A PCR product of the expected size (360 bp) was cloned into pCR II (Invitrogen, San Diego, CA, U.S.A.). Anchored PCR was used to amplify the 5' and 3' ends of the cDNA from a human kidney λgt 11 library (Clontech). The entire coding region was amplified and cloned into pBluescript (Stratagene, La Jolla, CA, U.S.A.). Clones were sequenced on both strands. cDNA encoding human PAR-2 was subcloned into pcDNA3 (Invitrogen), and stably expressed in KNRK cells (a line of Kirsten murine sarcoma virus-transformed rat kidney epithelial cells) [7]. For immunofluorescence localization, a receptor construct with the 12CA5 haemagglutinin epitope (YPYDVPDYA) at the C-terminus was generated by PCR.

Immunofluorescence

PAR-2 was localized by immunofluorescence with antibody to the haemagglutinin epitope [HA.11 (Berkeley Antibody Co., Richmond, CA, U.S.A.), 1:5000 overnight at 4 °C] and a secondary antibody labelled with Texas Red [8]. The Golgi apparatus was localized with antibody to mannosidase II (Berkeley Antibody Co.) (1:2000) and a secondary antibody labelled with fluorescein isothiocyanate. In controls, antibodies were incubated with non-transfected KNRK cells.

Northern hybridization

A 320 bp probe was amplified from human PAR-2 by using primers to transmembrane domain II (5'-GGCCAATCTGGCCTTGGCTGAC-3') and extracellular loop II (5'-GGGCAGGAATGAAGATGGTCTGC-3'), and labelled with [α -³²P]dCTP by random priming [9]. RNA was separated on a 1.2% (w/v)

Abbreviations used: AP, activating peptide; [Ca²⁺]_i, cytosolic calcium ion concentration; h, human; PAR-2, proteinase-activated receptor-2; RP, reverse peptide; SBTI, soybean trypsin inhibitor.

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The sequence in this paper has been deposited in GenBank with the Accession Number U34038.

