Proteolytic Activation of the Protease-activated Receptor (PAR)-2 by the Glycosylphosphatidylinositol-anchored Serine Protease Testisin*

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**Background:** PAR-2 is a protease-activated, G-protein-coupled receptor involved in inflammation, development, and immunity.

**Results:** The GPI-anchored serine protease testisin activates PAR-2, inducing PAR-2 loss from the cell surface, internalization, and cellular signaling.

**Conclusion:** PAR-2 activation by testisin reveals a unique mode of cell surface PAR-2 regulation.

**Significance:** This is the first report of GPI-anchored protease-mediated cleavage and activation of a PAR.

Protease-activated receptors (PARs) are a family of seven-transmembrane, G-protein-coupled receptors that are activated by multiple serine proteases through specific N-terminal proteolytic cleavage and the unmasking of a tethered ligand. The majority of PAR-activating proteases described to date are soluble proteases that are active during injury, coagulation, and inflammation. Less investigation, however, has focused on the potential for membrane-anchored serine proteases to regulate PAR activation. Testisin is a unique trypsin-like serine protease that is tethered to the extracellular membrane of cells through a glycosylphosphatidylinositol (GPI) anchor. Here, we show that the N-terminal domain of PAR-2 is a substrate for testisin and that proteolytic cleavage of PAR-2 by recombinant testisin activates downstream signaling pathways, including intracellular Ca²⁺ mobilization and ERK1/2 phosphorylation. When testisin and PAR-2 are co-expressed in HeLa cells, GPI-anchored testisin specifically releases the PAR-2 tethered ligand. Conversely, knockdown of endogenous testisin in NCI/ADR-Res ovarian tumor cells reduces PAR-2 N-terminal proteolytic cleavage. The cleavage of PAR-2 by testisin induces activation of the intracellular serum-response element and NFκB signaling pathways and the induction of IL-8 and IL-6 cytokine gene expression. Furthermore, the activation of PAR-2 by testisin results in the loss and internalization of PAR-2 from the cell surface. This study reveals a new biological substrate for testisin and is the first demonstration of the activation of a PAR by a serine protease GPI-linked to the cell surface.

Protease-activated receptors (PARs)² are unique G-protein-coupled receptors that allow cells to sense specific proteases in their environment. These receptors have emerged as attractive therapeutic targets for several inflammatory diseases, including cardiovascular diseases, arthritis, colitis, asthma, neurogenerative conditions, and cancer (1–3). PAR activation has been shown to regulate processes important for hemostasis, inflammation, pain, and tissue repair (4). PARs may be irreversibly activated upon proteolytic cleavage by trypsin-like serine proteases at a specific amino residue in the N terminus of the receptor, which generates a tethered ligand that binds intramolecularly with an extracellular domain of the receptor to cause a conformational change and the activation of site-specific G-proteins. Through the recruitment and phosphorylation of adaptor proteins, the activation of PARs leads to the initiation of distinct intracellular signaling pathways important in a variety of proliferative, survival, and motility-promoting processes (5).

Four PARs are found in humans and mice. PARs 1, 3, and 4 primarily mediate cellular responses to the coagulation protease thrombin (6). Human PAR-1 is activated following cleavage at Arg⁴¹ by thrombin, Factor Xa (FXa), and activated protein C (APC) (6). Human PAR-2 has been shown to mediate cellular responses in vitro following cleavage at Arg⁴⁶ by several serine proteases, including trypsin, trypsin IV, trypptase, kallikrein 4, and Factors VIIa (FVIIa) and FXa. The FVIIa-FXa complex must be anchored to the cell surface bound to tissue factor-FVIIa-FXa-TF to activate PAR-2 (4, 7). The physiological in vivo activators of PAR-2 are not clearly defined, but there are thought to be several. In the laboratory setting, 6-amino acid-activating peptides (AP) that mimic the tethered ligand of the cleaved PARs are often utilized to study the activation of PARs.

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2 The abbreviations used are: PAR, protease-activated receptor; GPI, glycosylphosphatidylinositol; SRE, serum-response element; PLAP, placential alkaline phosphatase; AlkPhos, alkaline phosphatase; AEBSF, 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochloride; AP, activating peptide; r, recombinant; h, human.
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(6). Activation of PARs by APs is independent of N-terminal cleavage and can lead to some of the same downstream signaling and receptor processing as is seen when PARs are processed by activating proteases.

The trypsin-like serine proteases represent a large family of proteolytic enzymes, which are historically recognized as soluble circulating proteins involved in digestion, blood coagulation, and homeostasis. In recent years, genome mining studies have revealed a novel subfamily of trypsin-like serine proteases that are directly tethered to the cell membrane (8–10). These membrane-anchored serine proteases are synthesized as type I transmembrane, type II transmembrane, or glycosylphosphatidylinositol (GPI)-anchored proteins. The truncated recombinant catalytic domains of several of the type II transmembrane serine proteases have been shown to proteolytically activate PAR-2 in vitro, including matriptase, HAT, and TMPRSS2 (11–15). Whether this activity occurs while these proteases are anchored on the cell surface has been less well studied, with the exception of matriptase, which has been shown to activate PAR-2 when expressed as a full-length protein in cells (16–19).

Testisin (also known as PRSS21 (20, 21)) is one of two reported human GPI-anchored serine proteases (22, 23), the other being prostasin (24). These proteases have been shown to be compartmentalized at plasma membranes within the microenvironment of specialized cholesterol-rich membrane microdomains or lipid rafts (23, 25). Prostasin does not activate PAR-2 directly but can induce matriptase zymogen activation resulting in PAR-2 activation and signaling (16, 18).

Among normal tissues testisin has a remarkably specific distribution, being detected only in abundance in testis (22), where its genetic deletion results in developmentally damaged spermatocytes (26–28). Testisin expression is lost in testicular germ cell tumors (22) and is found aberrantly overexpressed in epithelial ovarian tumors, where its expression has been shown to promote malignant transformation (29, 30). To date, little is known regarding the identity of testisin’s proteolytic substrates that mediate its functions in spermatocytes and tumor cells. Here, we report that testisin functions as a cell surface activator of PAR-2, inducing PAR-2 N-terminal cleavage and internalization and the activation of several PAR-2-dependent intracellular signaling pathways.

EXPERIMENTAL PROCEDURES

Cell Culture and Plasmid Transfections—HeLa cells were cultured and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37 °C in 5% CO2. Serum-free incubations were performed in Opti-MEM medium (Invitrogen). HeLa cells were transfected using Lipofectamine 2000. Lentivirus-containing supernatants were mixed with 6 μg/ml Polybrene (American Bioanalytical) and applied to NCI/ADR-Res cells. Pools of stably transfected cells were obtained via fluorescence cell sorting based on GFP expression (FACS Aria II, BD Biosciences). Knockdown of testisin protein expression was analyzed by SDS-PAGE and immunoblot.

Reagents—Recombinant serine proteases include trypsin (Sigma) and human α-thrombin (American Diagnostica Inc., Stamford, CT). The recombinant serine protease domain of human matriptase (rMatriptase) was kindly provided by R. Leduc (31). Recombinant human prostasin (rProstasin) and mouse testisin (rTestisin) were obtained from R&D Biosystems and activated according to the manufacturer’s instructions. The molar active site concentrations of all recombinant proteases were determined by active-site titration with the burst titrant 4-methylumbelliferyl 4-guanidinobenzoate (Sigma) (data not shown) (32). Human PAR-1 (SFLLRN) and PAR-2 (SLIGKV) APs were purchased from Bachem. Protease inhibitors used include leupeptin, aprotinin, and AEBSF (Sigma) and EDTA (Invitrogen).

