Biochemical and Biophysical Research Communications **275,** 77–83 (2000)
doi:10.1006/bbrc.2000.3267, available online at http://www.idealibrary.com on IDEAL®

Extrapancreatic Trypsin-2 Cleaves Proteinase-Activated Receptor-2

Anna-Karin Alm,* Rebecca Gagnemo-Persson,* Timo Sorsa,† and Johan Sundelin*
**Division of Molecular Neurobiology, Department of Physiological Sciences, Lund University, Lund, Sweden; and †Faculty of Medicine, University of Helsinki, Helsinki, Finland*

Received July 17, 2000

Proteinase-activated receptors (PARs) are activated by proteolytic removal of a short amino terminal peptide, thus exposing a new amino terminus that functions as a tethered ligand that activates the receptor. With the aim to identify and study potential activators of PAR-2 we have developed a new method to measure proteolytic cleavage of PARs. PAR-2 was tagged with the insulin C-peptide that upon receptor cleavage is released and quantified using an ELISA. The modified receptor, shown to be functional in mouse 3T3 cells, was expressed in an insect cell line and the ability of different proteinases to cleave PAR-2 was studied. Two different mast cell tryptases cleaved PAR-2 in a concentration dependent manner, but were much less potent than pancreatic trypsin and trypsin-2 isolated from a carcinoma cell line. Pancreatic trypsin and trypsin-2 were almost equally effective at cleaving PAR-2 suggesting that extrapancreatic trypsins are potential *in vivo* activators of PAR-2. © 2000 Academic Press

Key Words: **proteinase-activated receptor-2; mast cell tryptase; trypsin-2.**

Serine proteinases are involved in a wide range of physiological processes. Recently, it has been shown that one of the many properties of proteinases is the ability to activate cell surface receptors. The most well studied proteinase in this regard is the serine proteinase thrombin that besides its classic role in coagulation also has the ability to activate blood platelets and to modulate the action of endothelial and smooth muscle cells (1). Most, if not all, of the effects are mediated via proteolytically activated G-protein-coupled receptors (PARs) on the cell surface. These receptors are activated by enzymatic cleavage of the extracellular portion of the receptor exposing a new amino terminus. The newly exposed amino terminus functions as a tethered peptide ligand, which by binding to a yet unknown region in the receptor itself causes its activation. So far four different PARs have been identified. PAR-1 as well as PAR-3 and PAR-4 are activated by thrombin (2–4). PAR-2, however, does not respond to thrombin (5, 6). The physiologic activator of PAR-2 has not yet been defined, although pancreatic trypsin as well as mast cell tryptase have been shown to activate the receptor *in vitro* (7).

PAR-2 is widely expressed particularly in cells of epithelial linings such as skin keratinocytes, the endothelial and smooth muscle cells of the blood vessel wall, and the gastric and intestinal mucosa (8). Thus, pancreatic trypsin seems unlikely to be the only physiologic activator of PAR-2. There are, however, at least four human trypsinogen genes (9–11), and production of extra-pancreatic trypsin has been observed in both vascular endothelial cells (12) and normal epithelia as well as in several tumor cell lines (13, 14). Mast cells are scattered throughout the tissues where PAR-2 is found and they release the serine proteinase tryptase together with other pro-inflammatory agents as part of an inflammatory reaction. Since the expression of PAR-2 in endothelial cells is up regulated as a response to inflammatory mediators such as tumour necrosis factor-α and interleukin-α (15), tryptase appears to be a likely PAR-2 activator. In several systems the efficacy and potency of tryptase is however low, when compared to trypsin (7, 16), and it has been suggested that tryptase may act at a subpopulation of PAR-2, or at a novel receptor (7, 16).

The search for PAR activators has been hampered by the absence of a suitable assay system. The identification of physiological activators of PAR-2 is complicated by the existence of at least three related PARs that also signals through the IP_3/Ca^{2+} pathway. This prompted us to establish a technique to measure PAR activation independent of intracellular signalling. We used PAR-2 as a model PAR and inserted a sequence corresponding to the human insulin C-peptide into the amino terminal part of the receptor. Upon activation by receptor cleavage, the C-peptide is released and may be quantified using a sensitive ELISA. We have stud-

Abbreviations used: PAR, proteinase-activated receptor.

0006-291X/00 $35.00

ied cleavage of PAR-2 by two different human tryptases isolated from lung (17) or skin (18) and by a human extrapancreatic trypsin-2 isolated from COLO-205, a colon carcinoma cell line (19). We found that the two human tryptases were virtually inactive whereas human trypsin-2 and bovine pancreatic trypsin were almost equally effective at cleaving PAR-2.