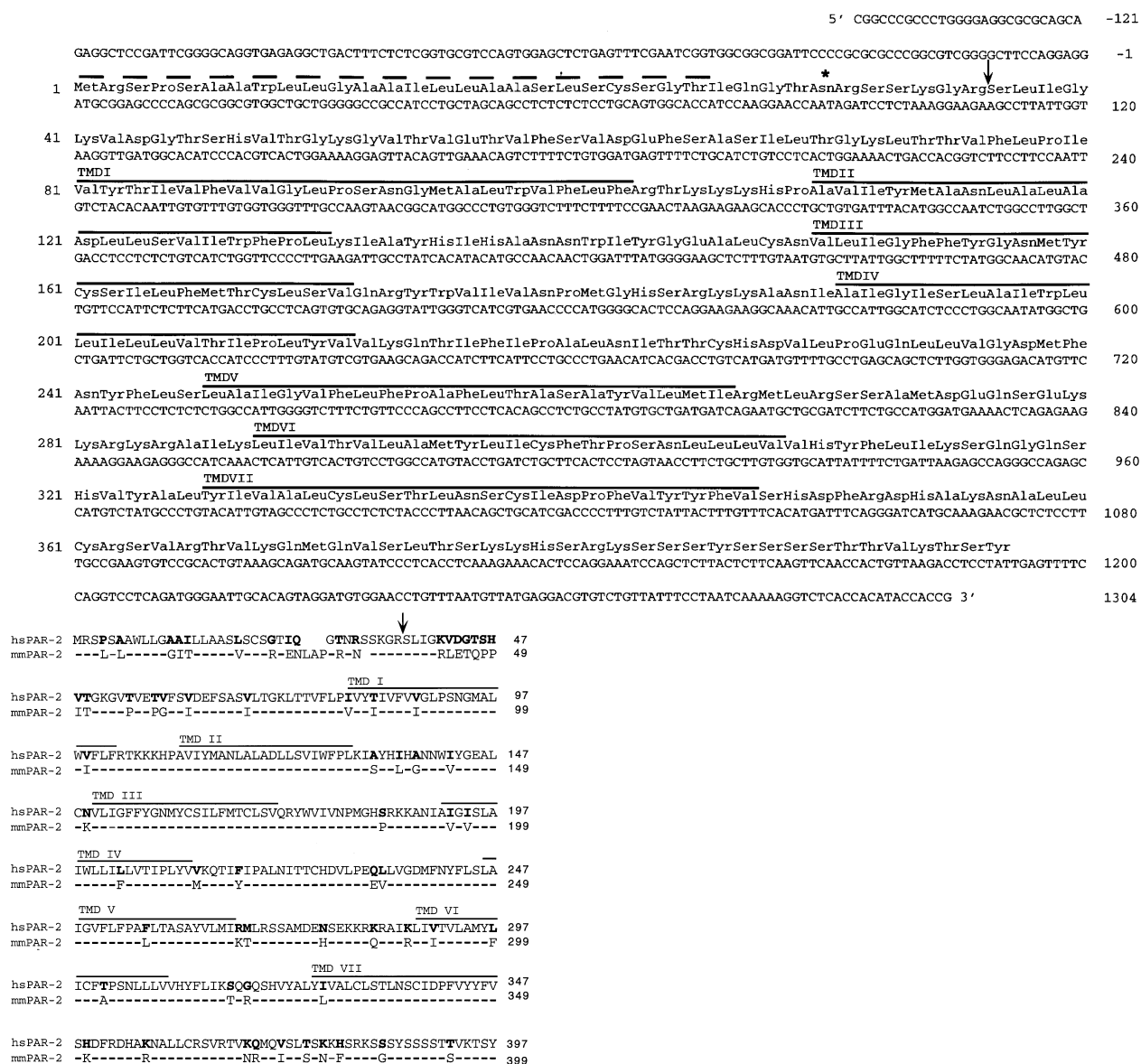


Figure 1 cDNA and predicted protein sequences of human PAR-2 (upper panel) and alignment of the predicted protein sequence of human (hs) and mouse (mm) PAR-2 (lower panel)

(Upper panel) Solid lines, predicted transmembrane domains; broken line, putative signal peptide; arrow, proposed trypsin cleavage site; *, putative glycosylation site; left-hand numbers, amino acids; right-hand numbers, nucleotides. (Lower panel) Identical residues are indicated by plain text and broken lines. Different residues are shown by bold text.

formaldehyde/agarose gel, transferred to nylon filters, cross-linked and hybridized overnight in $5 \times$ SSPE, $10 \times$ Denhardt's reagent, 50% formamide, 2% (w/v) SDS, and 1 μ g/ml salmon sperm DNA at 42 °C [9]. Filters were washed in $2 \times$ SSC, 0.1% SDS for 20 min at room temperature and for 40 min at 68 °C, and exposed to film. Filters were hybridized with a cDNA probe for β -actin as a control for the amount of loaded RNA.

In situ hybridization

Six-week-old inbred C57BL/6 mice were restricted overnight to a liquid diet and perfused with 4% (w/v) paraformaldehyde in PBS. Organs were removed and fixed by immersion in 4% paraformaldehyde for 2–4 h. Tissues were dehydrated in ethanol, cleared in toluene and embedded in paraffin. Sections 10 μ m

thick were mounted on slides, deparaffinized, fixed in 4% paraformaldehyde, treated with proteinase K and acetylated with 0.25% (v/v) acetic anhydride. Hybridization *in situ* was performed as previously described [10]. RNA probes were transcribed from plasmids containing a 1.2 kb fragment comprising most of the coding region of mouse PAR2 and a 0.8 kb *BspEI*–*ClaI* fragment of the mouse thrombin receptor coding region. The mouse PAR2 fragment was cloned by PCR from mouse genomic DNA by using primers, 5'-CGTAAAGGAAGAAGTCTTATTGGC-3' and 5'-CACATCTCAGGTACAGCTCAGTA-3', based on published sequences [4,5]. Sense and antisense probes were transcribed from linearized plasmids by using T3 or T7 RNA polymerases with 35 S-labelled UTP. Sections were prehybridized in 50% deionized formamide, 0.3 M NaCl, 20 mM Tris buffer, pH 8, 5 mM EDTA, 10% (w/v)

dextran sulphate and 10 mM dithiothreitol for 1–3 h at 55 °C. RNA probes (600 000 c.p.m. per slide) were added and incubation continued for 12–18 h at 55 °C. Sections were washed for 20 min in $2 \times$ SSC, 10 mM 2-mercaptoethanol and 1 mM EDTA, treated with RNase A (20 mg/ml) for 30 min at room temperature, then washed as before except at high stringency with $0.1 \times$ SSC at 55 °C for 2 h. Sections were dehydrated, dipped in photographic emulsion, stored at 4 °C, developed after 4 or 8 weeks of exposure, and counterstained with haematoxylin and eosin.

Measurement of cytosolic calcium ion concentration

Cytosolic calcium ion concentration ($[Ca^{2+}]_i$) was measured by spectrofluorometry with fura-2/AM [7]. Cells were exposed to bovine trypsin (Worthington Biochemical Corporation, Freehold, NJ, U.S.A., or Boehringer Mannheim, Indianapolis, IN, U.S.A.) or activating peptide (AP) corresponding to the tethered ligand exposed after trypsin cleavage [SLIGKV-NH₂ for human PAR-2 (hPAR-2) or SLIGRL-NH₂ for mouse PAR-2]. For controls, cells were exposed to inactivated trypsin (preincubated with 10 μ g/ml soybean trypsin inhibitor for 60 min at 4 °C) or reverse peptide (RP) VKGILS-NH₂ (hPAR-2) or LRGILS-NH₂ (mouse PAR-2).

Measurement of growth of A549 cells

A549 cells, maintained in F12K modified Kaighn's medium, were dispersed non-enzymically, triturated to form a single cell suspension, and plated at 10^3 cells per 35 mm well. Cells were grown in medium containing human AP or RP, which was replaced daily. After 6 d, cells were fixed in 10% formalin, stained with 0.2% Crystal Violet, and colonies of more than 25 cells were counted.

