Protease-Activated Receptor-1 Can Mediate Responses to SFLLRN in Thrombin-Desensitized Cells: Evidence for a Novel Mechanism for Preventing or Terminating Signaling by PAR1's Tethered Ligand†

Stephen R. Hammes‡,§,|| and Shaun R. Coughlin*,‡,§,⊥

Cardiovascular Research Institute, Department of Medicine, Division of Endocrinology, and Department of Cellular and Molecular Pharmacology, University of California, San Francisco, San Francisco, California 94143

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ABSTRACT: The thrombin receptor PAR1 is activated when thrombin cleaves the receptor's amino-terminal exodomain to reveal the new N-terminal sequence SFLLRN which then acts as a tethered peptide ligand. Free SFLLRN activates PAR1 independent of receptor cleavage and has been used to probe PAR1 function in various cells and tissues. PAR1-expressing cells desensitized to thrombin retain responsiveness to SFLLRN. Toward determining the mechanism of such responses, we utilized fibroblasts derived from a PAR1-deficient mouse. These cells were unresponsive to thrombin and SFLLRN and became sensitive to both ligands after transfection with human PAR1 cDNA. Moreover, PAR1-transfected cells responded to SFLLRN after thrombin-desensitization, indicating that signaling of thrombin-desensitized cells to SFLLRN was mediated by PAR1 itself. SFLLRN caused signaling in thrombin-desensitized cells when no uncleaved PAR1 was detectable on the cell surface; however, cleaved PAR1 was present. To determine whether the cleaved receptors could still signal, fibroblasts were transfected with a PAR1 mutant containing a trypsin site/SFLLRN sequence carboxyl terminal to the native thrombin site. These cells retained responsiveness to trypsin after thrombin-desensitization. Conversely, fibroblasts expressing a PAR1 mutant with the trypsin site/SFLLRN sequence amino terminal to the native thrombin site retained responsiveness to thrombin after trypsin-desensitization. This suggests that a population of thrombin-cleaved PAR1 can respond both to exogenous SFLLRN and to a second tethered ligand. In this population, the tethered ligand unmasked by thrombin cleavage must not be functional, suggesting the possibility of a novel mechanism of receptor shutoff involving sequestration or modification of the tethered ligand to prevent or terminate its function.

The thrombin receptor protease-activated receptor-1 (PAR1)[1] is a seven-transmembrane domain G protein-coupled receptor (GPCR) that is activated by an unusual proteolytic mechanism (*1*−*3*). Thrombin binds to and cleaves the receptor's amino-terminal exodomain to unmask a new amino-terminal sequence, SFLLRN. This new amino terminus then serves as a tethered peptide agonist, binding intramolecularly to the body of the receptor to effect receptor activation. The free synthetic peptide SFLLRN also acts as a PAR1 agonist, activating PAR1 in the absence of thrombin and without the need for receptor cleavage.

Thrombin-mediated cleavage presents PAR1 with a ligand that cannot diffuse away. Cleaved receptors might therefore be expected to signal indefinitely. In fact, each activated receptor appears to signal only briefly (*4*). As for other GPCRs, the acute uncoupling of PAR1 from signaling appears to be mediated at least in part by receptor phosphorylation (*5*−*7*). However, for classical GPCRs this uncoupling mechanism is reversible (*5*, *7*), implying that other mechanisms must exist to prevent persistent signaling by thrombin-cleaved PAR1 molecules. One such mechanism involves PAR1 trafficking. Upon activation by thrombin, most cleaved cell surface PAR1 is internalized and degraded in lysosomes (*8*, *9*). However, in some cell types expressing PAR1, cleaved receptors were detected on the cell surface even after prolonged exposure to thrombin (*4*, *10*). Such cells failed to respond to a second thrombin challenge, consistent with the lack of detectable uncleaved receptors on their surface. Surprisingly, however, such thrombin-desensitized cells still signaled in response to exogenous SFLLRN peptide (*4*). This second signal has been attributed to mechanisms independent of PAR1 (*11*). For example, SFLLRN is known to be capable of activating PAR2 (*12*, *13*). PAR2 is thrombin-insensitive and could well mediate SFLLRN signaling after thrombin-desensitization in cell types which express both PAR1 and PAR2. Here we report studies using fibroblasts derived from PAR1-deficient mice which suggest that a second signal to SFLLRN can be mediated by PAR1 itself, apparently by receptors previously cleaved by thrombin. In

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* Address correspondence to this author at HSW-1300, Box 0130, University of California, San Francisco, 505 Parnassus Ave., San Francisco, CA 94143-0130. Telephone: 415-476-6174. FAX: 415-476-8173. E-mail: shaun_coughlin@quickmail.ucsf.edu.

‡ Cardiovascular Research Institute.

§ Department of Medicine.

|| Division of Endocrinology.

⊥ Department of Cellular and Molecular Pharmacology.

[1] Abbreviations: PAR, protease-activated receptor; GPCR, G protein-coupled receptor; CM, complete media; LPA, lysophosphatidic acid; PBS, phosphate-buffered saline.

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addition, these cleaved receptors, while no longer responding to the tethered ligand unmasked by thrombin, appear capable of responding to the unmasking of a second tethered ligand carboxyl to the first. Together, these data suggest the possibility of a novel mechanism to prevent or terminate the function of PAR1's tethered ligand domain.

MATERIALS AND METHODS

Plasmid Construction. Mutated PAR1 cDNAs were derived from cDNA encoding human PAR1 bearing a FLAG epitope at its amino terminus (*4*, *14*) by oligonucleotide-directed mutagenesis (*15*). Wild-type and mutant receptor cDNAs were subcloned into the mammalian expression vector pBJ1 (provided by Mark Davis, Stanford University, Palo Alto, CA).