DNA Constructs—The alkaline phosphatase reporter plasmid for human PAR-1, pCMV.SEAP.PAR-1 (AlkPhos-PAR-1), was a kind gift from J. Trejo (33). The alkaline phosphatase reporter plasmid for murine PAR-2 and the PAR-2 cleavage site mutant, pCMV.SEAP.PAR-2 (AlkPhos-PAR-2 and pCMV.SEAP.PAR-2csm containing R38A and S39P mutations (AlkPhos-PAR-2csm) respectively, were kindly provided by S. Coughlin (16). The plasmid for N-terminally FLAG-tagged PAR-1 pcDNA3.1.FLAG.PAR-1 (FLAG.PAR-1) and N-terminally FLAG-tagged human PAR-2 pBJ1.FLAG.PAR-2 (FLAG.PAR-2) were provided by J. Trejo (34). The pBJ1.FLAG.PAR-2 plasmid was used as the template for the generation of a PAR-2 cleavage site mutant, pBJ1.FLAG.PAR-2 (R36A) (called FLAG.PAR-2csm), using the QuikChange mutagenesis kit from Stratagene (La Jolla, CA) according to the manufacturer’s instructions and the following primers: forward 5′-GGTAAGGTTGAT-3′ and reverse 5′-ATCAACCTTACC-AATAGGTGTTGAT-3′ and reverse 5′-ATCAACCTTACC-AATAGGTGTTGAT-3′. The C-terminal GFP-tagged PAR-2 plasmids, pcDNA3.1.BAB-PAR-2.EGFP (PAR-2.GFP) and pcDNA3.1.BAB-PAR-2csm.EGFP containing the R36A mutation (called PAR-2csm.GFP), were kind gifts from M. Hollenberg (35).
To generate an expression plasmid encoding full-length GPI-anchored testisin with an HA tag at the N terminus, full-length human testisin cDNA (22) was amplified by PCR using the following primers, forward 5'-GGGCGGAGATCTAAACGCCGAGTCCGAG-3' and reverse 5'-GCGGGATATGTCGAC-TATCAGACCGGCCCCAG-3', and cloned into the BglI and SalI restriction enzyme sites of the pDisplay vector (Invitrogen). The resulting plasmid (pDisplay.Testisin) encodes the mammalian Ig-k chain signal sequence with an N-terminal HA tag inserted in-frame with the testisin cDNA sequence to express HA-tagged GPI-Testisin. pDisplay.Testisin was used as the template for mutating Ser238 to Ala238 (pDisplay.TestisinSA) using the QuikChange mutagenesis kit (Stratagene, La Jolla CA) to express the catalytically inactive mutant testisin (GPI-TestisinSA). Primers used were as follows: forward 5'-CCTGCTTTCCGGTGACGGCGCCGACC-3' and reverse 5'-CAGGGCAAGGGTCCGCTGGTCAC-3'. All plasmids were verified by DNA sequencing. When expressed in cells, GPI-Testisin and GPI-TestisinSA could be released from the cell surface using exogenous bacterial phospholipidsinositol-specific phospholipase C (data not shown), demonstrating that these proteins were processed normally and tethered to the cell surface by GPI anchors. The luciferase reporter plasmids pSRE-firefly luciferase and pRL- Renilla luciferase were the kind gift from T. Bugge (18) and pNFR8-firefly luciferase (BD Biosciences and Clontech) was a kind gift from J. Winkles (36).

Peptide Assays—Chromogenic peptides were purchased from Bachem (Torrance, CA) or synthesized by Peptide 2.0 Inc. (Chantilly, VA). Kinetic assays were performed with 4 nM active rTestisin and 200 μM chromogenic peptides. Changes in absorbance were measured at 420 nm using a Tecan GeniosPro plate reader for 30 cycles over 15 min. Protease inhibitors were pre-incubated (10 μM leupeptin, aprotinin, AEBFS, and 1 mM EDTA) with the rTestisin (4 nM) for 10 min, and activity was assayed using the chromogenic succinyl-AAPR-p-nitroaniline peptide. Residual activity was compared with the activity in the absence of inhibitor.

Cell Lysis and Immunoblotting—Cells lysates of transiently transfected cells were prepared using ice-cold lysis buffer containing 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholic acid, and complete protease inhibitor mixture (Roche Applied Science), or using NuPAGE LDS Sample Buffer (Invitrogen). Cells were lysed 48 h post-transfection unless otherwise indicated. Protein concentrations were estimated using the Bio-Rad protein assay kit. Protein lysates, in the absence (nonreducing) or presence (reducing) of 7% 2-mercaptoethanol, were resolved by SDS-PAGE, and immunoblots were probed with the indicated primary antibody and detected with HRP-conjugated secondary antibody using standard techniques.

Quantitation of Soluble Alkaline Phosphatase Released by PAR Cleavage—The release of soluble alkaline phosphatase (AlkPhos) as a measure for PAR N-terminal cleavage was performed as described previously (33). Briefly, HeLa or NCI/ADR-Res control and shRNA-treated cells were transiently transfected with pCMV.SEAP.PAR-1, pCMV.SEAP.PAR-2, or pCMV.SEAP.PAR-2csm. After 48 h, cells were washed and then either treated with the indicated proteases in serum-free media (HeLa cells) or were incubated in serum-free media alone (NCI/ADR-Res lines) for 4 h. The conditioned medium was assayed for AlkPhos activity using the Applied Biosystems Phospa-light detection system and chemiluminescence measured on a Berthold Technologies Centro LB-960 plate reader.

Intracellular Calcium Release—HeLa cells were cultured in 6-well plates on No. 1 glass coverslips and transfected with pcDNA3.1 vector, pcDNA3.1.FLAG.PAR-1, pBJ1.FLAG.PAR-2, or pBJ1.FLAG.PAR-2csm. After 48 h, cells were washed and loaded with Fluo-3 calcium indicator by incubation with 2 μM Fluo-3 AM in growth medium for 30 min at room temperature in 5% CO₂. Thereafter, the coverslip bearing the cells was mounted in an open bottom stainless steel Petri dish containing serum-free growth medium maintained at 27–28 °C. Fluorescence images were acquired on an inverted epifluorescence microscope (Eclipse TE200; Nikon Corp., Tokyo, Japan) equipped with a x40 oil immersion objective (SuperFluor, NA 1.4; Nikon). Excitation light (488 nm) was delivered by a xenon source coupled to a monochromator (PolyChrome II; TILL Photonics, Gräfelfing, Germany).

Fluorescence was passed through a bandpass filter before capture by a CCD camera (CoolSnap HQ; Roper Scientific, Tucson, AZ). MetaFluor software (Molecular Devices, Downingtown, PA) was used for instrument control and image acquisition and analysis. Image acquisition rate was between 0.33 and 1 Hz. To monitor calcium signaling, cells were imaged as 4 nM rTestisin, and 1 μM PAR APs were bath-applied; 300 nM ionomycin (Calbiochem) was used as positive control. Background fluorescence was recorded after end-of-experiment cell lysis with 40 μM digitonin (Sigma). At least 100 cells were analyzed from each imaged field; a region-of-interest was defined for each cell, and the mean intensity within each region-of-interest was determined for each image frame and analyzed as described previously (37, 38).