Measurement of amylase secretion

Pancreatic acini were prepared from male Sprague–Dawley rats (200–250 g) as described [11]. Acini were incubated in 2 ml of Krebs–Henseleit bicarbonate buffer with trypsin, inactivated trypsin, mouse AP or human RP for 30–45 min at 37 °C with shaking (120 per min). At beginning of each experiment, an aliquot of acini was removed, centrifuged (10 min at 10000 g), and amylase was assayed in the supernatant. This initial value was subtracted from values obtained after incubation. After incubation with agonists, acini were centrifuged and amylase was assayed in the supernatant. The total amylase content of homogenized acini was also measured. Amylase was measured with an amylase reagent kit (Sigma Chemical Co., St. Louis, MO, U.S.A.).

RESULTS AND DISCUSSION

Molecular cloning of human PAR-2

A human PAR-2 cDNA clone of 1451 bp was obtained with an open reading frame of 1191 bp (Figure 1, upper panel). Part of the 5' untranslated sequence (–70 to –24) was highly conserved (81%) between human and mouse PAR-2, and may play a role in translational regulation. The ATG codon was surrounded by a consensus sequence for initiation of translation [12]. The reading frame encoded a protein of 397 residues (calculated M_r 44125) with seven hydrophobic, presumably membrane-spanning, domains and an N-terminal hydrophobic region that is compatible with a signal peptide with the most likely cleavage site between Thr²⁵ and Ile²⁶ [5,13]. Putative signal peptides in the G-protein coupled receptor family have been found only in proteinase-activated receptors and those with long N-terminal

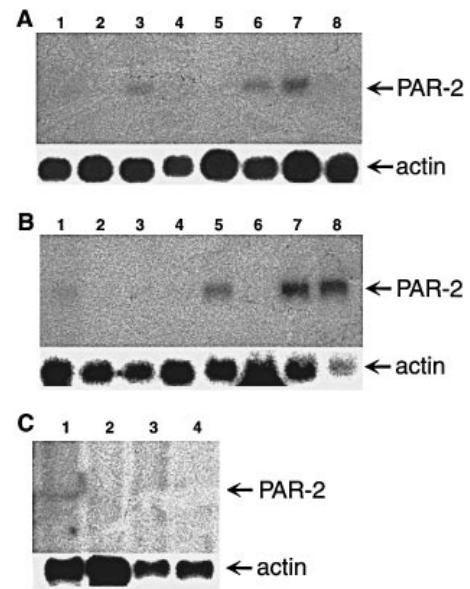


Figure 2 Northern blots of human tissues hybridized with a human PAR-2 probe and a probe for β -actin, as a control for loaded RNA

(A) Lane 1, spleen; lane 2, thymus; lane 3, prostate; lane 4, testis; lane 5, ovary; lane 6, small intestine; lane 7, colon; lane 8, leucocytes. (B) Lane 1, heart; lane 2, brain; lane 3, placenta; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, pancreas. (C) Lane 1, trachea; lane 2, aorta; lane 3, salivary gland; lane 4, stomach. Each lane contains 2 μ g mRNA.

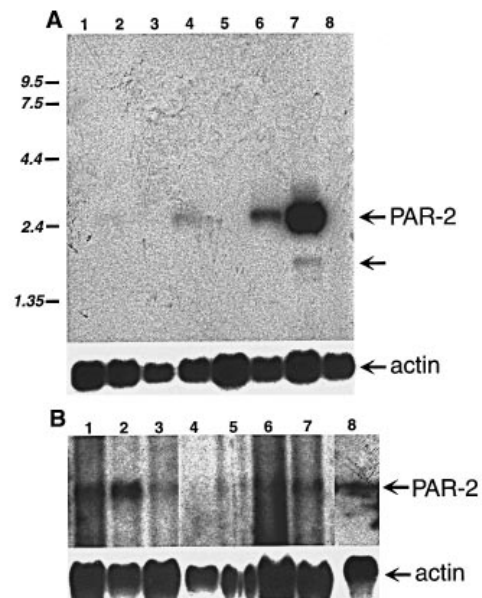


Figure 3 Northern blots of cell lines hybridized with a human PAR-2 probe and a probe for β -actin, as a control for loaded RNA

(A) Lane 1, HL-60; lane 2, HeLa S3; lane 3, K562; lane 4, MOLT-4; lane 5, Raji; lane 6, SW480; lane 7, A549; lane 8, G361. (B) Lane 1, Caco 2 (2 d post-confluence); lane 2, Caco 2 (14 d post-confluence); lane 3, COLO 205; lane 4, MIA PaCa; lane 5, PANC 1; lane 6, DU 145; lane 7, PC-3; lane 8, KNRK. Each lane contains 2 μ g mRNA.