MATERIALS AND METHODS

Materials. Cell culture media were purchased from Life Technologies Inc. and all other reagents were from Sigma unless otherwise specified. DAKO C-peptide, an enzyme immunoassay for the quantitative measurement of C-peptide in human clinical samples, was from DAKO and the chromogenic substrate S2765 was from Chromogenix AB, Mölndal, Sweden. Trypsin (bovine pancreatic type III, 10000-13000 BAEE units/mg protein) was purchased from Sigma, and α-Thrombin, (3800 NIH units/mg of protein) was from Haematologic Technologies Inc. (River Road Essex Junction, VT). Tryptase isolated from human lung (17) and tryptase isolated from human skin (18) were kind gifts from Dr. Lawrence B. Schwartz and Dr. Ilkka T. Harvima, respectively. Trypsinogen-2 was purified from serum-free conditioned media from COLO 205 colon carcinoma cells and activated with enteropeptidase as described previously (19, 14). Tumour associated trypsin inhibitor (TATI) was purified from the urine of cancer or pancreatitis patients as described elsewhere (20).

Determination of enzyme activity. Enzymatic activities were determined using the chromogenic substrate S2765, *Z*-D-Arg-Gly-Arg-pNA · 2HCl. Assays were carried out at room temperature in 0.05 M Hepes, 0.12 M NaCl, pH 7.6 (21) with 0.2 mM S2765 and the hydrolysis reaction was recorded at 405 nm using an Ultrospec 3000 spectrophotometer from Pharmacia Biotech Ltd. Specific activity is defined as U/μmol enzyme where 1 unit is defined as the amount of enzyme that hydrolyses 1 μmol of substrate/minute under the above conditions.

Construction of the PAR-2/insulin C-peptide reporter. Construction of the PAR-2/insulin C-peptide reporter (PAR-2/C-pep) was done in three steps. First, PCR mediated mutagenesis was used to remove an endogenous *Pst*I site in the region corresponding to the signal sequence of mouse PAR-2, and to create a new *Pst*I site further downstream in a region encoding the mature protein. Briefly, with PAR-2 cDNA cloned in the *Bam*HI site of the eukaryotic expression vector pcDNA3 from Invitrogen (mPAR-2/pcDNA3) as template, two mutagenesis primers L184 (5′-TAGCCGGACCCTGCAGGAGAA-CCTTGCACCG-3′) and L185 (5′-CTGCAGGGTCCGGCTACAGG-AGACCGAGGC-3′), (mutant positions are underlined) were used in separate PCRs. The primer T7 from pcDNA3 was used with L185 in one reaction, and L184 with a PAR-2 specific oligonucleotide (L54, 5′-GGGGGAACCAGATGACA-3′) in the second reaction. The obtained fragments were purified and used as templates in a subsequent PCR with the primers T7 and L54. After purification and digestion with *Bam*HI and *Cla*I, the amplified fragment was subcloned in the plasmid cloning vector pBluescriptSKII from Stratagene. A clone with the appropriate mutation (PAR-2/Pst mutant) was identified by sequence analysis.

A fragment encoding the insulin C-peptide (residues 1–27) was generated by partial *Pst*I cleavage of a 300 bp *Eco*RI-*Rsa*I fragment from a human insulin full length clone kindly provided by Dr. Helena Edlund, Umeå University, Sweden. The 75 bp *Pst*I fragment was purified following electrophoresis in a 3% metaphor (FMC Bioproducts, Rockland, ME) agarose gel, and then ligated to *Pst*I digested PAR-2/Pst mutant. One clone harbouring the insulin C-peptide fragment in the correct orientation was identified by sequence analysis. Finally, the *Bam*HI-*Cla*I fragment of this clone, which encoded the 5′-part of PAR-2 with the inserted C-peptide fragment, was excised and used to replace the wild-type *Bam*HI-*Cla*I fragment of mPAR-2/pcDNA3.

For expression in High Five insect cells, PAR-2/C-pep was excised from pcDNA3 using *Bam*HI and *Not*I and ligated to a *Bgl*II-*Not*I digested pIE1-3 vector (Novagen).

Cell culture. 3T3 Clone A31 Balb/c mouse embryo fibroblast cells, No. 86110401, were obtained from the European Collection of Animal Cell Cultures (ECACC), Salisbury, Wiltshire, England, and cultured in DMEM with 10% fetal bovine serum, penicillin (50 U/ml) and streptomycin (50 μg/ml) at 37°C with 5% CO_2. *Trichoplusia ni* derived High Five cells from Invitrogen were cultured at 27°C in ExCell-401 medium (JRH Biosciences, Lenexa, USA) supplemented with 10% fetal bovine serum for insect cell culture (Life Technologies Inc.), penicillin (50 U/ml) and streptomycin (50 μg/ml).

The construct DNA was introduced into 3T3 cells by the calcium-phosphate precipitation method and stable cell lines expressing PAR-2/C-pep were established using G418 selection (400 μg/ml). High Five cells were transiently transfected with the expression construct using Lipofectin (Life Technologies Inc.) and used in assays 48 hours after transfection.