ERK1/2 Activation—HeLa cells were transiently transfected with pBJ1.FLAG.PAR-2 or pBJ1.FLAG.PAR-2csm. After 36 h, cells were washed and cultured in serum-free media overnight. Cells were then left untreated or treated for 2, 5, 15, 30, and 60 min with 4 nM rTestisin, 4 nM trypsin, or 100 μM PAR-2 AP. Whole cell lysates were prepared and analyzed for p-ERK1/2 and total ERK1/2 expression by immunoblot.

Flow Cytometry—HeLa cells were co-transfected with pBJ1.FLAG.PAR-2 or pBJ1.FLAG.PAR-2csm and pDisplay.Testisin, pDisplay.TestisinSA, or pDisplay vector. 48 h post-transfection, cells were gently lifted with Versene (Lonza) at room temperature. For detection of surface expression, cells were washed with ice-cold FACS buffer (phosphate-buffered saline (PBS) containing 0.5% FBS and 0.025% sodium azide) and incubated with primary antibodies for 30 min on ice. Cells were washed and then incubated with fluorochrome-conjugated secondary antibodies for 15 min on ice. For detection of whole cell PAR-2 and PAR-2csm expression, cells were fixed in 4% formaldehyde for 10 min at room temperature, and antibody incubations were performed in buffer containing 0.5% Triton X-100 to permeabilize cells. Flow cytometry data were acquired on a FACS Calibur (BD Biosciences) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

Surface Protein Bisination—HeLa cells were co-transfected with pcDNA3.1.BAB-PAR-2.EFGP, pcDNA3.1.BAB-PAR-2, EFGPcsm, or pcDNA3.1 and pDisplay.Testisin, pDisplay.TestisinSA, or pDisplay vector to equal DNA concentrations.
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After 36 h, cells were serum-starved overnight and then labeled with 1 mg/ml EZ-link™ Sulfo-NHS-SS-Biotin (Thermo Scientific) per the manufacturer’s instructions. Lysates were prepared in ice-cold lysis buffer, and equal protein concentrations were incubated overnight with immobilized NeutrAvidin (Pierce) at 4 °C. The following day, the NeutrAvidin Sepharose-bound proteins were collected by centrifugation and boiled in 4 × SDS sample buffer (NuPAGE) containing 7% β-mercaptoethanol for immunoblot analysis.

PAR-2 Internalization—The PAR-2 internalization assay was performed as described previously (39). Briefly, HeLa cells were transfected with either pcDNA3.1.BAB-PAR-2-EGFP or pcDNA3.1.BAB-PAR-2csm-EGFP. After 48 h, cells were labeled with 0.5 mg/ml EZ-link™ Sulfo-NHS-SS-Biotin (Thermo Scientific) per the manufacturer’s instructions. Cells were then incubated with culture media containing vehicle, 4 mM rTestisin, or 100 μM PAR-2 AP and 20 μM MG132 for 5 and 15 min to allow receptor activation and internalization. Surface biotin was removed by incubating cells at 4 °C in reducing buffer (50 mM MeSNA, 100 mM Tris, pH 8.6, 100 mM NaCl) for 30 min. Excess MeSNA was quenched with 60 mM iodoacetamide for 15 min in PBS. Whole cell lysates were collected in ice-cold lysis buffer, and equal protein concentrations were incubated overnight with immobilized NeutrAvidin (Pierce) at 4 °C to isolate internalized PAR-2. The following day, the NeutrAvidin-Sepharose bound proteins were collected by centrifugation and boiled in 4 × SDS sample buffer (NuPAGE) containing 7% β-mercaptoethanol for immunoblot analysis.

SRE-Luciferase and NFκB-Luciferase Assays—The luciferase assays were performed as described previously (18, 36). Briefly, HeLa cells were co-transfected with pSRE-firefly luciferase or NFκB-firefly luciferase (150 ng) and pRL-Renilla luciferase (20 ng), in combination with pDisplay vector, pBJ1.FLAG.PAR-2, or pBJ1.FLAG.PAR-2csm (300 ng) and either pDisplay vector, pDisplay.Testisin, or pDisplay.TestisinSA (300 ng). After 12 h, the cells were serum-starved overnight and lysed, and luciferase activity was measured using the Dual-Luciferase assay kit (Promega, Madison, WI) according to the manufacturer’s instructions. Chemiluminescence was measured using a Berthold Technologies Centro LB-960 plate reader. SRE and NFκB activation was assessed as the ratio of firefly to Renilla luciferase counts.

Cytokine Expression—HeLa cells were transiently transfected with pB1.FLAG.PAR-2 or pB1.FLAG.PAR-2csm and pDisplay.Testisin, or pDisplay alone, and RNA purified using the RNeasy kit (Qiagen) per the manufacturer’s instructions. Reverse transcription was performed using TaqMan reverse transcription reagents (Applied Biosystems). Quantitative PCR was performed with TaqMan primers for hIL-8 (catalog no. Hs99999034_m1) and hIL-6 (catalog no. Hs00985641_m1) along with control hGAPDH (catalog no. Hs9999905_1_m1). Cytokine mRNA levels were calculated relative to GAPDH.

Statistics—Data are presented as means ± S.E. Unpaired Student’s t test was used to compare experimental groups that were normally distributed (GraphPad software). p ≤ 0.05 was defined as statistical significance.

RESULTS

Catalytic Specificity of rTestisin—In common with other serine proteases, the testisin active site consists of a catalytic triad of amino acid residues His, Asp, and Ser (22). The presence of the Asp residue at the bottom of the conserved binding pocket predicts that testisin has trypsin-like specificity with proteolytic cleavage after basic amino acid residues, P1-Arg or P1-Lys, in target substrates (22). To experimentally investigate testisin substrate specificity, the activity of purified rTestisin was determined using a panel of chromogenic peptide substrates (Fig. 1A). Comparing the rate of cleavage of peptide 1 versus peptides 2–4 showed that rTestisin prefers to cleave after P1-Arg compared with P1-Lys, and it has little preference for hydrophobic amino acids Val and Phe at the P1 position (Fig. 1A). To further analyze the enzymatic properties of testisin, the effects of various protease inhibitors on rTestisin activity were investigated using peptide 1. The serine protease inhibitors AEBSF, leupeptin, and aprotinin significantly inhibited rTestisin proteolytic activity, whereas the metalloproteinase inhibitor EDTA had no effect on rTestisin activity (Fig. 1B).

PAR-2 Is a Testisin Substrate—PAR-activating serine proteases cleave after specific Arg amino acids within the extracellular N-terminal domains of PARs to expose a tethered ligand. When the activity of rTestisin was determined using chromogenic peptide substrates derived from the amino acid sequences of the N-terminal cleavage activation sites of PAR-1 (peptide 5) and PAR-2 (peptide 6), rTestisin was able to effectively cleave...
the PAR-2 activation sequence, showing preference over the PAR-1 activation sequence (Fig. 1A).