Cell Culture. The lung fibroblast cell lines from both wild-type mice and PAR1 knockout mice (*16*) were previously described (*17*). Cell lines were cultured in complete media (CM), which consisted of Dulbecco's modified Eagle's medium H-16: 3 g/L glucose, 0.584 g/L L-glutamine, 0.11 g/L sodium pyruvate, 3.7 g/L $NaHCO_3$, supplemented 10% bovine calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL fungizone. The fibroblast lines derived from PAR1-deficient mice showed no increases in cytosolic calcium in response to thrombin, trypsin, SFLLRN, or SLIGRL [(*17*) and data not shown], suggesting that none of the known PARs (PAR1, PAR2, PAR3, or PAR4) mediate signaling in these cells.

Transfection. Fibroblasts derived from PAR1 knockout mice were stably transfected with the recombinant human thrombin receptors using LipofectAMINE (Life Technologies, Inc.) as described (*9*). Briefly, cells were grown to 75% confluency in a 100 mm tissue culture dish. Then 3.1 μg of the plasmid containing the recombinant thrombin receptor was cotransfected with 0.4 μg of a plasmid containing the hygromycin resistance gene, 30 μL of lipofectamine, and 5 mL of OptiMem. After 5 h at 37 °C, the medium was removed from cells and replaced with CM. Cells were split into larger flasks on the following day, and hygromycin was added at a concentration of 200 μg/mL on the third day. Individual colonies of cells were then picked after antibiotic selection and expanded. Stable cell lines were selected by cell-surface ELISA using M1 antibody (Kodak) to the FLAG epitope at the amino terminus of wild-type PAR1, Tr/Th, and Th/Tr (Figure 3). Lines were chosen to have relatively similar expression levels (Figure 4).

Measurement of Cell-Surface Expression. PAR1 on the surface of cells was detected by cell-surface ELISA (*9*). Cells were incubated at 25 °C in RPMI containing 1 mg/ml BSA (RPMI/BSA) in the presence or absence of 10 nM α-thrombin (Enzyme Research Labs). Cells were then fixed after various times with 4% PFA in PBS for 5 min at 25 °C, followed by 2 rinses with PBS alone. Fixed cells were then incubated for 1 h at 25 °C with receptor antibodies in RPMI/BSA. Uncleaved receptors were detected using the mouse monoclonal M1 anti-FLAG antibody (Kodak). Total cell-surface receptors (both cleaved and uncleaved) were measured using a rabbit anti-human PAR1 antibody (*4*) raised to the peptide YEPFWEDEEKNESGLTEYC (*18*), which represents a sequence carboxyl to the thrombin cleavage site in the receptor's amino-terminal exodomain. After incubation with primary antibodies, cells were washed twice with PBS and incubated for 30 min with horseradish peroxidase (HRP)-coupled goat anti-mouse or goat anti-rabbit antibodies (Bio-Rad) in RPMI/BSA. Cells were again washed twice with PBS and then incubated in One Step ABTS solution (Pierce) for 10 min. Finally, the absorbance of the supernatant was measured at a wavelength of 405 nm using a Molecular Devices microplate spectrophotometer.

Calcium Mobilization Assay. Increases in cytoplasmic calcium in response to agonists were measured using the fluorescent probe fura-2AM (Molecular Probes) (*4*, *16*, *19*). Adherent fibroblasts were removed from the surface of flasks using Cell Dissociation Buffer (GibcoBRL) and were loaded with fura-2AM at 37 °C for 30 min in RPMI/BSA. Cells were washed with RPMI/BSA twice before resuspension at 10^6 cells/ml in RPMI/BSA at 25 °C. Calcium mobilization was measured at 25 °C in a Hitachi F2000 fluorometer. Agonists included 10 nM α-thrombin (Enzyme Research Labs), 20 nM trypsin (Sigma), 1 μM LPA (Sigma), and 100 μM SFLLRN peptide. Duration and order of agonist addition is described in the figure legends.

Phosphoinositide Hydrolysis Assay. The release of [^{3}H]-inositol phosphates in response to various agonists was measured as described (*4*, *20*). Briefly, cells were incubated overnight with [^{3}H]inositol (NEN), washed, and treated with the specific agonist as described in the figure legends. Then 20 mM lithium chloride was added to block metabolism of [^{3}H]inositol monophosphate. Cells were lysed, and total [^{3}H]-inositol phosphates were quantitated (*21*).

RESULTS

Thrombin-Desensitized Fibroblasts Remain Sensitive to Activation by the Agonist Peptide SFLLRN. Activation of PAR1-expressing cells by thrombin or exogenous agonist peptide was followed by measuring intracellular calcium mobilization using the fluorescent calcium indicator fura-2. Wild-type mouse lung fibroblasts responded rapidly to thrombin with an increase in intracellular calcium concentration (Figure 1A). Intracellular calcium returned to base line by 5 min after thrombin addition. Subsequent thrombin challenge did not yield a second increase in intracellular calcium. Surprisingly, while these cells were refractory to rechallenge with thrombin, they were responsive to the PAR1 agonist SFLLRN (Figure 1A). Similar results are seen using other cell lines (*4*, *11*).

PAR1 Can Mediate the Response to Agonist Peptide SFLLRN in Cells Desensitized to Thrombin. To determine whether the response of thrombin-desensitized cells to exogenous SFLLRN agonist peptide was mediated by PAR1 itself or by another receptor (*4*, *11*), signaling was studied in lung fibroblasts derived from knockout mice lacking the PAR1 gene. Unlike the cognate fibroblasts derived from wild-type mice (Figure 1A), the PAR1 knockout fibroblasts did not respond to either thrombin or SFLLRN agonist peptide (Figure 1B) (*17*). Lysophosphatidic acid (LPA) did cause calcium mobilization in these cells, indicating that they are still capable of mobilizing calcium upon activation of appropriate G protein-coupled receptors.