Intracellular Ca^{2+} measurements. 3T3 cells expressing PAR-2/C-pep were seeded onto poly-D-lysine coated glass cover slips and grown for 3–5 days until confluent. Cells were loaded with 2 mM fura 2-acetoxymethylester in culture medium for 30 minutes at 37°C in the presence of 2.5 mM probenecid. Cover slips were mounted in a chamber on the stage of a Nikon inverted microscope fitted to a digital camera. After three washes with extracellular medium buffer (ECB; 135 mM NaCl, 4.6 mM KCl, 1.2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 11 mM glucose, 11 mM HEPES, pH 7.4) at 37°C, emitted fluorescence at 510 nm was measured with alternate excitation at 340 and 380 nm. Measurements were made in a final volume of 500 μl ECB, and 50 μl of selected agonists (diluted in the same buffer), were added using a micropipette. Data were analyzed using Image Master software (Photon Technology International).

Enzyme assay using whole cells. Cells, seeded in 96 well culture plates in sufficient numbers to create a confluent cell layer, were allowed to attach to the plastic over night. Cells were washed once in ECB and then placed on ice. Proteinases were diluted in ice-cold ECB and added to cells in a final volume of 30 μl. The cleavage reaction, performed at 37°C for 5 minutes was terminated with phenylmethylsulfonyl fluoride (PMSF) at a final concentration of 0.5 mM. The resulting solution was transferred to microcentrifuge tubes and centrifuged at 16000*g* and 4°C to remove potential cell debris. The concentration of free C-peptide was determined using DAKO C-peptide enzyme immunoassay according to the manufacturer's recommendations.

Cell membrane preparation. High Five cells, expressing PAR-2/C-pep, were washed with PBS. The cells grow loosely attached to the plastic and they could be gently flushed off and suspended in ice-cold 50 mM Tris–HCl, pH 7.4. All the following steps were performed on ice. The cell suspension was homogenised by hand using a Potter pestle and then centrifuged, at 20000*g* and 4°C for 15 minutes. The resulting pellet was washed once with 50 mM Tris–HCl, pH 7.4, and then resuspended in the same buffer. The protein concentration was determined using BCA Protein Assay Reagent Kit (Pierce, Rockford, USA) and then aliquoted into microcentrifuge tubes before the final centrifugation, at 16000*g* and 4°C for 15 minutes. The resulting pellet was resuspended in 100 μl 50 mM Tris–HCl, pH 7.4, with 0.32 M sucrose and frozen at −80°C.

Enzyme assay using isolated cell membranes. The membrane preparation was thawed on ice, washed once with ice-cold 50 mM Tris–HCl, pH 7.4, diluted in the same buffer to a final concentration of 4 μg protein/μl and homogenized with a micro pestle. The proteinase assay was carried out in microcentrifuge tubes in which the membrane protein solution and the proteinase solution were mixed on ice to a final volume of 40 μl. The cleavage reaction was performed

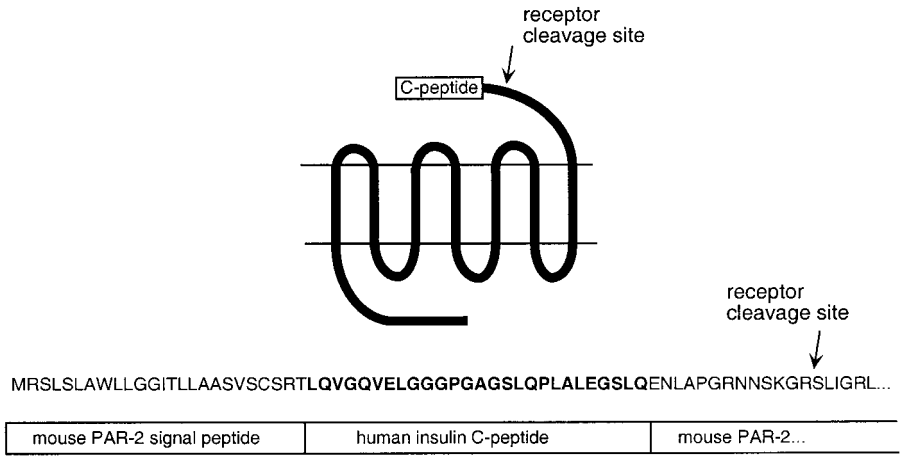

FIG. 1. A fragment encoding part of the human insulin C-peptide (residues 1–27, shown in bold type) was ligated to the extracellular amino terminal part of the mouse PAR-2. Proteolytic cleavage of the receptor at the activation site (indicated by the arrow) liberates the C-peptide that can be quantified.

at 37°C for 5 minutes and terminated using PMSF at a final concentration of 0.5 mM. The tubes were centrifuged at 16000*g* and 4°C for 5 minutes and the C-peptide content of the supernatant was determined using a DAKO C-peptide enzyme immunoassay. In all experiments presented here we used 80 μg protein per sample from the same cell membrane preparation. The proteinases were dissolved in 50 mM Tris–HCl, pH 7.4. In addition, the assay buffer for tryptase contained heparin to a final concentration of 0.05 mg/ml.