To further assess the ability of testisin to productively cleave PARs, we transiently transfected HeLa cells with PAR-1 and PAR-2 expression constructs that had secreted human placental AlkPhos fused to their N-terminal exodomains such that receptor cleavage released AlkPhos into the culture medium (Fig. 2) (16, 33). Addition of 7.5 and 15 nM rTestisin to HeLa cells expressing AlkPhos-PAR-2 was incubated with the indicated active soluble recombinant protease domains as follows: 7.5 and 15 nM rProstasin; 4 nm rMatriptase; and 2 nm trypsin. After 4 h, the AlkPhos activity released was quantified as fold change over untreated cells. 7.5 and 15 nM rTestisin specifically cleaved AlkPhos-PAR-2 in a dose-dependent manner, similar to the AlkPhos release seen with matriptase and trypsin treatment, whereas prostasin did not release AlkPhos activity. B, rTestisin does not cleave the N terminus of PAR-1 as compared with thrombin or trypsin. HeLa cells transiently expressing AlkPhos-PAR-1 were incubated with the indicated active soluble recombinant protease domains as follows: 7.5 nM rTestisin, 2 nm trypsin, and 1 nm thrombin. C, rTestisin cleaves PAR-2 at the P1-Arg activating cleavage site. The addition of rTestisin and the PAR-2 agonist proteases, trypsin and matriptase, to HeLa cells expressing PAR-2 activation sequence mutations, AlkPhos-PAR-2csm (containing a two amino acid substitution (R38A/S39P)) that abolishes cleavage at the activating P1-Arg cleavage site (16, 33), does not induce AlkPhos release. D, expression of cell surface and total AlkPhos-PAR proteins in transfections. Cell surface biotinylation of HeLa cells transiently expressing AlkPhos-PAR-2, AlkPhos-PAR-2csm, AlkPhos-PAR-1, or vector alone was performed at 48 h post-transfection. Equal protein from whole cell lysates or streptavidin-captured cell surface proteins were resolved by SDS-PAGE and immunoblotted. AlkPhos-PAR-1 was not detected by this anti-PLAP antibody, but it was detected in both the whole cell lysates and on the cell surface using anti-PAR-1, H111 antibody. The lower molecular weight band in the cell surface sample may represent PAR-1 that has lost the P1-Arg activating cleavage site due to cleavage by an endogenous protease. Graphs show the mean ± S.E. from duplicate samples and is representative of two independent experiments. *, p < 0.05; ***, p < 0.001.

PAR-2 Cleavage by rTestisin Induces Transient Intracellular Calcium Mobilization—Productive N-terminal cleavage of PARs generates a tethered ligand domain that induces transmembrane signal activation. Like other G-protein-coupled receptors, the activation of PAR-1 or PAR-2 results in a rapid and transient increase in intracellular calcium (6, 40). To investigate the ability of testisin to regulate PAR-2 activation, HeLa cells transiently transfected with the PAR expression plasmids, FLAG.PAR-1, FLAG.PAR-2, FLAG.PAR-2csm or vector alone, were loaded with the calcium indicator Fluo-3, which fluoresces brightly when bound to calcium ions (Ca2+) and is almost nonfluorescent in the Ca2+-free form. Treatment of cells expressing FLAG.PAR-2 with 4 nm rTestisin induced a transient rise of cytosolic free Ca2+ concentration (Fig. 3A), indicating receptor activation. Importantly, rTestisin treatment of cells expressing the PAR-2 cleavage site mutant FLAG.PAR-2csm did not induce calcium release (Fig. 3B), indicating that rTestisin specifically activates and cleaves PAR-2 at the P1-Arg activating cleavage site that is utilized by other PAR-2 agonists.

FIGURE 2. Testisin productively cleaves PAR-2 to release the activating tethered ligand. A, rTestisin cleaves PAR-2 to release the tethered ligand. HeLa cells expressing AlkPhos-PAR-2 were incubated with the indicated active soluble recombinant protease domains as follows: 7.5 and 15 nM rTestisin; 4 nm rProstasin; 4 nm rMatriptase; and 2 nm trypsin. After 4 h, the AlkPhos activity released was quantified as fold change over untreated cells. 7.5 and 15 nM rTestisin specifically cleaved AlkPhos-PAR-2 in a dose-dependent manner, similar to the AlkPhos release seen with matriptase and trypsin treatment, whereas prostasin did not release AlkPhos activity. B, rTestisin does not cleave the N terminus of PAR-1 as compared with thrombin or trypsin. HeLa cells transiently expressing AlkPhos-PAR-1 were incubated with the indicated active soluble recombinant protease domains as follows: 7.5 nM rTestisin, 2 nm trypsin, and 1 nm thrombin. C, rTestisin cleaves PAR-2 at the P1-Arg-activating cleavage site. The addition of rTestisin and the PAR-2 agonist proteases, trypsin and matriptase, to HeLa cells expressing the PAR-2 activation sequence mutant, AlkPhos-PAR-2csm (containing a two amino acid substitution (R38A/S39P)) that abolishes cleavage at the activating P1-Arg cleavage site (16, 33), does not induce AlkPhos release. D, expression of cell surface and total AlkPhos-PAR proteins in transfections. Cell surface biotinylation of HeLa cells transiently expressing AlkPhos-PAR-2, AlkPhos-PAR-2csm, AlkPhos-PAR-1, or vector alone was performed at 48 h post-transfection. Equal protein from whole cell lysates or streptavidin-captured cell surface proteins were resolved by SDS-PAGE and immunoblotted. AlkPhos-PAR-2 results in a 2- and 3-fold release of AlkPhos activity by rTestisin, similar to the inhibition of AlkPhos release by anti-PLAP, because PAR-2 is poorly detected by immunoblotting using commercially available PAR-2 antibodies (48). AlkPhos-PAR-1 was not detected by this anti-PLAP antibody, but it was detected in both the whole cell lysates and on the cell surface using anti-PAR-1 H111 antibody. The lower molecular weight band in the cell surface sample may represent PAR-1 that has lost the 70-kDa AlkPhos domain due to cleavage by an endogenous protease. Graphs show the mean ± S.E. from duplicate samples and is representative of two independent experiments. *, p < 0.05; ***, p < 0.001.

Calcium Mobilization—
receptor independent of N-terminal proteolytic cleavage, did evoke a robust Ca^{2+}/H11001 signal in cells that express either FLAG.PAR-2 or FLAG.PAR-2csm, showing that both the expressed PAR-2s retain the ability to trigger Ca^{2+}/H11001 signaling when non-proteolytically activated (Fig. 3, A and B). No intracellular Ca^{2+} response was detected following treatment of vector alone-transfected cells with 4 nM rTestisin or PAR-2 activating peptide (SLIGKV) and 300 nM ionomycin (Iono) as indicated. Treatment with rTestisin evoked an intracellular calcium signal in cells expressing FLAG.PAR-2, evidenced by a transient rise of Fluo-3 fluorescence, which did not occur in cells expressing FLAG.PAR-2csm or FLAG.PAR-1. Expression of PAR-1 and PAR-2 also was verified by the calcium signal evoked by the respective activating peptides. Ionomycin, a calcium ionophore that facilitates Ca^{2+} transport across cellular membranes, served as positive control in all experiments. All reagents were bath-applied. Graphs are representative of two independent experiments. a.u., arbitrary units.

FIGURE 3. PAR-2 cleavage by rTestisin induces intracellular calcium mobilization. HeLa cells expressing FLAG.PAR-2 (A), FLAG.PAR-2csm (B), FLAG.PAR-1 (C), and vector alone (D) were loaded with Fluo-3, a fluorescent calcium indicator, and challenged sequentially with 4 nM rTestisin (rTs), 1 μM PAR-1 activating peptide (SFLLRN), or PAR-2 activating peptide (SLIGKV) and 300 nM ionomycin (Iono) as indicated. Treatment with rTestisin evoked an intracellular calcium signal in cells expressing FLAG.PAR-2, evidenced by a transient rise of Fluo-3 fluorescence, which did not occur in cells expressing FLAG.PAR-2csm or FLAG.PAR-1. Expression of PAR-1 and PAR-2 also was verified by the calcium signal evoked by the respective activating peptides. Ionomycin, a calcium ionophore that facilitates Ca^{2+} transport across cellular membranes, served as positive control in all experiments. All reagents were bath-applied. Graphs are representative of two independent experiments. a.u., arbitrary units.