Transfection of wild-type PAR1 into PAR1 knockout fibroblasts restored calcium signaling to both thrombin and SFLLRN. Moreover, these cells now showed continued

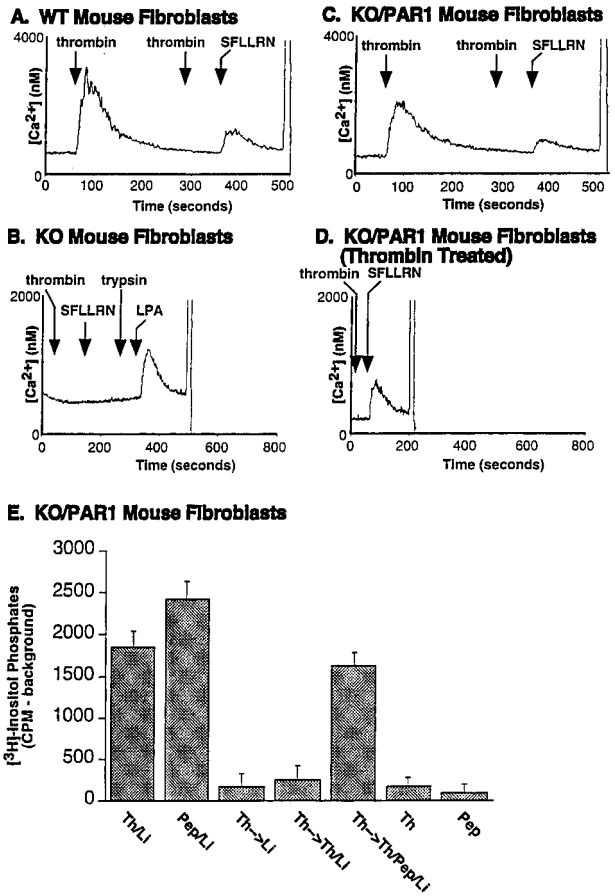

FIGURE 1: PAR1-mediated signaling in mouse fibroblast cell lines. Lung fibroblasts derived from wild-type mice (A) and PAR1 knockout mice (B) were examined for their ability to respond to either thrombin (10 nM), SFLLRN (100 μM), trypsin (20 nM), or LPA (1 μM). PAR1-transfected knockout fibroblasts stably expressing human PAR1 were also tested, both without (C) and with (D) thrombin pretreatment (10 nM for 60 min at 37 °C). Cells were loaded with fura-2AM, and calcium mobilization was examined using a fluorometer as described under Materials and Methods. The ordinate represents the concentration of intracellular calcium. Agonists were added sequentially at the indicated time points. All experiments were reproduced at least 3 times. Agonist-triggered inositol phosphate release from [^{3}H]inositol-labeled cells was measured as described under Materials and Methods (E). The ordinate represents counts recovered in total inositol phosphates after the indicated agonist treatment minus background. Background was defined as counts recovered in total inositol phosphates after 60 min at room temperature with LiCl (Li) and no agonist, and was 764 cpm in the experiment shown. Cells were incubated at room temperature for 60 min with 10 nM thrombin (Th) or 100 μM SFLLRN peptide (Pep) in the presence or absence of 20 mM LiCl (Li), and then washed (→) and further incubated as indicated. No agonist-stimulated accumulation of inositol phosphates was detected in the absence of LiCl nor in untransfected PAR1 knockout fibroblasts treated with either thrombin or SFLLRN in the presence of lithium. Data shown are mean ± SD (n = 3). This experiment was performed 3 times with similar results.

responsiveness to SFLLRN after desensitization to thrombin (Figure 1C), even after an hour of thrombin treatment at 37 °C (Figure 1D).

Similar experiments were performed using phosphoinositide hydrolysis as a more quantitative measurement of PAR1 signaling. As in the calcium mobilization studies, PAR1 knockout mouse fibroblasts showed no response to either thrombin or SFLLRN agonist peptide in this assay [not shown, (*17*)]. In contrast, knockout fibroblasts transfected with PAR1 showed robust phosphoinositide hydrolysis in response to saturating concentrations of either thrombin or agonist peptide (Figure 1E, Th/Li and Pep/Li, respectively). To examine second responses in these cells, cultures were initially exposed to thrombin in the absence of LiCl. Under these conditions, inositol phosphates generated by thrombin-triggered phosphoinositide hydrolysis are rapidly metabolized and therefore not detected. Cells were then washed with media and incubated with either LiCl alone, LiCl and thrombin, or LiCL, thrombin, and peptide. After 60 min of exposure to thrombin, addition of LiCl in the continued presence or absence of thrombin caused little accumulation of inositol phosphates. (Figure 1E, Th→Li and Th→Th/Li). In contrast, exposure of thrombin-pretreated cells to the SFLLRN agonist peptide in the continued presence (Figure 1E, Th→Th/Pep/Li) or absence of thrombin (data not shown) yielded a signal nearly 70% of that observed with stimulation by SFLLRN and LiCl without pretreatment (Figure 1E, Pep/Li). These data confirm the results from the calcium mobilization experiment, demonstrating a PAR1 transfection-dependent response to agonist peptide in thrombin-desensitized cells. The loss of both thrombin and SFLLRN responses in PAR1 knockout fibroblasts and the reconstitution not only of thrombin and SFLLRN signaling but also of SFLLRN signaling after thrombin desensitization suggest that the second peptide-induced signal does not require a second receptor like PAR2. Rather, SFLLRN signaling after thrombin desensitization can be mediated solely by PAR1.