RESULTS AND DISCUSSION

Despite the current great interest in PARs, it is still unclear what is, or are, the endogenous activator(s) of PAR-2. The identification of activators of PAR-2 has been complicated by the existence of related PARs that utilise similar signal transduction pathways (22). To circumvent this difficulty we established an assay that is independent of intracellular signalling, and takes advantage of the activation mechanism of PARs, i.e., the proteolytic removal of a peptide.

We used the murine PAR-2 (23) and inserted 27 amino acids corresponding to the human insulin C-peptide (24) in the amino terminal part of the receptor (PAR-2/C-pep) as shown in Fig. 1. The choice of insulin C-peptide as receptor tag was based on its limited size, which reduces the risk for perturbing receptor function, its stability, and the availability of commercial assays for quantification. The construct cDNA encoding PAR-2/C-pep, was transfected into murine 3T3 cells in a vector carrying a resistance to geneticin (G418) and stable clonal cell lines were isolated.

Characterisation of the Recombinant PAR-2/C-pep

In order to verify that the inserted C-peptide did not interfere with receptor function we studied Ca^{2+} mobilization in PAR-2/C-pep expressing 3T3 cells. Cells were stimulated with 20 μM of the mouse PAR-2 agonist peptide SLIGRL (Fig. 2A) and with bovine pancreatic trypsin, 10 nM (Fig. 2B). Both the agonist peptide and trypsin caused an increased intracellular calcium concentration. The responses are similar to results obtained in previous experiments performed with native PAR-2 (25). The same cells were also stimulated with 20 μM of the PAR-1 agonist peptide SFLLRNP as a control for an endogenous receptor. As expected, SFLLRNP caused an increased intracellular calcium concentration, despite previous PAR-2 activation (Fig. 2A). Preincubation of the trypsin solution with a specific trypsin inhibitor, tumor associated trypsin inhibitor (TATI), in 4 fold molar excess for ten minutes, completely abolished the intracellular calcium release (data not shown).

To directly study cleavage of the PAR-2/C-pep, we examined 3T3 cells expressing the recombinant receptor. Cells were grown in 96 well culture plates until confluent and the cells were stimulated with trypsin to induce cleavage of PAR-2/C-pep. The content of free C-peptide was measured using an ELISA specific for human insulin C-peptide. Detectable, but low, amounts of C-peptide were indeed released by trypsin in a dose-dependent manner (data not shown). We speculated that the 3T3 cells expressed too few recombinant receptors on the cell surface to be used in the 96-well format. To increase the expression level, the recombinant receptor was transiently expressed in the insect cell line High Five derived from the cabbage looper, *Trichoplusia ni*. These cells are known to be able to express high levels of recombinant protein.

Assay of Receptor Cleavage Using PAR-2/C-pep

High Five cells expressing PAR-2/C-pep were stimulated with various concentrations of trypsin as described. The cleavage reaction time, 5 minutes, was chosen to give a representative dose-response curve

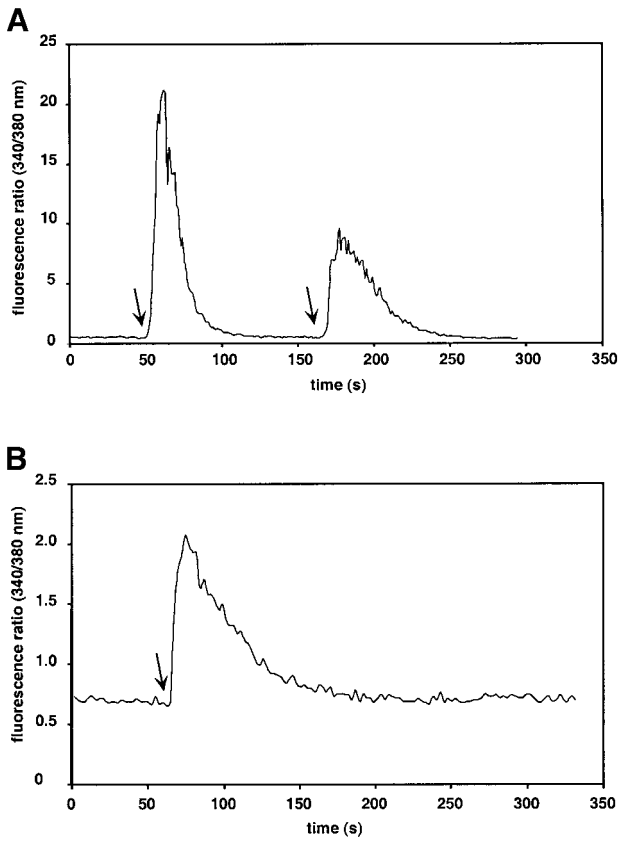

FIG. 2. Agonist peptide and trypsin induced Ca^{2+} mobilization in 3T3 cells expressing PAR-2/C-pep. Cells were loaded with fura 2-acetoxymethylester and emitted fluorescence was measured at 510 nm with alternate excitations at 340 and 380 nm. In (A) a group of 10 cells were stimulated with 20 μM murine PAR-2 agonist peptide SLIGRL (first arrow) followed by 20 μM PAR-1 agonist peptide SFLLRNP (second arrow). In (B) A single cell was stimulated with 10 nM bovine pancreatic trypsin. Representative curves from several experiments are shown.

within the maximum limit of the assay. As shown in Fig. 3, dose-dependent receptor cleavage by pancreatic trypsin was detected down to 1 nM, with an EC_{50} of approximately 50 nM.