Cleavage of PAR-2 by rTestisin Activates PAR-2

PAR-2 activation by trypsin and PAR-2 AP SLIGKV has been shown by others to induce transient phosphorylation of extracellular signal-related kinases (ERK1/2) (40, 41). When HeLa cells expressing FLAG.PAR-2 or FLAG.PAR-2csm were treated with 4 nM rTestisin, transient phosphorylation of ERK1/2 in cells expressing FLAG.PAR-2 was detected, similar to the signal generated by the PAR-2 activators trypsin and SLIGKV (Fig. 4A). ERK1/2 phosphorylation was not observed after treatment with rTestisin in cells expressing the PAR-2 cleavage mutant FLAG.PAR-2csm, whereas the addition of SLIGKV resulted in ERK1/2 activation through FLAG.PAR-2csm (Fig. 4B), showing that the PAR-2csm retains
the ability to signal when activated. Expression of FLAG.PAR-2 and FLAG.PAR-2csm was verified by immunoblot analysis with an anti-PAR-2 antibody (data not shown). These data show that the productive cleavage of PAR-2 by rTestisin induces receptor activation and the stimulation of downstream signaling pathways documented for other known PAR-2-activating proteases.

**PAR-2 N Terminus Is Cleaved by Membrane-anchored Testisin**—Testisin is tethered to the cell surface via a GPI anchor (22, 30). Unlike many other membrane-anchored serine proteases, including prostasin (42), testisin has not been found to be naturally shed from the cell surface. To investigate membrane-tethered testisin activation of PAR-2 on the cell surface, plasmids encoding N-terminally HA-tagged human testisin (GPI-Testisin) and an active-site mutant testisin, in which the active site Ser238 was changed to alanine (GPI-TestisinSA), were generated. These plasmids were co-transfected into HeLa cells, which express a low level of endogenous testisin (43). Nonpermeabilized HeLa cells expressing FLAG.PAR-2 or vector alone, together with GPI-Testisin or GPI-TestisinSA, were stained with anti-FLAG antibody and analyzed by flow cytometry. Cells co-expressing FLAG.PAR-2 and GPI-Testisin or GPI-TestisinSA, were stained with anti-FLAG antibody and analyzed by flow cytometry. Cells co-expressing FLAG.PAR-2 and GPI-Testisin showed an 8-fold reduction in cell-surface FLAG compared with cells expressing FLAG.PAR-2 alone or expressing FLAG.PAR-2 and the GPI-TestisinSA mutant (Fig. 5), indicating that co-expression of active testisin induces release of the N-terminal PAR-2 peptide.

**PAR-2 Is Cleaved by Endogenous Testisin**—To investigate the cleavage of PAR-2 by testisin in a natural system, we investigated PAR-2 cleavage using NCI/ADR-Res ovarian cancer cells, which constitutively express endogenous testisin. NCI/ADR-Res cells in which testisin expression was silenced by stable expression of three different testisin shRNAs and NCI/ADR-Res cells expressing shRNA scrambled control (Fig. 6A), immunoblotting the expression of endogenous testisin protein in NCI/ADR-Res ovarian carcinoma cells stably expressing an shRNA scrambled control (1st lane) or each of three different testisin-specific shRNAs (2nd to 4th lanes). B, shRNA knockdown of endogenous testisin reduces PAR-2 AlkPhos release by NCI/ADR-Res cells. The four NCI/ADR-Res cell lines were transiently transfected with a plasmid expressing AlkPhos-PAR-2. Forty-eight h post-transfection, conditioned media collected over a 4-h period were analyzed for AlkPhos activity. Data are expressed as a % of AlkPhos release compared with control cells and represents the mean ± S.E. of duplicate wells from two independent experiments. ***, p < 0.001.**

PAR-2 surface expression was also analyzed by flow cytometry using an anti-PAR-2 antibody that detects total PAR-2 (full-length and cleaved). Cells co-expressing FLAG.PAR-2 and GPI-Testisin exhibit significantly less PAR-2 protein on the cell surface than cells expressing PAR-2 alone or PAR-2 and the GPI-TestisinSA mutant (Fig. 7A), consistent with detection of reduced FLAG on the cell surface (Fig. 5). The PAR-2 expression present in permeabilized cells was similar irrespective of the presence or absence of testisin (Fig. 7, B and D), suggesting
that the reduced PAR-2 on the cell surface is not due to a difference in total cellular PAR-2 expression. These data demonstrate that testisin facilitates PAR-2 loss from the cell surface. HeLa cells co-expressing the activation site mutant FLAG.PAR-2csm and GPI-Testisin (Fig. 7C), or FLAG.PAR-2 and GPI-TestisinSA (Fig. 7B), showed no significant change in PAR-2 cell-surface expression, demonstrating that the testisin-induced PAR-2 loss requires specific cleavage at the P1-Arg-activating cleavage site by proteolytically active testisin.

The cleavage of PAR-2 by surface-anchored testisin was further confirmed by cell surface biotinylation studies using HeLa cells expressing C-terminally GFP-tagged PAR-2 (PAR-2.GFP). The C-terminal GFP tag allows for the detection of full-length and cleaved PAR-2 with high antibody affinity by immunoblot. The presence of cell surface PAR-2.GFP detected in bead eluates after biotin labeling was significantly reduced in cells co-expressing PAR-2.GFP and active testisin (GPI-Testisin) compared with that seen with the PAR-2 cleavage mutant PAR-2.GFPcsm (Fig. 8A). The reduction in cell surface PAR-2 was dependent on expression of active testisin as no reduction was observed with the GPI-TestisinSA mutant (Fig. 8A). Analyses of whole cell lysates show that the total levels of PAR-2.GFP present under each condition were similar (Fig. 8B), confirming that the reduction in cell-surface expression was not due to a change in total cellular PAR-2 expression.

HeLa cells expressing wild type GPI-Testisin or GPI-TestisinSA showed similar total testisin protein levels as detected by immunoblot using an anti-Testisin antibody (Fig. 8B). When the blots were re-probed with an anti-HA antibody, however, a significantly increased signal for the GPI-TestisinSA mutant was detected compared with wild type testisin (Fig. 8B). Detection of the HA tag is diagnostic for the single chain testisin zymogen, because when analyzed under reducing conditions by immunoblot, the HA tag, which is fused to the N-terminal testisin pro-domain, will be dissociated from the two-chain catalytically active testisin. The increased levels of the zymogen form of testisin SA compared with wild type testisin may indicate that catalytically active testisin facilitates testisin zymogen activation.