Cleaved but Not Uncleaved PAR1 Can Be Detected on the Cell Surface after Prolonged Exposure to Thrombin. The SFLLRN-mediated response in thrombin-desensitized cells may be due to incomplete cleavage and activation of PAR1 by thrombin. To test the extent of PAR1 cleavage by thrombin during the desensitization period used in the above studies, the relative amounts of cleaved and uncleaved PAR1 present on the surface of knockout fibroblasts stably expressing PAR1 were determined at various times after exposure to thrombin (Figure 2). The PAR1 cDNAs used in this study encoded a FLAG epitope amino terminal to PAR1's thrombin cleavage site such that the epitope is cleaved from the receptor by thrombin (*4*) (Figure 3). Antibodies to the FLAG epitope thus detect only uncleaved receptors on the cell surface. Total (cleaved and uncleaved) cell-surface PAR1 was detected using a rabbit polyclonal antibody against the human PAR1 hirudin-like domain, which is located carboxyl terminal to the thrombin cleavage site (Figure 3). Within 5 min of exposure to thrombin, over 80% of cell-surface PAR1 was cleaved; by 40 min no uncleaved receptors bearing the FLAG epitope were detected on the cell surface (Figure 2). By contrast, the level of hirudin-like domain antibody binding decreased by half within 10 min of exposure to thrombin, but at 40 min the level of binding was still 30% of the original level. Together, these data suggest SFLLRN had caused signaling (Figure 1) at a time when cleaved PAR1, but no uncleaved PAR1, could be detected on the cell surface.

Thrombin-Desensitized Cells Expressing PAR1 Containing an Additional Proteolytic Cleavage Site and Tethered Ligand Are Capable of Resignaling in Response to the Second Tethered Ligand. It was possible that SFLLRN-triggered signaling in thrombin-desensitized cells was mediated by a pool of PAR1 accessible to neither thrombin nor antibody. If so, then such a pool should not be accessible to other

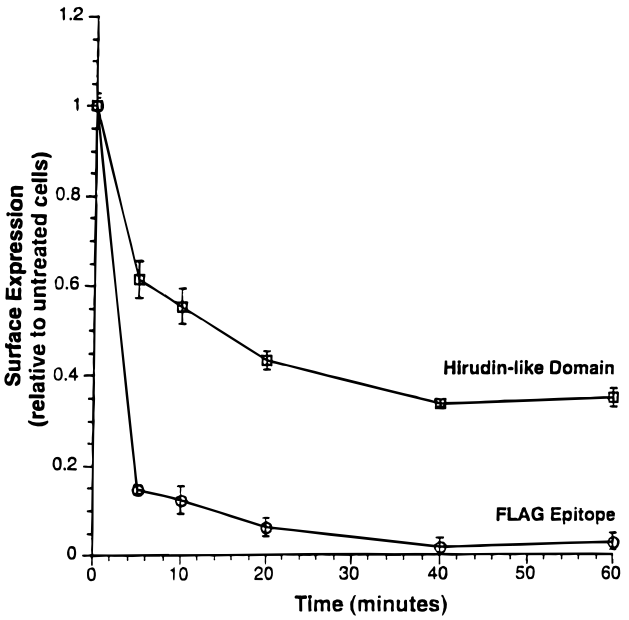

FIGURE 2: Detection of cleaved and uncleaved PAR1 on the surface of mouse fibroblasts exposed to thrombin. PAR1 knockout fibroblasts stably expressing human PAR1 bearing a FLAG epitope amino terminal to PAR1's thrombin cleavage site (see Figure 3) were exposed to 10 nM thrombin for the indicated times. Uncleaved receptors were detected by cell-surface binding of M1 antibody to the FLAG epitope. Both cleaved and uncleaved receptors were detected by surface binding of 1809 antibody to PAR1's hirudin-like domain, which is located carboxyl terminal to the thrombin cleavage site (Figure 3; WT). The ordinate represents the amount of antibody binding relative to untreated cells. Background binding defined as the amount of antibody bound to the untransfected PAR1 knockout fibroblasts was subtracted from all points. Background for M1 binding was 14% of the binding to untreated PAR1-expressing cells, while background for 1809 binding was 16% of binding to untreated PAR1-expressing cells. Data shown are mean ± SD ($n = 3$). Similar experiments were performed 2 times with comparable results.

proteases of size similar to thrombin. To test this prediction, PAR1 mutants designated Th/Tr and Tr/Th were generated. In Th/Tr, a trypsin cleavage site/SFLLRN sequence was introduced carboxyl terminal to the native thrombin cleavage site/SFLLRN sequence and hirudin-like domain (Figure 3). In Tr/Th, the trypsin site/SFLLRN cassette was introduced amino terminal to the native thrombin site/SFLLRN sequence. With the caveat that trypsin can act weakly at PAR1's native thrombin cleavage site (Figure 4), thrombin and trypsin should independently unmask a PAR1 tethered ligand in Th/Tr and Tr/Th. In the case of Th/Tr, trypsin should unmask a second SFLLRN sequence even in Th/Tr receptors previously cleaved by thrombin; in the case of Tr/Th, the converse should hold (Figure 3).

Cleavage of Th/Tr and Tr/Th on the surface of stably transfected PAR1 knockout mouse fibroblasts was examined. Exposure to either 10 nM thrombin or 20 nM trypsin for 5 min resulted in complete removal of the FLAG epitope from the surface of Th/Tr and Tr/Th expressing cells (Figure 4A). In contrast, wild-type PAR1 was relatively insensitive to trypsin cleavage. The small decrease in FLAG epitope after trypsin treatment of wild-type PAR1-expressing cells was likely secondary to some trypsin cleavage at the thrombin site. These data suggested that the new trypsin sites in Th/Tr and in Tr/Th were efficiently cleaved by trypsin. Thrombin treatment of cells expressing wild-type PAR1, Th/Tr, and Tr/Th resulted in a decrease in cell-surface binding of antibodies to PAR1's hirudin-like domain, consistent with internalization of activated receptors (Figure 4B). Treatment of Th/Tr-expressing cells with trypsin eliminated detectable hirudin-like domain from the cell surface, consistent with the trypsin sites being carboxyl terminal to the binding site for this antibody in Th/Tr (Figure 4B). Similar trypsin treatment of Tr/Th-expressing cells caused only an approximately 30% decrease in cell-surface expression of the hirudin-like domain, consistent with the amino-terminal location of the trypsin site relative to the antibody binding site in Tr/Th and with some internalization of trypsin-activated receptors.