Since proteinase activated receptors are for single use only, cells may keep an intracellular pool of spare receptors that are transported to the cell surface when needed (26). To include the intracellular receptor pool in the assay Triton X-100 was added to the extra cellular medium buffer during the cleavage. Detergent addition increased the signal by a factor of two suggesting that in High Five cells only half the receptor pool is exposed on the cell surface (see Fig. 3). The EC_{50}, however, was not affected by the addition of detergent. We attempted to study intracellular Ca^{2+} mobilization in High Five cells as well, but we were not able to detect calcium signals from PAR-2. This might be due to a lack of suitable G-proteins in these insect cells. In order to include the intracellular receptor pool in the assay without addition of detergent and to obviate the need for cell culture and transfection for each assay, we isolated total cell membranes of PAR-2/C-pep expressing High Five cells. In the initial experiments, bovine pancreatic trypsin was used to validate this method. The amount of membrane protein per sample (80 μg) was chosen to give a good detection level in the ELISA. This parameter, however, have to be adjusted for each membrane preparation, because of variable cell transfection efficiency. As seen in Fig. 4, the responses to trypsin were similar to the whole cell approach (EC_{50} approximately 50 nM) (see Fig. 3 for comparison). The serine proteinase α-thrombin, which does not activate PAR-2, was used as control (6). Minor cleavage of PAR-2/C-pep was detected but only at very high concentrations ($\geq$500 nM) of thrombin (Fig. 4).

Previous studies analysing PAR activation independently of intracellular signalling have been based on the cleavage of soluble synthetic peptides of different lengths. Fox and co-workers have studied PAR-2-proteinase interaction using a peptidyl chloromethane inhibitor based on four peptides upstream from the cleavage site in PAR-2 (27). They proposed acrosin, tryptase and trypsin as the most potent activators of PAR-2. Recently, Altrogge and Monard presented a study in which they utilised a peptide representing the extracellular part of different PARs fused to β-galactosidase (28). The bacterially expressed fusion protein was linked via a biotin module to streptavidin-coated paramagnetic beads. The advantage of this approach was the high sensitivity conferred by the introduction of β-galactosidase as reporter of receptor cleavage. However, neither this method nor our assay, based on the recombinant PAR-2/C-pep expressed in High Five cells, can discriminate between receptor-activating and -inactivating proteinases. It is possible

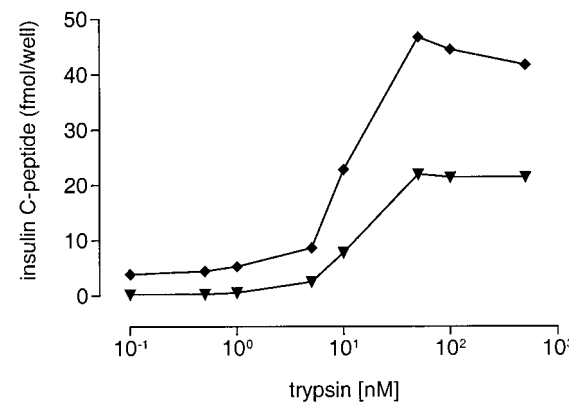

FIG. 3. Cleavage of PAR-2/C-pep expressed in High Five cells by pancreatic trypsin. Cells grown in 96-well plates were treated with pancreatic trypsin at the indicated concentrations. After incubation at 37°C for 5 minutes, soluble C-peptide was quantified using a C-peptide ELISA. (▼) denotes cleavage of PAR-2/C-pep in intact cells (◆) denotes cleavage of PAR-2/C-pep in cells permeabilized with 0.1% Triton X-100. The curves display the result from single determinations representative for several experiments.

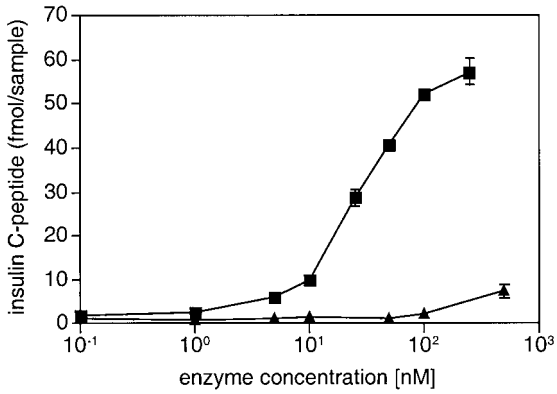

FIG. 4. C-peptide release by pancreatic trypsin and α-thrombin in isolated membranes from High Five cells expressing PAR-2/C-pep. A total membrane preparation was made from High Five cells expressing PAR-2/C-pep. The membrane protein solution and the proteinase solution were mixed in microcentrifuge tubes and the cleavage reaction was performed at 37°C for 5 minutes. The tubes were centrifuged and the C-peptide content of the supernatant was determined using a C-peptide ELISA. The experiment was performed in triplicates and the data are the mean ± S.D. (■) pancreatic trypsin and (▲) α-thrombin.