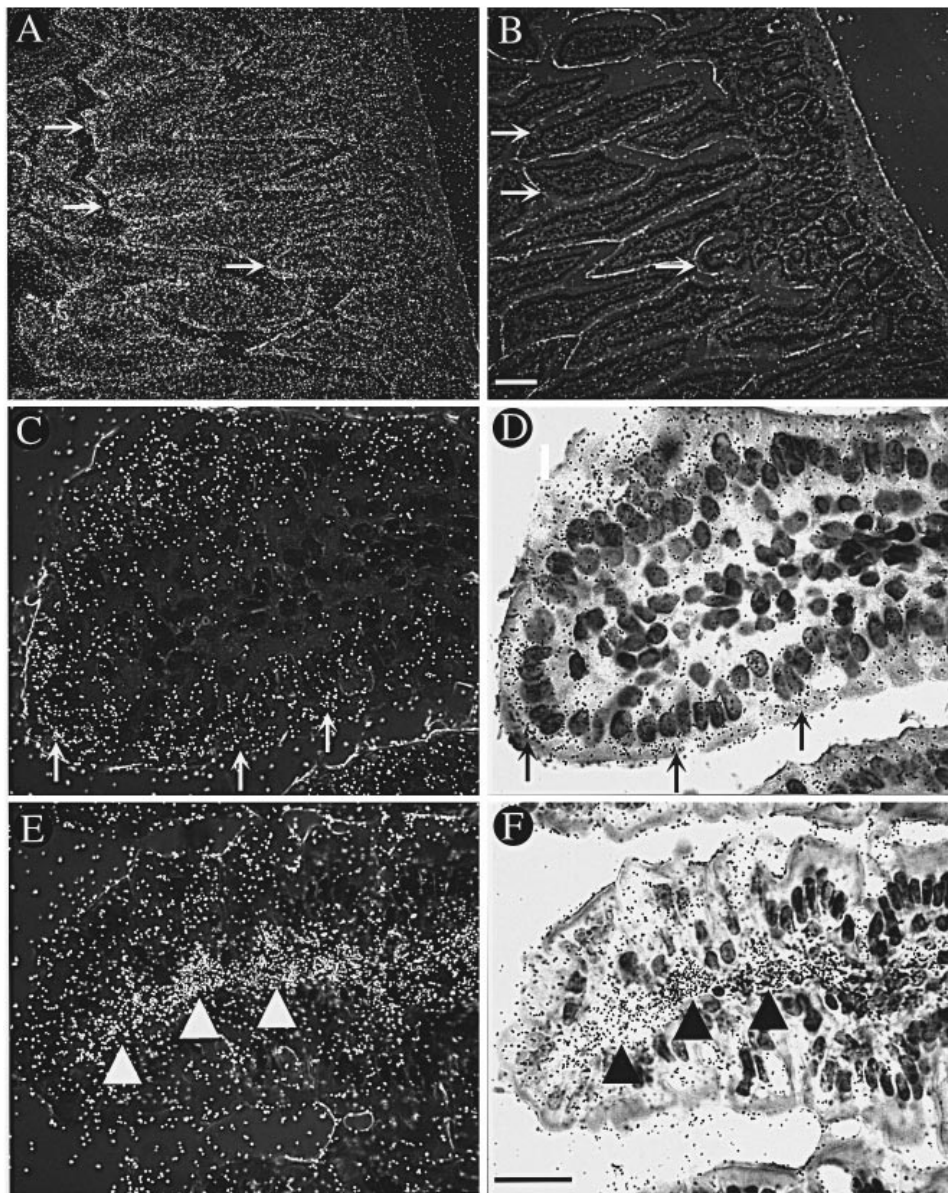


Figure 4 Photomicrographs showing the localization of PAR-2 mRNA (A–D) and thrombin receptor mRNA (E–F) in the proximal small intestine of the mouse, detected by hybridization *in situ*

(A) Dark-field photomicrograph of intestine hybridized with antisense PAR-2 probe. (B) Dark-field photomicrograph of intestine hybridized with sense PAR-2 probe. (C) Dark-field and (D) bright-field photomicrographs of villus hybridized with antisense PAR-2 probe. (E) Dark-field and (F) bright-field photomicrographs of villus hybridized with antisense thrombin receptor probe. Arrows indicate accumulation of silver grains in surface epithelial cells of intestinal villi in sections hybridized with the antisense PAR-2 probe. Arrow heads indicate accumulation of silver grains in endothelial cells in the center of the intestinal villi in sections hybridized with the antisense thrombin receptor probe. Scale bar = 20 μm (A, B) or 5 μm (C–F).

tails, such as the thyrotropin receptor [14]. The N-terminus of human PAR-2 contained one consensus site for N-linked glycosylation. It is not known whether signal peptide cleavage and receptor glycosylation affect receptor activation and function.

The amino acid sequence of human PAR-2 was 83% and 35% identical to mouse PAR-2 and human thrombin receptor respectively (Figure 1, lower panel). The extracellular N-terminus was least conserved between human and mouse PAR-2, with only 65% identity. However, the eight residues surrounding the putative trypsin cleavage site (SKGR↓SLIG) were conserved, suggesting that this region is important for cleavage site recognition and receptor activation. Although the two residues

immediately C-terminal to the putative trypsin cleavage sequence SKGR↓SLIG were different (KV in human and RL in mouse PAR-2), these are conservative substitutions. Another area of high sequence similarity between human and mouse PAR-2 was immediately N-terminal to transmembrane domain I, corresponding to a region of the thrombin receptor that is important for ligand binding [15].