After confirming that Th/Tr was cleaved appropriately, its signaling in response to thrombin and trypsin was examined by measuring calcium mobilization. Untransfected mouse PAR1 knockout fibroblasts did not respond to trypsin, thrombin, or agonist peptide (Figure 1B). As expected, cells expressing wild-type PAR1 or Th/Tr responded to 10 nM thrombin (Figure 5A, B, respectively). Th/Tr-expressing cells demonstrated robust signaling to 20 nM trypsin (Figure 5B). Wild-type PAR1-expressing cells also responded to trypsin, most likely due to some cleavage at the thrombin cleavage site by trypsin (Figures 4A and 5A).

After initially responding to thrombin, wild-type PAR1-expressing cells became refractory to both thrombin and trypsin (Figure 5A). This is consistent with the receptor cleavage data indicating that thrombin cleaved nearly all the cell-surface receptors within 5 min. As expected, these cells responded to agonist peptide after both thrombin and trypsin treatment. In addition, cells treated first with trypsin still responded to thrombin (Figure 5A), consistent with trypsin cleaving only a minority of PAR1 molecules at the thrombin cleavage site, thereby leaving uncleaved receptors on the cell surface for activation by thrombin (Figure 4A).

Unlike wild-type PAR1-expressing cells, Th/Tr-expressing cells responded to trypsin after desensitization to thrombin, but did not respond to thrombin after trypsin activation (Figure 5B). Mutation of the tethered ligand sequence carboxyl to the trypsin cleavage site in Th/Tr abolished its ability to convey trypsin responsiveness in thrombin-desensitized cells (Figure 5C). This confirmed that trypsin was activating receptors via the expected cleavage site in thrombin-desensitized cells. These data suggested that Th/Tr receptors that were cleaved by thrombin could signal again if cleaved a second time by trypsin and thereby again presented with a functional tethered ligand. However, it was still possible that trypsin signaling in Th/Tr-expressing cells that were desensitized to thrombin was mediated by a small population of receptors not cleaved by thrombin—a population too small to be detected in the antibody binding studies. In this scenario, trypsin must be viewed as a "better" enzyme than thrombin, and thus capable of causing calcium signaling by acting upon a small cohort of receptors, or as able to access a receptor pool not seen by thrombin.

To test this possibility, we constructed the Tr/Th mutant in which the trypsin cleavage site/tethered ligand sequence was inserted amino rather than carboxyl terminal to PAR1's native thrombin cleavage site (Figures 3 and 5E). Tr/Th-expressing cells exposed to thrombin became refractory to both thrombin and trypsin, implying that thrombin did cleave all available thrombin cleavage sites; no cohort of receptors