though that mammalian cells expressing PAR-2/C-pep may be employed for this purpose in analogy with the studies by Ishii and co-workers on PAR-1 cleavage in relation to phosphoinositide hydrolysis (29). In addition, the PAR-2/C-pep construct could be used in quantitative cell biological experiments to address questions regarding for example receptor trafficking, and possibly also to analyse PAR-2 activation *in vivo* by using transgenic mice.

Analysis of Receptor Cleavage by Trypsin and Tryptase

Our intention was to use the PAR-2/C-pep construct to compare different proteinases that have been proposed as activators of PAR-2 and to identify novel activators. Mast cell tryptase and pancreatic trypsin are the most well studied proteinases in this regard but there are other candidate PAR-2 activators. Trypsinogen expression and trypsin release has been reported in several types of human cancer cells. Trypsinogen expression has also been seen in various healthy epithelial tissues such as in the gastrointestinal tract, kidney, liver, endothelial cells as well as in leukocytes and neuronal cells (12, 14), which are tissues also known to express PAR-2. We studied cleavage of PAR-2/C-pep by two different human mast cell tryptases isolated from skin or lung, bovine pancreatic trypsin and extra-pancreatic trypsin-2, isolated from the human colon carcinoma cell line COLO-205.

In order to compare receptor cleavage by the different proteinases, the enzymatic activities were determined using the chromogenic substrate S-2765, *Z*-D-Arg-Gly-Arg-pNA · 2HCl. The C-terminal positions, P2 and P1 (Gly-Arg) of the substrate are identical to the sequence of PAR-2 just before the extracellular cleavage site. The commercial bovine pancreatic trypsin possessed the highest enzymatic activity, 3600 ± 290

TABLE 1

Enzymatic Activity of the Proteinases

Proteinase	Enzymatic activity (U/μmol)[a]	Species
Pancreatic trypsin	3600 ± 290	Bovine
Trypsin-2 (COLO-205)	470 ± 20	Human
Tryptase, skin	400 ± 10	Human
Tryptase, lung	740 ± 40	Human

[a] The experiments were repeated at least three times and two different concentrations of proteinase were used. The data are shown as the mean value ± S.D.

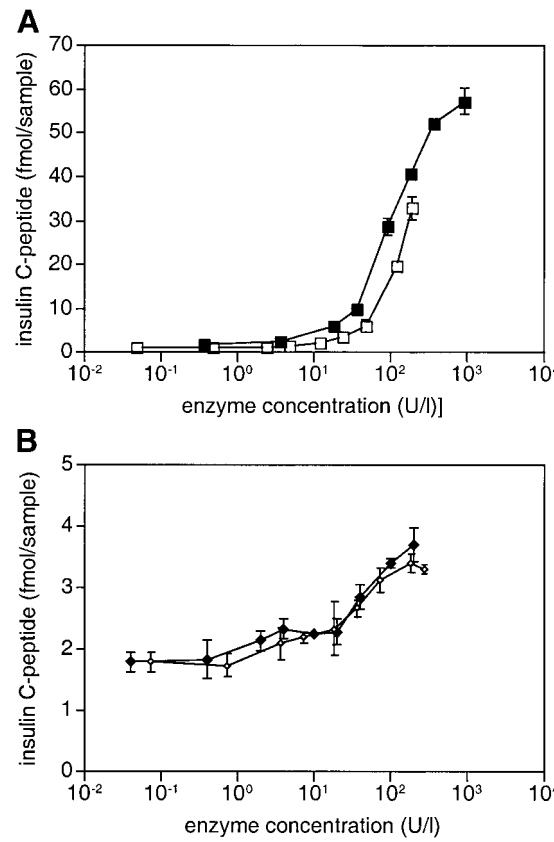

FIG. 5. C-peptide release by different proteinases in isolated membranes from High Five cells expressing PAR-2/C-pep. Total cell membranes were incubated with the different proteinases in microcentrifuge tubes for 5 minutes at 37°C after which the released C-peptide was quantified. For comparison of the different proteinases, the enzymatic activity was determined for each proteinase using the chromogenic substrate S-2765. The proteinase concentrations are displayed as enzymatic activity per litre (U/l). The experiments were performed in triplicates and the data are the mean ± S.D. In (A), (■) bovine pancreatic trypsin, (□) human trypsin-2. In (B), (◇) human lung tryptase, (◆) human skin tryptase.