Expression of PAR-2 in tissues and cell lines

Northern blots of normal human tissues and cell lines showed a single transcript of approx. 3.0 kb (Figures 2 and 3). A549 cells

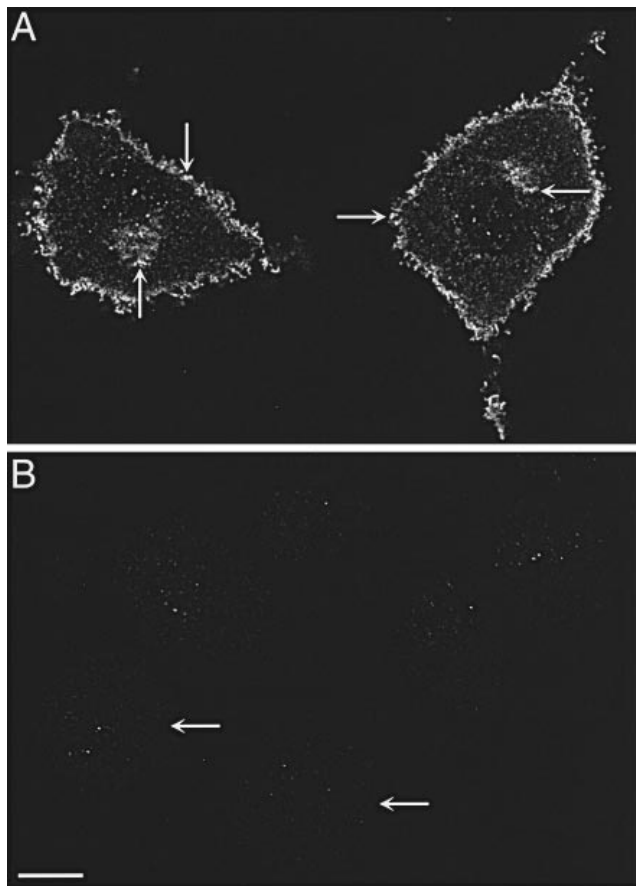


Figure 5 Confocal photomicrographs of KNRK-PAR-2 cells (A) and untransfected KNRK cells (B) stained with the HA.11 antibody to the C-terminal 12CA5 epitope of PAR-2

Scale bar = 5 μ m.

had an additional transcript of approx. 2.2 kb. Human PAR-2 was expressed in pancreas, kidney (+ + +), colon, small intestine, liver (+ +), prostate, heart, lung and trachea (+) (Figures 2A–2C) (explanation of symbols: hybridization signals were raked and grouped in four categories, with + + + + representing the strongest, and + the weakest, symbol). Message was undetectable in spleen, thymus, testis, ovary, leucocytes, brain, placenta, skeletal muscle, aorta, salivary gland and stomach. In mouse tissues PAR-2 mRNA was detected in stomach in addition to small intestine and kidney [4]. Human PAR-2 was expressed by the tumour cell lines A549 (lung adenocarcinoma) (+ + + +), Caco 2, SW480 (colon adenocarcinoma) (+ + +), COLO 205 (colon adenocarcinoma), DU 145, PC-3 (prostatic carcinoma), MOLT-4 (lymphoblastic leukaemia) (+ +) and PANC-1 (pancreatic duct cell carcinoma) (+) (Figures 3A and 3B). Message was undetectable in HL-60 (promyelocytic leukaemia), HeLa S3 (epithelioid carcinoma), K562 (chronic myelogenous leukaemia), Raji (Burkitt lymphoma), G361 (melanoma) and MIA-PaCa (pancreatic duct cell carcinoma). PAR-2 transcripts were detected in KNRK (+ +) and AR4-2J (rat pancreatic acinar carcinoma) (results not shown; +) cells.

Of all the tissues that express PAR-2, only the intestine is normally exposed to pancreatic trypsin. To determine whether PAR-2 is expressed by surface epithelial cells, where it might be activated by pancreatic trypsin during digestion, we used *in situ*

hybridization. Mouse intestine was used because human tissue was not readily available. The antisense PAR-2 probe hybridized strongly to surface epithelial cells of the entire small intestine (Figures 4A, 4C and 4D), as well as the colon and stomach. The sense probe did not hybridize, confirming specificity (Figure 4B). The signal was strongest in epithelial cells lining the upper two-thirds of the intestinal villi, and was weaker in the crypt regions. This localization was distinct from that of the thrombin receptor, which was expressed by endothelial cells in the lamina propria and in the submucosa (Figures 4E and 4F), as expected from previous studies [16].

Thus PAR-2 is preferentially expressed by organs of epithelial origin, and particularly those with an extensive brush border such as the kidney and small intestine. This is in contrast with the thrombin receptor, which is expressed by endothelial cells and platelets [17]. The high expression of PAR-2 in gastrointestinal tissues that secrete trypsin (pancreas) or are exposed to trypsin (intestine) suggests the possibility that this receptor participates in the reflex inhibition of pancreatic enzyme secretion by luminal trypsin, perhaps by regulating release of cholecystokinin [18]. However, the demonstration that PAR-2 is located at the apical membrane of intestinal epithelial cells is required to confirm that PAR-2 can be activated by trypsin in the intestinal lumen. Elsewhere, potential roles of PAR-2 are less obvious. However, trypsin-like enzymes and pancreatic secretory trypsin inhibitors are present in non-pancreatic tissues, tumours and tumour cell lines, where PAR-2 may regulate growth [19–22].