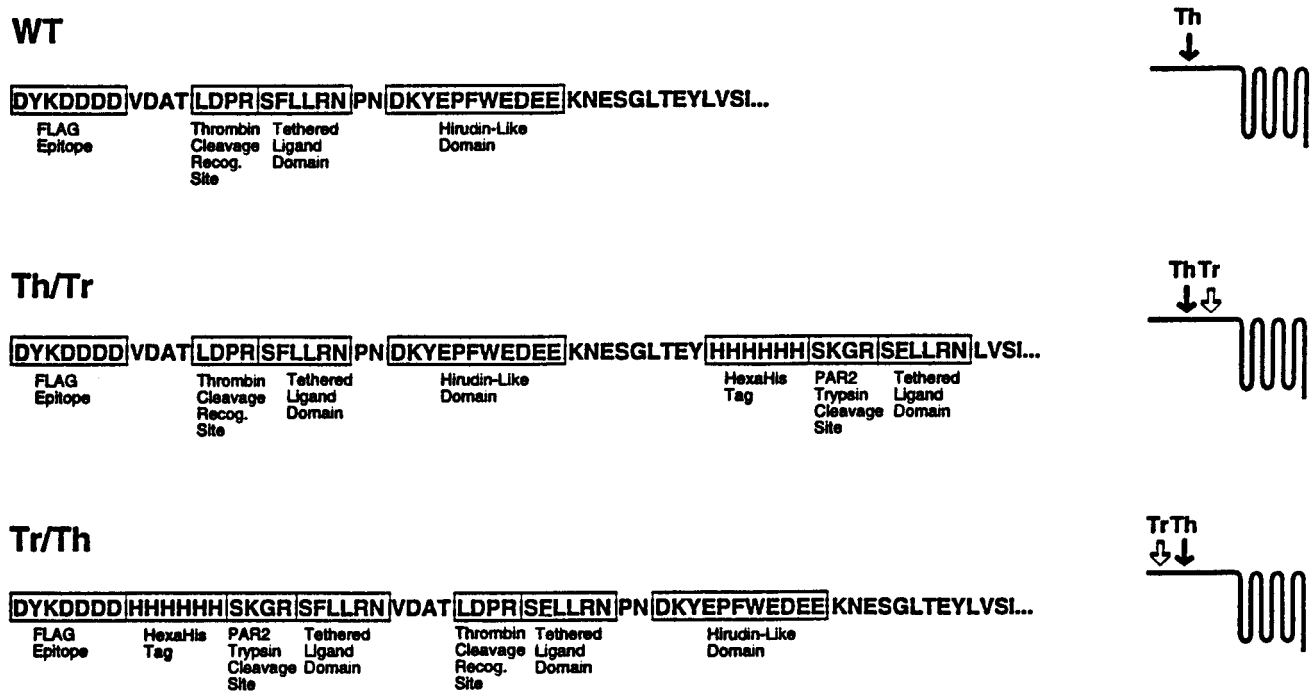

FIGURE 3: Amino-terminal exodomains of epitope-tagged wild-type PAR1 and mutants. Amino-terminal exodomain sequences encoded by wild-type and mutant cDNAs are shown. The FLAG epitope, thrombin and trypsin cleavage sites, tethered ligand domains, hirudin-like domain, and other features are indicated. Note that the Th/Tr PAR1 mutant contains a trypsin cleavage site poised to unmask a second SFLLRN tethered ligand domain which is carboxyl terminal to the endogenous thrombin cleavage site and SFLLRN sequence. The trypsin site was derived from PAR2 (*27*). The Tr/Th PAR1 mutant contains the same trypsin cleavage site/SFLLRN sequence inserted amino terminal to the endogenous thrombin cleavage site and tethered ligand. Two additional mutants in which the inserted tethered ligand was rendered nonfunctional by substitution of alanine for phenylalanine in SFLLRN (underlined F) were also utilized (Figure 5C, D).

that could signal in response to trypsin was detected. By contrast, Tr/Th-expressing cells exposed first to trypsin became refractory to trypsin but remained responsive to thrombin (Figure 5E). Cells expressing Tr/Th containing a nonfunctional tethered ligand adjacent to the thrombin cleavage site (Tr/ThF→A) still responded to an initial exposure to tryspin but were unresponsive to subsequent challenge with thrombin (Figure 5D), confirming that trypsin caused signaling by cleavage at the trypsin site and not at the thrombin cleavage site, and that signaling to thrombin after desensitization to trypsin required cleavage at the thrombin site. To explain thrombin signaling in trypsin-desensitized Tr/Th cells as due to thrombin acting at a minor receptor population not activated by trypsin, one would now have to postulate that thrombin was a "better" enzyme than trypsin. This is inconsistent with the behavior of the Th/Tr mutant described above.

DISCUSSION

In these studies, we set out to characterize the mechanism by which the PAR1 agonist peptide SFLLRN triggers reponses in thrombin-desensitized cells (*4*, *10*, *11*). In endothelial cells, such responses have been attributable to SFLLRN possibly activating other protease-activated receptors; SFLLRN can activate PAR2, and PAR2 is indeed expressed in endothelial cells (*11*). Our studies used fibroblasts derived from PAR1-deficient mice, which do not respond to either thrombin or SFLLRN peptide. Transfection of these cells with human PAR1 cDNA reconstituted signaling to thrombin and the SFLLRN response in thrombin-desensitized cells. While we cannot exclude the formal possibility that SFLLRN acts at an unknown receptor that is distinct from PAR1 but requires PAR1 for function, a more parsimonious explanation is that SFLLRN responses in the PAR1-transfected knockout fibroblasts are mediated by PAR1 itself. This finding is important for interpretation of pharmacological studies in untransfected cell lines and tissues; persistent responsiveness to SFLLRN after desensitization to thrombin in such systems does not necessarily reflect the action of a receptor distinct from PAR1 (*22*).

How is PAR1 capable of mediating a response to SFLLRN in thrombin-desenstized cells? SFLLRN signaling in thrombin-desensitized cells occurred when only cleaved PAR1 was detectable on the cell surface (Figures 1D, 1E, and 2). This result left the possibilities that SFLLRN signaling in thrombin-desensitized cells might be mediated by a small population of uncleaved PAR1 remaining on the cell surface but not detected by ELISA, by uncleaved PAR1 in a compartment accessible to SFLLRN but not to the larger thrombin molecule or antibody, or, most interestingly, by previously cleaved PAR1. We addressed these possibilities by expressing PAR1 mutants containing a trypsin site/tethered ligand sequence (SKGR/SFLLRN) both carboxyl terminal (Th/Tr) and amino terminal (Tr/Th) to the native thrombin site/tethered ligand sequence in PAR1-deficient fibroblasts. Th/Tr-expressing cells made refractory to thrombin still responded to trypsin, while Tr/Th-expressing cells made refractory to trypsin still responded to thrombin. Responses to a second protease occurred only when the cleavage site/tethered ligand insert was carboxyl to the first protease's site, while initial cleavage at the carboxyl-terminal site eliminated signaling by subsequent addition of the