U/μmol, while the activity of both the lung and skin tryptase as well as trypsin-2 was about 10 times lower (Table 1). As shown in Fig. 5, where the proteinase concentrations are displayed as enzymatic activity per litre (U/l), tryptase from both lung and skin were able to cleave PAR-2/C-pep in a concentration dependent manner, but with low potency (Fig. 5B). At the highest concentration of tryptase used, (lung tryptase; 273 U/l (corresponding to 369 nM)), the number of receptors cleaved was only 10% of the number of receptors cleaved by the same amount (units) of trypsin. Most studies describing PAR-2 activation by mast cell tryptase have been performed on different *in vitro* systems in which the actions of these two enzymes have been compared. In several of the studies the responses to mast cell tryptase have been slower and the efficacy has been lower, compared to pancreatic trypsin (7, 16). This is well in agreement with our findings where both lung and skin tryptase could activate PAR-2 in a concentration dependent manner albeit with a low potency.

Due to a shortage of isolated trypsin-2, a complete dose-response curve could not be produced. Nonetheless, the results from our experiments (Fig. 5A), using the recombinant PAR-2/C-pep expressed in High Five cells, show that trypsin-2 is almost as potent as pancreatic trypsin when cleaving PAR-2/C-pep. The extrapancreatic trypsins thus seem to be potential *in vivo* activators of PAR-2.

ACKNOWLEDGMENTS

We thank Dr. Lawrence B. Schwartz, Department of Medicine, Medical College of Virginia, Richmond, Virginia, and Gunnar Nilsson, Department of Genetics and Pathology, University of Uppsala, Sweden, for giving us the human lung tryptase, Dr. Ilkka Harvima, Department of Dermatology, University of Kuopio, Finland, for providing the human skin tryptase, and Dr. Helena Edlund, Umeå University, Sweden, for the human insulin cDNA. This work was supported by the Swedish Medical Research Council Project B96-13X-09467 and the Medical Faculty, Lund University.

REFERENCES

1. Déry, O., Corvera, C. U., Steinhoff, M., and Bunnett, N. W. (1998) Proteinase-activated receptors: Novel mechanisms of signaling by serine proteases. *Am. J. Physiol.* **274,** C1429–C1452.
2. Vu, T-K. H., Hung, D. T., Wheaton, V. I., and Coughlin, S. R. (1991) Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* **64,** 1057–1068.
3. Ishihara, H., Connolly, A. J., Zeng, D., Kahn, M. L., Zheng, Y. W., Timmons, C., Tram, T., and Coughlin, S. R. (1997) Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature* **386,** 502–506.
4. Xu, W-F., Andersen, H., Whitmore, T. E., Presnell, S. R., Yee, D. P., Ching, A., Gilbert, T., Davie, E. W., and Foster, D. C. (1998) Cloning and characterization of human protease-activated receptor 4. *Proc. Natl. Acad. Sci. USA* **95,** 6642–6646.
5. Nystedt, S., Emilsson, K., Wahlestedt, C., and Sundelin, J. (1994) Molecular cloning of a potential proteinase activated receptor. *Proc. Natl. Acad. Sci. USA* **91,** 9208–9212.
6. Blackhart, B. D., Emilsson, K., Nguyen, D., Teng, W., Martelli, A. J., Nystedt, S., Sundelin, J., and Scarborough, R. M. (1996) Ligand cross-reactivity within the protease-activated receptor family. *J. Biol. Chem.* **271,** 16466–16471.
7. Molino, M., Barnathan, E. S., Numerof, R., Clark, J., Dreyer, M., Cumashi, A., Hoxie, J. A., Schechter, N., Woolkalis, M., and Brass, L. F. (1997) Interactions of mast cell tryptase with thrombin receptors and PAR-2. *J. Biol. Chem.* **272,** 4043–4049.
8. D'Andrea, M. R., Derian, C. K., Leturcq, D., Baker, S. M., Brunmark, A., Ling, P., Darrow, A. L., Santulli, R. J., Brass, L. F., and Andrade-Gordon, P. (1998) Characterization of protease-activated receptor-2 immunoreactivity in normal human tissues. *J. Histochem. Cytochem.* **46,** 157–164.
9. Emi, M., Nakamura, Y., Ogawa, M., Yamamoto, T., Nishide, T., Mori, T., and Matsubara, K. (1986) Cloning, characterization and nucleotide sequences of two cDNAs encoding human pancreatic trypsinogens. *Gene* **41,** 305–310.
10. Tani, T., Kawashima, I., Mita, K., and Takiguchi, Y. (1990) Nucleotide sequence of the human pancreatic trypsinogen III cDNA. *Nucleic Acids Res.* **18,** 1631.
11. Wiegand, U., Corbach, S., Minn, A., Kang, J., and Muller-Hill, B. (1993) Cloning of the cDNA encoding human brain trypsinogen and characterization of its product. *Gene* **136,** 167–175.
12. Koshikawa, N., Nagashima, Y., Miyagi, Y., Mizushima, H., Yanoma, S., Yasumitsu, H., and Miyazaki, K. (1997) Expression of trypsin in vascular endothelial cells. *FEBS Lett.* **409,** 442–448.
13. Koivunen, E., Saksela, O., Itkonen, O., Osman, S., Huhtala, M-L., and Stenman, U-H. (1991) Human colon carcinoma, fibrosarcoma and leukemia cell lines produce tumor-associated trypsinogen. *Int. J. Cancer.* **47,** 592–596.
14. Koshikawa, N., Hasegawa, S., Nagashima, Y., Mitsuhashi, K., Tsubota, Y., Miyata, S., Miyagi, Y., Yasumitsu, H., and Miyazaki, K. (1998) Expression of trypsin by epithelial cells of various tissues, leukocytes, and neurons in human and mouse. *Am. J. Pathol.* **153,** 937–944.
15. Nystedt, S., Ramakrishnan, V., and Sundelin, J. (1996) The proteinase-activated receptor 2 is induced by inflammatory mediators in human endothelial cells. *J. Biol. Chem.* **271,** 14910–14915.
16. Corvera, C. U., Déry, O., McConalogue, K., Böhm, S. K., Khitin, L. M., Caughey, G. H., Payan, D. G., and Bunnett, N. W. (1997) Mast cell tryptase regulates rat colonic myocytes through Proteinase-activated receptor 2. *J. Clin. Invest.* **100,** 1383–1393.
17. Schwartz, L. B., Bradford, T. R., Lee, D. C., and Chlebowski, J. F. (1990) Immunologic and physicochemical evidence for conformational changes occuring on conversion of human mast cell tryptase from active tetramer to inactive monomer. Production of monoclonal antibodies recognizing active tryptase. *J. Immunol.* **144,** 2304–2311.
18. Harvima, T. I., Schechter, N. M., Harvima, R. J., and Fräki, J. E. (1988) Human skin tryptase: purification, partial characterization and comparison with human lung tryptase. *Biochim. Biophys. Acta* **957,** 71–80.
19. Sorsa, T., Salo, T., Koivunen, E., Tyynelä, J., Konttinen, Y. T., Bergmann, U., Tuuttila, A., Niemi, E., Teronen, O., Heikkilá, P., Tschesche, H., Leinonen, J., Osman, S., and Stenman, U-H. (1997) Activation of type IV procollagenases by human tumor-associated trypsin-2. *J. Biol. Chem.* **272,** 21067–21074.
20. Koivunen, E., Ristimáki, A., Itkonen, O., Osman, S., Vuento, M., and Stenman U-H. (1991) Tumor-associated trypsin participates