Expression of functional human PAR-2 in KNRK cells

Expression of PAR-2 in KNRK cells was confirmed by immunofluorescence by using an antibody to the 12CA5 epitope at the C-terminus (Figure 5A). Non-transfected cells were unstained (Figure 5B). In KNRK-PAR-2 cells, PAR-2 was detected at the plasma membrane and in the Golgi apparatus, where it colocalized with the resident Golgi protein mannosidase II (Figures 6A–6C). This distribution resembles that of the thrombin receptor [23] but differs from that of the substance P or neurokinin 1 receptor, where there are no large intracellular stores [8]. Although both receptors are endocytosed after activation, substance P receptors efficiently recycle and recycling is required for resensitization [8], whereas thrombin receptors are mostly degraded and resensitization requires trafficking of preformed receptors from intracellular stores to the plasma membrane [23,24].

Trypsin and AP, but not inactivated trypsin or RP, stimulated a transient increase in $[Ca^{2+}]_i$ in KNRK-PAR-2 cells, although trypsin was 7800-fold more potent than AP (EC_{50} for trypsin, 2.3 nM; EC_{50} for AP, 18 μ M) (Figures 7A and 8A). Similarly, thrombin and trypsin are more potent activators of the thrombin receptor and mouse PAR-2 respectively than activating peptides corresponding to the new N-terminus [2,4,5]. Presumably the tethered ligand is maintained at a high concentration and is favourably presented to the binding sites by its physical attachment to the receptor, which it can activate with high affinity. The Ca^{2+} responses to trypsin and AP were unaffected when cells were assayed in Ca^{2+} -free medium, indicating that Ca^{2+} derives from intracellular pools (results not shown). The ability of AP to activate PAR-2 suggests that trypsin cleaves human PAR-2 at SKGR↓SLIG, as it does for mouse PAR-2, which is also coupled to mobilization of intracellular Ca^{2+} [2,4,5]. High concentrations of trypsin (over 100 nM) and AP (over 100 μ M) induced a small Ca^{2+} response in non-transfected KNRK cells, suggesting a low level of PAR-2 expression.

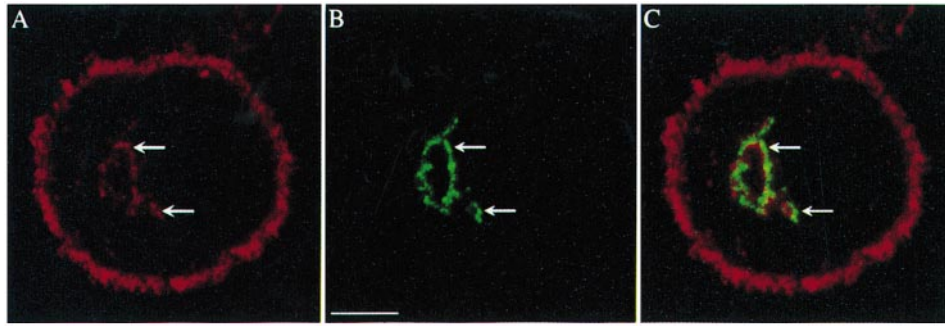


Figure 6 Confocal photomicrographs of KNRK-PAR-2 cells simultaneously stained with the HA.11 antibody to the C-terminal 12CA5 epitope of PAR-2 (A), and the Golgi apparatus marker mannosidase II (B); C is a superimposition of A and B

Images are z projections of three serial sections at 0.54 μm intervals. Scale bar = 5 μm .

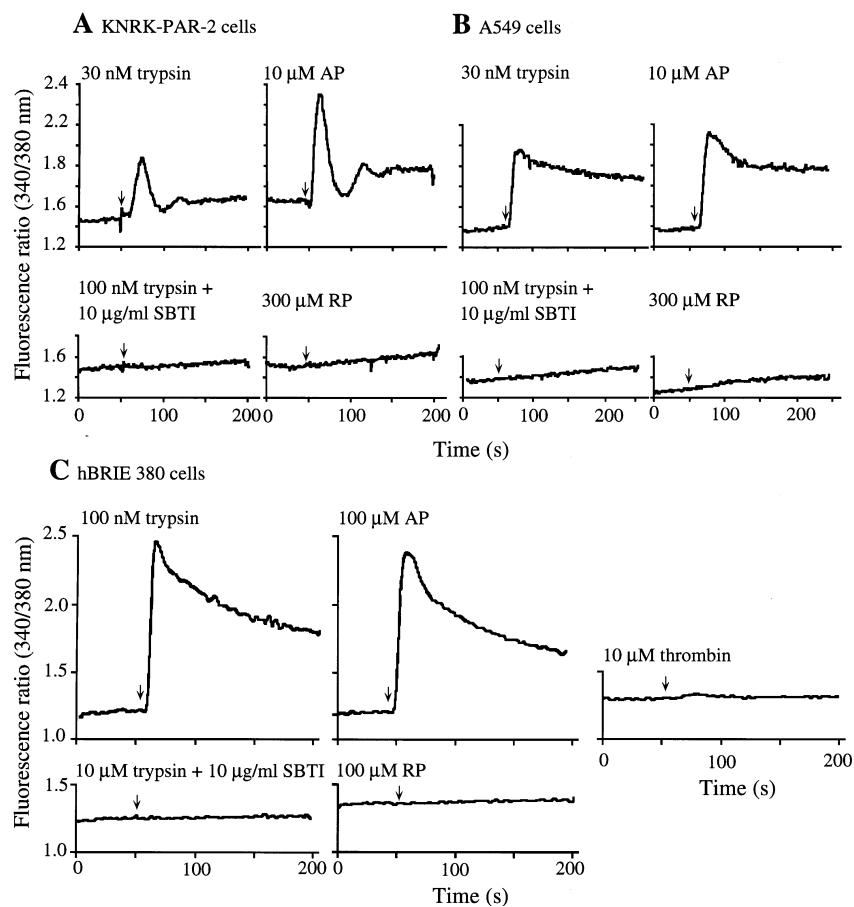


Figure 7 Ca^{2+} responses to trypsin, trypsin inhibited by incubation with SBTI, AP and RP in KNRK-PAR-2 cells (A), A549 cells (B) and hBRIE 380 cells (C)

KNRK-PAR-2 cells and A549 cells were treated with human AP and hBRIE 380 cells were treated with mouse AP.