**Cleavage of PAR-2 by Testisin Induces PAR-2 Internalization**—Cleavage of PAR-2 by other PAR-2-activating proteases, such as trypsin, is known to induce PAR-2 phosphorylation, internalization, and the initiation of specific intracellular signaling pathways (5). The reduction in the level of cell surface PAR-2.GFP when co-expressed with wild type testisin was similar to the reduced levels seen when the cells were treated with the PAR-2-activating protease trypsin (Fig. 8A), suggesting that PAR-2 may be internalized from the cell surface when activated by cell surface testisin. To specifically test this, HeLa cells transiently expressing PAR-2.GFP, PAR-2csm.GFP, or vector alone were surface biotin-labeled and treated with vehicle, rTestisin, or PAR-2 AP (SLIGKV) to allow endocytosis of surface-labeled PAR-2 induced by receptor activation (Fig. 9). At the indicated times, residual cell surface biotin was removed with a mild reducing agent, biotin-labeled proteins precipitated from whole cell lysates with streptavidin beads, and internalized biotin-labeled PAR-2 detected by anti-GFP immunoblot. The data showed that PAR-2.GFP internalization steadily increased over 15 min in response to both rTestisin and PAR-2 AP, with the internalized receptor detected by 5 min (Fig. 9). Under the same conditions however, there was no increase in internalization of PAR-2csm.GFP when treated with rTestisin, suggesting spe-

![FIGURE 7. Testisin induces the loss of the PAR-2 N terminus from the cell surface through cleavage at the PAR-2 activation site. Flow cytometry analysis of PAR-2 expression in HeLa cells co-expressing FLAG.PAR-2 (A and B) or FLAG.PAR-2csm (C and D) together with GPI-Testisin, GPI-TestisinSA, or vector alone. Cells were stained using anti-PAR-2 antibody either unpermeabilized (A and C) to detect cell surface PAR-2 or permeabilized (B and D) to detect whole cell PAR-2 expression. Reduced PAR-2 is detected on the cell surface when cells express active testisin, although this is not seen with the PAR-2 activation cleavage site mutant. The vector control cells show PAR-2 expression levels above the unstained (Unst) cells, indicating that HeLa cells express a low level of endogenous PAR-2. Graphs represent the mean ± S.E. from two independent experiments. *, p < 0.05.](image-url)
Specific activation and internalization of PAR-2 following activation cleavage by testisin.

Proteolytic Activation by Co-expressed Testisin Induces PAR-2-dependent Intracellular Signaling—Productive downstream PAR-2 signaling induced by testisin was investigated using gene promoter reporter assays and by cytokine gene induction. HeLa cells were co-transfected with expression vectors encoding PAR-2 and testisin and either an SRE-luciferase reporter plasmid or an NFκB-luciferase reporter plasmid to measure PAR-2 signaling activities (Fig. 10, A and B). Cells expressing PAR-2 show large increases in both SRE- and NFκB-luciferase reporter gene activities when co-expressing GPI-Testisin but not when co-expressing GPI-TestisinSA, the cleavage-resistant PAR-2 mutant (PAR-2csm) or the empty vector (Fig. 10, A and B).

The proteolytic activation of PAR-2 in a variety of epithelial cells has been found to induce the expression of several cytokines, specifically IL-8 and IL-6 (41, 44, 45). HeLa cells co-expressing PAR-2 and GPI-Testisin similarly show a significant induction of IL-8 and IL-6 mRNA compared with cells expressing the cleavage-resistant PAR-2csm or GPI-Testisin alone, suggesting that the induction of these cytokines is due to the specific activation of PAR-2 by surface-expressed testisin (Fig. 10, C and D). Taken together, the data show that testisin specifically induces functional signaling responses upon proteolytic activation of PAR-2.

DISCUSSION

PARs are important cellular signaling receptors for many diverse physiological processes. The mechanisms that regulate PAR surface expression and receptor activation are not yet well understood. In this study, we provide the first evidence for the activation of PAR-2 by a GPI-anchored serine protease. Testisin is able to specifically activate PAR-2 on the cell surface, inducing PAR-2 internalization and the activation of PAR-2-dependent intracellular signaling pathways and cytokine expression.

We find that both the soluble recombinant catalytic domain of testisin, endogenous testisin, and exogenous full-length GPI-anchored testisin specifically cleave PAR-2 expressed on the surface of HeLa cells. Testisin cleavage of PAR-2 requires the Arg activation cleavage site within the N-terminal extracellular domain. This activation cleavage site has been shown to be utilized by other PAR-2 protease agonists to expose the tethered ligand, namely trypsin (46), tryptase (47), matriptase (16), and TMPRSS2 (15) when analyzed in vitro. Although multiple proteases may activate PAR-2 through cleavage at the same activation site, it is becoming evident that PAR activation by
different agonist proteases, or even the activation of a PAR by the same agonist but in a different subcellular location, can result in the coupling and activation of distinct signaling pathways leading to diverse physiological outcomes, a phenomenon known as “biased agonism” (5, 49). Here, we have used several different assay systems to evaluate the nature of the PAR-2-activated signaling pathways induced by testisin cleavage in HeLa cells. Recombinant testisin was able to stimulate PAR-2-dependent transient calcium release and ERK1/2 activation, which was not observed by treatment of cells expressing PAR-1 or the PAR-2 cleavage site mutants (Figs. 3 and 4). Additionally, co-expressed surface-bound GPI-anchored testisin was able to specifically promote PAR-2 activation, triggering intracellular signaling pathways that lead to SRE and NFκB transcriptional activation, and the induction of IL-6 and IL-8 gene expression (Fig. 10). Other aspects of known PAR-2-mediated signaling pathways and the ultimate downstream effects on cellular physiology imparted by testisin-mediated PAR-2 activation in comparison with other

**FIGURE 10. Activation of PAR-2 by GPI-anchored testisin induces PAR-2-dependent intracellular signaling and cytokine gene induction.** Luciferase reporter assays of lysates from HeLa cells transiently co-expressing pSRE-firefly luciferase and pRL-Renilla luciferase (A) or NFκB-firefly luciferase and pRL-Renilla luciferase (B) are shown, with FLAG.PAR-2 or FLAG.PAR-2csm and GPI-Testisin or GPI-TestisinSA. Signals were quantified as the ratio of SRE- or NFκB-firefly luciferase to Renilla-luciferase activity, and graphs represent mean ± S.E. calculated from triplicate wells from two independent experiments. Cells co-expressing PAR-2 and GPI-Testisin show an ~3-fold increase in SRE-Luc/Renilla-Luc activity, and cells co-expressing PAR-2 and GPI-Testisin show ~4.5-fold activation of NFκB signaling. Testisin expression was verified by immunoblot analysis of whole cell lysates using an anti-HA antibody. For analysis of cytokine gene induction, cDNA prepared from HeLa cells co-expressing FLAG.PAR-2 or FLAG.PAR-2csm and GPI-Testisin was analyzed by quantitative PCR for induction of IL-8 (C) and IL-6 (D) mRNA expression. The presence of PAR-2 and GPI-Testisin resulted in an over 50-fold increase in IL-8 mRNA expression and a 2.5-fold increase in IL-6 mRNA expression. **Graphs** show relative mRNA expression ± S.E. from triplicate wells in two independent experiments. **, p < 0.005; ***, p < 0.001.
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PAR-2-activating proteases remains to be determined, and they are likely to be cell type- and context-specific.