in cancer cell-mediated degradation of extracellular matrix. *Cancer Res.* **51,** 2107–2112.

21. Schwartz, L. B. (1994) Tryptase: A mast cell serine protease. *Methods Enzymol.* **244,** 88–100.
22. Molino, M., Woolkalis, M. J., Reavey-Cantwell, J., Praticó, D., Andrade-Gordon, P., Barnathan, E. S., and Brass, L. F. (1997) Endothelial cell thrombin receptors and PAR-2; two protease-activated receptors located in a single cellular environment. *J. Biol. Chem.* **272,** 11133–11141.
23. Nystedt, S., Larsson, A-K., Åberg, H., and Sundelin, J. (1995) The mouse proteinase-activated receptor-2 cDNA and gene. *J. Biol. Chem.* **270,** 5950–5955.
24. Bell, G. I., Swain, W. F., Pictet, R., Cordell, B., Goodman, H. M., and Rutter, W. J. (1979) Nucleotide sequence of cDNA clone encoding human preproinsulin. *Nature* **282,** 525–527.
25. Nystedt, S., Emilsson, K., Larsson, A-K., Strömbeck, B., and Sundelin, J. (1995) Molecular cloning and functional expression of the gene encoding the human proteinase-activated receptor-2. *Eur. J. Biochem.* **232,** 84–89.
26. Déry, O., Thoma, M. S., Wong, H., Grady, E. F., and Bunnett, N. W. (1999) Trafficking of Proteinase-activated receptor-2 and β-arrestin-1 tagged with green flourescent protein. β-arrestin-dependent endocytosis of a proteinase receptor. *J. Biol. Chem.* **274,** 18524–18535.
27. Fox, M. T., Harriot, P., Walker, B., and Stone, S. R. (1997) Identification of potential activators of proteinase-activated receptor-2. *FEBS Lett.* **417,** 267–269.
28. Altrogge, L. M., and Monard, D. (2000) An assay for high-sensitivity detection of thrombin activity and determination of proteases activating or inactivating protease-activated receptors. *Anal. Biochem.* **277,** 33–45.
29. Ishii, K., Hein, L., Kobilka, B., and Coughlin, S. R. (1993) Kinetics of thrombin receptor cleavage in intact cells. Relation to signaling. *J. Biol. Chem.* **268,** 9780–9786.