Expression of functional PAR-2 in tumour cells and intestinal epithelial cells

The A549 cell line is derived from a lung adenocarcinoma and expresses PAR-2 mRNA highly. To determine whether PAR-2 is functional in these cells, we measured trypsin- and AP-induced Ca^{2+} mobilization. Trypsin and AP, but not inactivated trypsin or RP, induced a rapid increase followed by a sustained plateau

in $[\text{Ca}^{2+}]_i$ (EC_{50} for trypsin, 5.2 nM; EC_{50} for AP, 4.6 μM), confirming expression of functional PAR-2 in A549 cells (Figures 7B and 8B). The rapid increase in $[\text{Ca}^{2+}]_i$ was maintained when cells were assayed in Ca^{2+} -free medium, suggesting that Ca^{2+} was from intracellular stores (results not shown). However, the plateau was less sustained when Ca^{2+} was omitted from the assay medium, indicating that an influx of extracellular Ca^{2+} also contributes to the plateau.

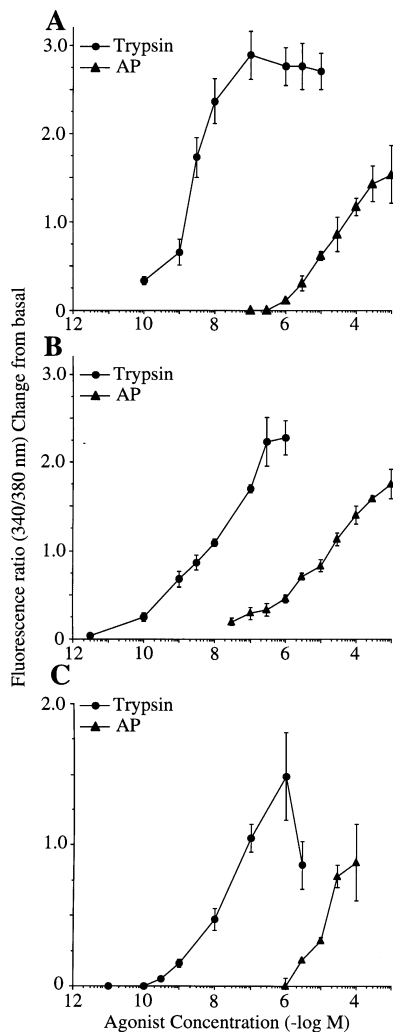


Figure 8 Dose–response curves for Ca²⁺ mobilization in response to trypsin and AP in KNRK-PAR-2 cells (A), A549 cells (B) and hBRIE 380 cells (C)

KNRK-PAR-2 cells and A549 cells were treated with human AP and hBRIE 380 cells were treated with mouse AP. Means \pm S.E.M., $n = 3$ observations.

We found that PAR-2 mRNA is highly expressed in the small intestine, where it is present in surface epithelial cells. To determine whether PAR-2 might be activated by pancreatic trypsin, we measured trypsin- and AP-induced Ca²⁺ mobilization in an intestinal epithelial cell line, hBRIE 380 [25,26]. This cell line was developed by fusing freshly isolated epithelial cells from the rat small intestine with a spontaneously transformed small-intestinal cell line (BRIE 291). The advantage of the hBRIE 380 cell line is that it retains the characteristics of intestinal epithelial cells more effectively than the transformed tumorigenic parental cell. Cells are cuboidal with microvilli, contain villin, are contact inhibited, anchorage-dependent, and require serum. hBRIE 380 cells were obtained from Dr. G. Aponte, University of California, Berkeley, and were grown on Matrigel to promote differentiation. Trypsin and AP, but not inactivated trypsin or RP, induced a sustained increase in [Ca²⁺]_i (EC₅₀ for trypsin, 25.3 nM; EC₅₀ for AP, 17.0 μ M), confirming PAR-2 expression in intestinal epithelial cells (Figures 7C and 8C).

Table 1 Effects human AP or RP on colony formation of A549 cells

Results are expressed as the numbers of colonies per 35 mm plate after 6 d in culture as a percentage of the untreated control. In the untreated control experiments, the number of colonies per 35 mm plate after 6 d was 97.2 ± 26.3 . Means \pm S.E.M., six observations per experiment, $n = 4$ experiments. * $P < 0.05$ compared with 100 μ M RP by ANOVA and Student's *t*-test.