As opposed to a transient signal that may occur following cleavage of PAR-2 by a soluble agonist, PAR-2 activation by a co-expressed membrane-localized activating protease on the cell surface may be important for inducing a sustained pattern of cellular signaling. We found that when abundant testisin is present on the cell surface, PAR-2 is constitutively internalized and intracellular signaling pathways are continuously activated. It is likely that following activation by testisin, PAR-2 is quickly phosphorylated, endocytosed, and subsequently degraded, similar to the rapid desensitization and internalization following activation by trypsin (50). In contrast to PAR-1, where stored intracellular pools of receptor move to the cell surface to replace activated and internalized PAR-1, repopulation of PAR-2 on the cell surface after activation requires de novo PAR-2 synthesis (3). This delay likely explains the reduced levels of cell surface PAR-2 in the presence of co-expressed testisin. The reduction of surface PAR-2 has several possible implications, because less PAR-2 is expected to be available for activation by other soluble proteases in the pericellular environment. Therefore, in cells expressing testisin, the impact of other PAR-2 agonists, such as trypsin or tryptase, could be diminished, potentially altering the signaling profile of the cell. In addition, the signaling through PAR-2 induced by testisin could affect cross-talk with other cell-signaling pathways.

The activation of PAR-1 or PAR-2 within the compartmentalized membrane microdomains is another mechanism of biased agonism, wherein differential intracellular signaling pathways are activated depending upon whether or not the PAR is localized within the cholesterol-rich lipid rafts on the cell membrane (51). Cell surface testisin is known to be localized within lipid raft microdomains (22, 52), and unlike other membrane-anchored serine proteases, testisin has not been found to be naturally shed from the cell surface, although we and others (23) find that testisin is released from cells in culture using exogenous bacterial phosphatidylinositol-specific phospholipase C. Lipid rafts provide signaling modules that can enhance interactions of proteins even at low concentrations within the rafts by bringing them into close proximity, as has been suggested for activation of PAR-2 by FXa-TF in lipid rafts (53, 54). Of note, activation of PAR-2 by soluble proteases is unlikely to be specific for receptors present in lipid rafts versus other microenvironments on the membrane, whereas a PAR-2 agonist co-localized with the receptor within a lipid raft provides the opportunity for biased signaling dependent on the presence of specific lipid raft-associated adaptor proteins. In different tissue contexts, signaling downstream of PAR-2 induced by surface-anchored testisin within lipid rafts may differ from the activation of PAR-2 by other soluble agonist proteases.

Because testisin is expressed endogenously by select cell types, the tissue specificity and cell-selective induction of testisin may be an important factor in the regulation of PAR-2-mediated cellular responses. Testisin is abundant in sperm where it has been shown to be required for processes of sperm cell maturation important for sperm motility and fertilizing ability in mice (26, 27). Both testisin and PAR-2 are expressed on the head region and midpiece of human spermatozoa (55). Whether testisin functions as an endogenous activator of PAR-2 in sperm is not known, but PAR-2 activation has been associated with the regulation of sperm motility following trypsin activation (55, 56).

Overexpression of testisin has been documented in several cancers, specifically ovarian cancers (29, 30). Testisin has been shown to promote malignant transformation when overexpressed in cultured tumor cells and also in mouse xenograft tumor models (30). siRNA-mediated knockdown of testisin in human tumor cell lines results in increased apoptosis and diminished ability to form colonies in soft agar (30). PAR-2 activation is implicated in the pathology of multiple tumors because downstream signals can promote cell proliferation, invasion, migration, and angiogenesis (6, 57, 58). Immunohistological staining of tumor specimens from ovarian cancer patients has shown that PAR-2 expression in tumor cells is increased with clinical tumor stage and correlates with reduced survival rate (59). In other tumor types, the activation of PAR-2 can induce the expression of anti-apoptotic proteins and prevents apoptosis induced by loss of cell adhesion (60, 61). The molecular mechanisms by which testisin increases tumor development are not known, but PAR-2 may represent a pathological substrate for further study.

In summary, the activation of PAR-2 by testisin reveals a novel biological substrate for testisin that may uniquely influence PAR-2 activation and the induction of downstream signaling pathways.