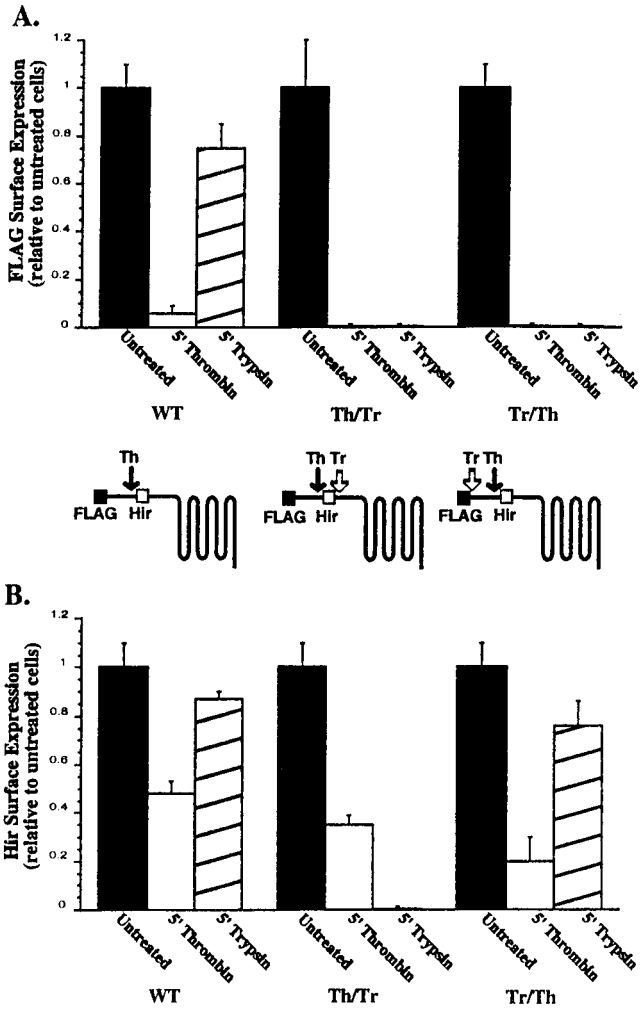

FIGURE 4: Expression and cleavage of wild-type PAR1 and mutants on the cell surface. Fibroblasts derived from a PAR1 knockout mouse were stably transfected with cDNAs encoding wild-type human PAR1 (WT) or the indicated mutants (see Figure 3 for key). Cultures were incubated for 5 min at room temperature in the presence or absence of thrombin (10 nM) or trypsin (20 nM) as indicated. Cultures were then washed and fixed, and cell-surface binding of M1 antibody to the FLAG epitope (A) or antibody 1809 to the hirudin-like domain (B) was measured as described under Materials and Methods. The receptor cartoons (center) indicate the relative positions of the FLAG (solid square) and hirudin-like domain (open square) epitopes relative to the thrombin (solid arrow) and trypsin (open arrow) cleavage sites. The ordinate represents antibody binding relative to that obtained before protease treatment. Background was defined as the amount of antibody binding to the untransfected PAR1 knockout fibroblasts and was subtracted from all points. Wild-type PAR1 was expressed approximately 5-fold above background, Th/Tr was expressed approximately 4-fold above background, and Tr/Th was expressed approximately 2.5-fold above background. Data represent mean ± SD ($n = 3$). This experiment was performed 3 times with similar results.

protease recognizing the amino-terminal site. This suggests complete cleavage of surface receptors by the first protease, and strongly suggests that the response to thrombin in trypsin-desensitized Tr/Th-expressing cells, as well as the response to trypsin seen in thrombin-desensitized Th/Tr-expressing cells, is mediated by previously cleaved receptors. The finding that cleaved receptors can signal when presented with a functional tethered ligand suggests that the response to free SFLLRN peptide seen in thrombin-desensitized cells expressing wild-type PAR1 is also likely to be mediated by previously cleaved receptors. Furthermore, the ability of a large protease molecule to access receptors and mediate signaling in cells previously cleaved and desensitized to an alternate protease argues against the hypothesis that the SFLLRN-mediated response in thrombin-desensitized cells was due to the activation of a pool of receptors accessible only to the small peptide.

How is it that previously cleaved receptors might be able to respond to unmasking of a new tethered ligand or to free SFLLRN peptide if the tethered ligand unmasked by the initial cleavage event is still functional? The ability of a second tethered ligand to mimic SFLLRN's ability to activate previously cleaved receptors militates against the hypothesis that free SFLLRN is an intrinsically better agonist than a tethered ligand and therefore able to cause additional signaling even after thrombin activates PAR1. Another possibility is that, while thrombin cleaves most PAR1 molecules productively at the R^{41}/S^{42} peptide bond to cause receptor activation, it or some other protease may cleave a fraction of receptors unproductively, perhaps at a site carboxyl terminal to R^{41}/S^{42}, such that the tethered ligand is removed. This would leave the receptor unactivated and refractory to thrombin stimulation but still responsive to free peptide. Removal of the receptor's amino-terminal exodomain by mutagenesis (*3*) or by cleavage with chymotrypsin (K. Ishii and S. R. Coughlin) or cathepsin G (*23*) indeed produced receptors refractory to thrombin but responsive to SFLLRN. Overdigestion of a polypeptide representing PAR1's amino-terminal exodomain with thrombin reveals cleavage only at the R^{41}/S^{42} site (*24*), and mutation of this site yields a receptor that is not detectably cleaved by thrombin (*6*). Thus, thrombin cleavage of PAR1's amino-terminal exodomain seems to occur virtually exclusively at the R^{41}/S^{42} site, making it unlikely that SFLLRN signaling is mediated by receptors cleaved by thrombin carboxyl to R^{41}/S^{42}. Still another possibility is that, independent of thrombin or receptor activation, a subset of PAR1 molecules may be cleaved by an alternative protease or otherwise modified so as to be refractory to thrombin but sensitive to SFLLRN.

If thrombin cleavage of PAR1 occurs only at the R^{41}/S^{42} site, then a subset of receptors must either never signal to thrombin (see last possibility above) or be cleaved, activated, and then cease responding to their tethered ligand yet remain capable of responding when presented with a new tethered ligand or free agonist peptide. This raises the intriguing possibility that a subset of cleaved thrombin receptors may have their tethered ligand somehow sequestered or inactivated. Sequestration might occur by binding an inhibitor. Inactivation might involve cellular proteases inactivating the tethered ligand either on the cell surface or after receptor internalization. A minor fraction of activated receptors returns to the cell surface in PAR1-transfected knockout fibroblasts (data not shown) and other cells (*10*); if these were modified in the manner postulated, they would be responsive to SFLLRN. Aminopeptidase-M has been reported to remove the N-terminal serine from free SFLLRN peptide, rendering it inactive (*25*), but the aminopeptidase inhibitors amastatin and bestatin and the dipeptidyl peptidase I inhibitor Gly-Phe-CHN_2 had no effect on PAR1 signaling (data not shown). Efforts to purify the peptide released by thrombin followed by trypsin cleavage of the Th/Tr molecule using the hexahistidine tag included in the recombinant protein