Treatment	Colonies per plate (% of control)
Control	100
0.1 μ M AP	92.5 \pm 1.1
1 μ M AP	76.1 \pm 6.2
10 μ M AP	67.6 \pm 8.8
100 μ M AP	55.5 \pm 13.7*
100 μ M RP	94.6 \pm 3.9

The observation that trypsin and AP stimulate Ca²⁺ mobilization by A549 cells and hBRIE 380 cells suggests that these cells express functional PAR-2 and that PAR-2 activation is coupled to phospholipase C. Indeed, trypsin and AP stimulated an increase in [Ca²⁺]_i with a similar EC₅₀ in KNRK-PAR-2 cells, A549 cells and hBRIE 380 cells. However, we cannot at present exclude the possibility that trypsin and AP also activate receptors other than PAR-2. This must await the development of specific agonists or antagonists of PAR-2.

Effects of PAR-2 activation on colony formation by A549 cells

The extremely high expression of functional PAR-2 by the rapidly growing tumour cell line A549 prompted us to examine whether activation of PAR-2 affected growth of these cells. This cell line has been used to examine the effects of growth factors such as gastrin releasing peptide, which is an autocrine growth stimulant in these cells [27,28]. We studied clonal growth because this is more sensitive than assays using mass culture, which rely on cell counts and incorporation of thymidine to measure growth. The colony-forming assay uses low numbers of widely dispersed cells and thus avoids the accumulation of large amounts of autocrine growth factors (such as gastrin releasing peptide) in the culture medium, which makes it very difficult to examine the effects of exogenously added growth factors [27,28]. Thus, in clonal assays, factors secreted by one cell are unlikely to influence others because of the low cell density. The colony-forming assay was used to demonstrate the role of gastrin releasing peptide as an autocrine growth factor in A549 cells [28].

Incubation of A549 cells with AP, but not RP, for 6 d inhibited colony formation by almost 50% (Table 1). This suggests that PAR-2 activation inhibits colony formation. Although colony formation is a widely used and very sensitive assay, the formation of colonies depends on cell division, cell survival and cell–cell interactions. Thus additional experiments will be required to establish the exact mechanism by which PAR-2 prevents colony formation by A549 cells. However, our results are supported by observations that trypsin inhibitors stimulate growth. For example, feeding the trypsin inhibitor camostate stimulates pancreatic growth, possibly by inhibition of luminal trypsin and consequent release of cholecystokinin [29]. Pancreatic secretory trypsin inhibitor directly stimulates growth of the pancreas-derived AR4-2J cell line [30]. Our results suggest that trypsin-like enzymes secreted from tumour cells could also directly regulate growth in an autocrine manner by interacting with PAR-2.

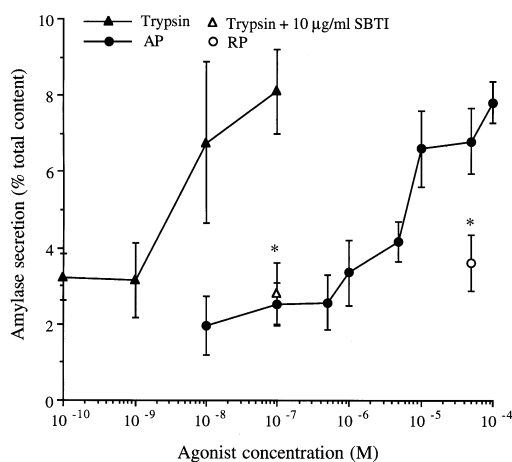


Figure 9 Effects of trypsin, trypsin inhibited by incubation with SBTI, mouse AP and human RP on amylase secretion from rat pancreatic acini

Means \pm S.E.M. for duplicate observations, $n = 5-9$ experiments. * $P < 0.05$ compared with controls, Student's t -test.

Effects of PAR-2 activation on amylase secretion by pancreatic acini

Because PAR-2 is expressed in high levels by the pancreas and the pancreas produces trypsin, we examined the effects of trypsin and AP on amylase secretion from isolated pancreatic acini. Basal amylase secretion ($2.94 \pm 0.57\%$ of total content per 30 min, mean \pm S.E.M., duplicate observations, $n = 11$ experiments) was stimulated by trypsin and mouse AP, but not by inactivated trypsin or RP (Figure 9). Preincubation of acini for 15 min with $50 \mu\text{M}$ AP, but not 100 nM trypsin, had a small additive effect on cholecystokinin-stimulated amylase secretion (results not shown). Thus PAR-2 may contribute to the regulation of pancreatic exocrine secretion. Although pancreatic acini are not usually exposed to active trypsin, activated trypsin is formed in the pancreas in pancreatitis, where it may activate PAR-2.

In conclusion, human and mouse PAR-2 are structurally similar, especially in the putative trypsin-cleavage domain. Human PAR-2 mRNA is highly expressed in the kidney, pancreas, intestine and A549 lung adenocarcinoma cells. In the intestine, PAR-2 is expressed by surface epithelial cells where it could be activated by luminal trypsin and mediate some of the physiological effects of trypsin in the small intestine. Activation of PAR-2 in transfected cell lines, A549 cells and hBRIE 380 cells by trypsin and AP mobilizes intracellular Ca^{2+} . Activation of PAR-2 inhibits colony formation by A549 cells and stimulates amylase secretion from pancreatic acini. Elucidation of the physiological role of PAR-2 will require development of specific agonists and antagonists.

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