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REFERENCES
1. Coughlin, S. R. (2005) Protease-activated receptors in hemostasis, thrombosis and vascular biology. J. Thromb. Haemost. 3, 1800–1814
2. Yu, T. K., 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. Soh, U. J., Dores, M. R., Chen, B., and Trejo, J. (2010) Signal transduction by protease-activated receptors. Br. J. Pharmacol. 160, 191–203
4. Cottrell, G. S., Amadesi, S., Schmidlin, F., and Bunnett, N. (2003) Protease-activated receptor 2: activation, signalling and function. Biochem. Soc. Trans. 31, 1191–1197
5. Adams, M. N., Ramachandran, R., Yau, M. K., Suen, J. Y., Fairlie, D. P., Hollenberg, M. D., and Hooper, J. D. (2011) Structure, function and physiological role of protease activated receptors. Pharmacol. Ther. 130, 248–282
6. Ososvskaya, V. S., and Bunnett, N. W. (2004) Protease-activated receptors: contribution to physiology and disease. Physiol. Rev. 84, 579–621
7. Ramsay, A. J., Dong, Y., Hunt, M. L., Linn, M., Samarutah, H., Clements, J. A., and Hooper, J. D. (2008) Kallikrein-related peptidase 4 (KLK4) initiates intracellular signaling via protease-activated receptors (PARs) KLK4 and PAR-2 are co-expressed during prostate cancer progression. J. Biol. Chem. 283, 12293–12304
8. Hooper, J. D., Clements, J. A., Quigley, J. P., and Antalis, T. M. (2001) Type II transmembrane serine proteases. Insights into an emerging class of cell surface proteolytic enzymes. J. Biol. Chem. 276, 857–860
9. Antalis, T. M., Bugge, T. H., and Wu, Q. (2011) Membrane-anchored serine proteases in health and disease. Prog. Mol. Biol. Transl. Sci. 99, 1–50
10. Antalis, T. M., Buzza, M. S., Hodge, K. M., Hooper, J. D., and Netzel-Arnett, S. (2010) The cutting edge: membrane-anchored serine protease
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activities in the pericellular microenvironment. *Biochem. J.* **428**, 325–346
11. Seitz, I., Hess, S., Schulz, H., Eckl, R., Busch, G., Montens, H. P., Brandl, R., Seidl, S., Schömig, A., and Ott, I. (2007) Membrane-type serine protease-1/matrisse induces interleukin-6 and -8 in endothelial cells by activation of protease-activated receptor-2: potential implications in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **27**, 769–775
12. Bocheva, G., Rattenholl, A., Kemptes, C., Goerge, T., Lin, C. Y., D’Andrea, M. R., Ständer, S., and Steinhoff, M. (2009) Role of matrisse and proteinase-activated receptor-2 in nonmelanoma skin cancer. *J. Invest. Dermatol.* **129**, 1816–1823
13. Takeuchi, T., Harris, J. L., Huang, W., Yan, K. W., Coughlin, S. R., and Craik, C. S. (2000) Cellular localization of membrane-type serine protease 1 and identification of protease-activated receptor-2 and single-chain urokinase-type plasminogen activator as substrates. *J. Biol. Chem.* **275**, 26333–26342
14. Liu, C., Li, Q., Zhou, X., Kolosov, V. P., and Perelman, J. M. (2013) Human airway trypsin-like protease induces mucins5AC hypersecretion via a protease-activated receptor 2-mediated pathway in human airway epithelial cells. *Arch. Biochem. Biophys.* **535**, 234–240
15. Wilson, S., Greer, B., Hooper, J., Zijlstra, A., Walker, B., Quigley, J., and Haw, M. R. (2008) The membrane-anchored serine protease, TMPRSS2, activates PAR-2 in prostate cancer cells. *Biochem. J.* **388**, 967–972
16. Camerer, E., Barker, A., Duong, D. N., Ganesan, R., Kataoka, H., Cornelisse, S., Tarragó, M. R., D’Andrea, C. D., and Chai, K. X. (2001) Prostasin is a glycosylphosphatidylinositol-anchored active serine protease.
17. Sales, K. U., Friis, S., Konkel, J. E., Godiksen, S., Hansen, K. K., Rogatto, S. R., Saabo, R., Vogel, L. K., Chen, W., Gutkind, J. S., and Bugge, T. H. (2012) Reduced prostasin (CAP1/PRSS8) activity eliminates HAI-1 and HAI-1 deficiency-associated developmental defects by preventing matrisse activation. *Plos Genet.* **8**, e1002937
18. Sales, K. U., Friis, S., Konkel, J. E., Godiksen, S., Hatakeyama, M., Hansen, K. K., Rogatto, S. R., Saabo, R., Vogel, L. K., Chen, W., Gutkind, J. S., and Bugge, T. H. (2010) Non-hematopoietic PAR-2 is essential for matrisse-driven pre-malignant progression and potentiation of ras-mediated squamous cell carcinogenesis. *Oncogene* **10.1038/3401061a**
19. Inoue, M., Isobe, M., Itoyama, T., and Kido, H. (1999) Structural analysis of the Fn14 cytoplasmic tail binds tumour-necrosis-factor-receptor-associated factors 1 and 2 and mediates nuclear factor-κB activation. *Biochem. J.* **371**, 395–403
20. Kao, J. P., Li, G., and Auston, D. A. (2010) Practical aspects of measuring intracellular calcium signals with fluorescent indicators. *Methods Cell Biol.* **99**, 113–152
21. Minta, A., Kao, J. P., and Tsien, R. Y. (1989) Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. *J. Biol. Chem.* **264**, 8171–8178
22. Adam, M. N., Christensen, M. E., He, Y., Waterhouse, N. J., and Hooper, J. D. (2011) The role of palmitoylation in signalling, cellular trafficking and plasma membrane localization of protease-activated receptor-2. *PLoS One* **6**, e28018
23. Koopman, W., Steffen, D., and Settmeier, U. (2011) Protease-activated receptor 2-mediated calcium signaling in hepatocellular carcinoma cells. *J. Cancer Res. Clin. Oncol.* **137**, 965–973
24. Tanaka, Y., Sekiguchi, F., Hong, H., and Wadayama, A. (2008) PAR2 triggers IL-8 release via MEK/ERK and PI3-kinase/Akt pathways in GI epithelial cells. *Biochem. Biophys. Res. Commun.* **377**, 622–626
25. Yu, J. X., Chao, L., and Chao, J. (1994) Prostasin is a novel human serine protease from seminal fluid. Purification, tissue distribution, and localization in prostate gland. *J. Biol. Chem.* **269**, 18843–18848
26. Yoshida, N., Katada, K., Vo, T., Naito, Y., Muñoz-Kuna, S., Shimada, Y., Yoshikawa, T., and Okanoue, T. (2007) Interaction of testisin with mmpin and its impact on invasion and cell death resistance of cervical cancer cells. *FEBS Lett.* **584**, 1469–1475
27. Yoshida, S., Katada, K., Handa, O., Takagi, T., Okanoue, T., Naito, Y., Muñoz-Kuna, S., Shimada, Y., Yoshikawa, T., and Okanoue, T. (2007) Interleukin-8 production via protease-activated receptor 2 in human esophageal epithelial cells. *Int. J. Mol. Med.* **19**, 335–340
28. Osuga, Y., Hirota, Y., Oshino, O., Hirata, T., Koga, T., and Taketani, Y. (2012) Protease-activated receptors in the endometrium and endometriosis. *Front. Biosci.* **4**, 1201–1212
29. Nystedt, S., Ramakrishnan, V., and Sundelin, J. (1996) The proteinase-activated receptor 2 is induced by inflammatory mediators in human en-
dothelial cells. Comparison with the thrombin receptor. J. Biol. Chem. 271, 14910–14915
47. Schechter, N. M., Brass, L. F., Lavker, R. M., and Jensen, P. J. (1998) Reaction of mast cell proteases tryptase and chymase with protease activated receptors (PARs) on keratinocytes and fibroblasts. J. Cell Physiol. 176, 365–373
48. Adams, M. N., Pagel, C. N., Mackie, E. J., and Hooper, J. D. (2012) Evaluation of antibodies directed against human protease-activated receptor-2. Naunyn Schmiedebergs Arch. Pharmacol. 385, 861–873
49. Ramachandran, R., Noorbakhsh, F., Defea, K., and Hollenberg, M. D. (2012) Targeting proteinase-activated receptors: therapeutic potential and challenges. Nat. Rev. Drug Discov. 11, 69–86
50. Ricks, T. K., and Trejo, J. (2009) Phosphorylation of protease-activated receptor-2 differentially regulates desensitization and internalization. J. Biol. Chem. 284, 34444–34457
51. Russo, A., Soh, U. J., and Trejo, J. (2009) Proteases display biased agonism at protease-activated receptors: location matters! Mol. Interv. 9, 87–96
52. Scarman, A. L., Hooper, J. D., Boucaut, K. J., Sit, M. L., Webb, G. C., Normyle, J. F., and Antalis, T. M. (2001) Organization and chromosomal localization of the murine Testisin gene encoding a serine protease temporally expressed during spermatogenesis. Eur. J. Biochem. 268, 1250–1258
53. Awasthi, V., Mandal, S. K., Papanna, V., Rao, L. V., and Pendurthi, U. R. (2007) Modulation of tissue factor-factor VIIa signaling by lipid rafts and caveolae. Arterioscler. Thromb. Vasc. Biol. 27, 1447–1455
54. Pendurthi, U. R., and Rao, L. V. (2008) Role of tissue factor disulfides and lipid rafts in signaling. Thromb. Res. 122, S14–S18
55. Weidinger, S., Mayerhofer, A., Frungieri, M. B., Meineke, V., Ring, J., and Kohn, F. M. (2003) Mast cell-sperm interaction: evidence for tryptase and proteinase-activated receptors in the regulation of sperm motility. Hum. Reprod. 18, 2519–2524
56. Miyata, H., Thaler, C. D., Hairoo, L. T., and Cardullo, R. A. (2012) Protease activation and the signal transduction pathway regulating motility in sperm from the water strider Aquarius remigis. Cytoskeleton 69, 207–220
57. Schaffner, F., and Ruf, W. (2009) Tissue factor and PAR2 signaling in the tumor microenvironment. Arterioscler. Thromb. Vasc. Biol. 29, 1999–2004
58. Del Rosso, M., Fibbi, G., Pucci, M., D’Alessio, S., Del Rosso, A., Magnelli, L., and Chiarugi, V. (2002) Multiple pathways of cell invasion are regulated by multiple families of serine proteases. Clin. Exp. Metastasis 19, 193–207
59. Jahan, I., Fujimoto, J., Alam, S. M., Sato, E., Sakaguchi, H., and Tamaya, T. (2007) Role of protease activated receptor-2 in tumor advancement of ovarian cancers. Ann. Oncol. 18, 1506–1512
60. Versteeg, H. H., Spek, C. A., Richel, D. J., and Peppelenbosch, M. P. (2004) Coagulation factors VIIa and Xa inhibit apoptosis and anoikis. Oncogene 23, 410–417
61. Sorensen, B. B., Rao, L. V., Tornehave, D., Gammeltoft, S., and Petersen, L. C. (2003) Antiapoptotic effect of coagulation factor VIIa. Blood 102, 1708–1715