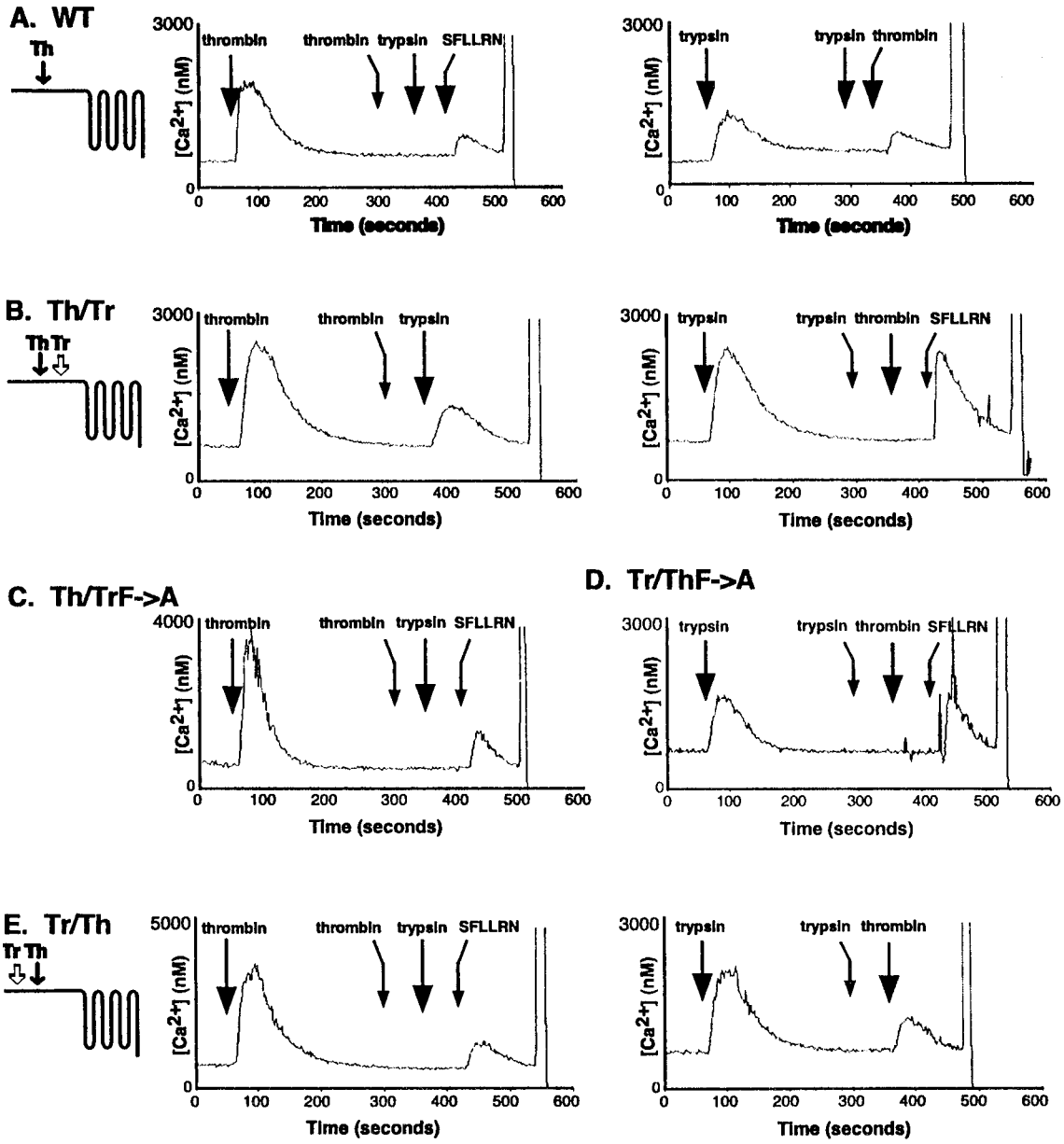

FIGURE 5: Calcium signaling by wild-type PAR1 and mutants. Agonist-triggered increases in cytoplasmic calcium were measured fluorometrically in transfected PAR1 knockout fibroblasts stably expressing either the wild-type PAR1 (WT) (A), Th/Tr (B), Th/Tr with a phenylalanine to alanine mutation in the tethered ligand carboxyl to the trypsin site (Th/TrF→A) (C), Tr/Th (E), or Tr/Th with a phenylalanine to alanine mutation in the tethered ligand adjacent to the thrombin cleavage site (Tr/ThF→A) (D). Cartoons indicating the relative positions of the thrombin (closed arrow) and trypsin (open arrow) cleavage site/SFLLRN cassettes are at left. Thrombin (10 nM), trypsin (20 nM), or SFLLRN (100 μM) were added at the indicated times. All experiments were repeated at least 3 times with similar results.

(Figure 3) have not yielded sufficient material for analysis; thus, it has not been possible to look for modification of the tethered ligand directly.

In summary, these studies strongly suggest that SFLLRN signaling in thrombin-desensitized PAR1-expressing cells can be mediated by PAR1. This observation is important for interpreting pharmacological experiments in which persistent responses to the PAR1 agonist peptide after thrombin desensitization might otherwise conjure the existence of distinct receptors. It would not be surprising if a similar phenomenon existed for other PARs. Our observations also suggest that such responses are likely mediated by receptors previously cleaved and activated by thrombin, raising the intriguing possibility of a novel mechanism of receptor shutoff that involves sequestration or modification of the tethered ligand agonist to render it nonfunctional. Endothelial cells and fibroblasts may be exposed to thrombin repeatedly over time, and maintaining the temporal fidelity of thrombin signaling is likely to be physiologically important. Modification or sequestration of PAR1's tethered ligand may provide a mechanism for terminating signaling by receptors that escape sorting to lysosomes (*26*).

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