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Article

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Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin

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Because of the role of thrombin and platelets in myocardial infarction and other pathological processes, identifying and blocking the receptors by which thrombin activates platelets has been an important goal. Three protease-activated receptors (PARs) for thrombin — PAR1, PAR3, and PAR4 — are now known. PAR1 functions in human platelets, and the recent observation that a PAR4-activating peptide activates human platelets suggests that PAR4 also acts in these cells. Whether PAR1 and PAR4 account for activation of human platelets by thrombin, or whether PAR3 or still other receptors contribute, is unknown. We have examined the roles of PAR1, PAR3, and PAR4 in platelets. PAR1 and PAR4 mRNA and protein were detected in human platelets. Activation of either receptor was sufficient to trigger platelet secretion and aggregation. Inhibition of PAR1 alone by antagonist, blocking antibody, or desensitization blocked platelet activation by 1 nM thrombin but only modestly attenuated platelet activation by 30 nM thrombin. Inhibition of PAR4 alone using a blocking antibody had little effect at either thrombin concentration. Strikingly, simultaneous inhibition of both PAR1 and PAR4 virtually ablated platelet secretion and aggregation, even at 30 nM thrombin. These observations suggest that PAR1 and PAR4 account for most, if not all, thrombin signaling in platelets and that antagonists that block these receptors might be useful antithrombotic agents.

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Introduction

Platelet activation is critical for normal hemostasis, and platelet-dependent arterial thrombosis underlies most myocardial infarctions. Thrombin is the most potent activator of platelets (1, 2). Characterization of the receptors that mediate thrombin's actions on platelets is therefore necessary for understanding hemostasis and thrombosis. Moreover, such receptors are potential targets for novel antiplatelet therapies.

Thrombin signaling is mediated at least in part by a family of G protein-coupled protease-activated receptors (PARs), for which PAR1 is the prototype (3, 4). PAR1 is activated when thrombin cleaves its NH₂-terminal exodomain to unmask a new receptor NH₂-terminus (3). This new NH₂-terminus then serves as a tethered peptide ligand, binding intramolecularly to the body of the receptor to effect transmembrane signaling (3, 5, 6). The synthetic peptide SFLLRN, which mimics the first six amino acids of the new NH₂-terminus unmasked by receptor cleavage, functions as a PAR1 agonist and activates the receptor independent of proteolysis (3, 7, 8). Such peptides have been used as pharmacological probes of PAR function in various cell types.

Our understanding of the role of PARs in platelet activation is evolving rapidly. PAR1 mRNA and protein were detected in human platelets (3, 9–11), SFLLRN activat-

ed human platelets (3, 7, 8), and PAR1-blocking antibodies inhibited human platelet activation by low, but not high, concentrations of thrombin (9, 10). These data suggested a role for PAR1 in activation of human platelets by thrombin but left open the possibility that other receptors might contribute.

Curiously, PAR1 appears to play no role in mouse platelets. PAR1-activating peptides did not activate rodent platelets (12–14), and platelets from PAR1-deficient mice responded like wild-type platelets to thrombin (14). This observation prompted a search for additional thrombin receptors and led to the identification of PAR3 (15). PAR3 is activated by thrombin and is expressed in mouse platelets. PAR3-blocking antibodies inhibited mouse platelet activation by low, but not high, concentrations of thrombin (16), and knockout of PAR3 abolished mouse platelet responses to low, but not high, concentrations of thrombin (17). These results established that PAR3 is necessary for normal thrombin signaling in mouse platelets but also pointed to the existence of another mouse platelet thrombin receptor. Such a receptor, PAR4, was recently identified (17, 18). PAR4 appears to function in both mouse and human platelets (17). Thus, available data suggest a testable working model in which PAR3 and PAR4 mediate thrombin activation

of mouse platelets and PAR1 and PAR4 mediate activation of human platelets. The role of PAR3, if any, in human platelets has not been determined. More broadly, the relative roles of PAR1, PAR3, and PAR4, and whether still other receptors also contribute to platelet activation by thrombin, are unknown.

To determine the roles of PAR1, PAR3, and PAR4 in activation of human platelets by thrombin, we examined expression of receptor mRNA and protein in platelets and probed receptor function with specific peptide agonists. We also examined the effect of receptor desensitization, receptor-blocking antibodies, and a PAR1 antagonist, used alone and in combination, on platelet activation. Our results suggest that PAR1 and PAR4 together account for most, if not all, thrombin signaling in human platelets. PAR3, while important for thrombin signaling in mouse platelets, appears to have little or no role in human platelets. These results are potentially important for the development of antiplatelet therapies.

Methods

Measurement of PAR mRNA levels by competitive reverse transcription-PCR. Dami cells (19) were grown in suspension in RPMI with 10% FBS. Platelet preparations (17) contained <0.1% leukocytes as assessed by light microscopic analysis. A discontinuous Per-

coll gradient was used to separate monocytes and lymphocytes from neutrophils, according to the manufacturer's instructions (Pharmacia Biotech Inc., Piscataway, New Jersey, USA). The monocyte/lymphocyte preparations contained <0.1% neutrophils, and the neutrophil preparations contained <0.1% monocytes or lymphocytes. Total RNA was prepared from all cells using Trizol Reagent (GIBCO BRL, Grand Island, New York, USA), treated with DNase (Boehringer Mannheim, Indianapolis, Indiana, USA), and quantified by OD 260.

Competitor RNA templates for each receptor were created by mutating the respective cDNA to ablate an endogenous restriction endonuclease site (see below), and competitor RNAs were generated by *in vitro* transcription. Reverse transcription (RT) reactions were performed using 200 ng of total cellular RNA mixed with varying amounts of competitor RNA in a 10- μ l reaction volume using a commercial kit (GIBCO BRL) and receptor specific primers (see below). RT product (2 μ l) was amplified by PCR in a 50- μ l volume containing a final concentration of 2 μ M primers (see below) and 5 U *Taq* polymerase (GIBCO BRL). Reaction conditions were 94°C for 4 min, 72°C for 1 min with addition of *Taq*, then 94°C for 45 s, 55°C for 1 min, 72°C for 1 min for 30–36 cycles (see below), and then 72°C for 8 min. Cycle numbers and concentration ranges for competitor RNAs were chosen for each sample in preliminary experiments. The number of cycles chosen for measurement of PAR1, PAR2, PAR3, and PAR4 mRNA levels, respectively, in RNA from the various sources follows.

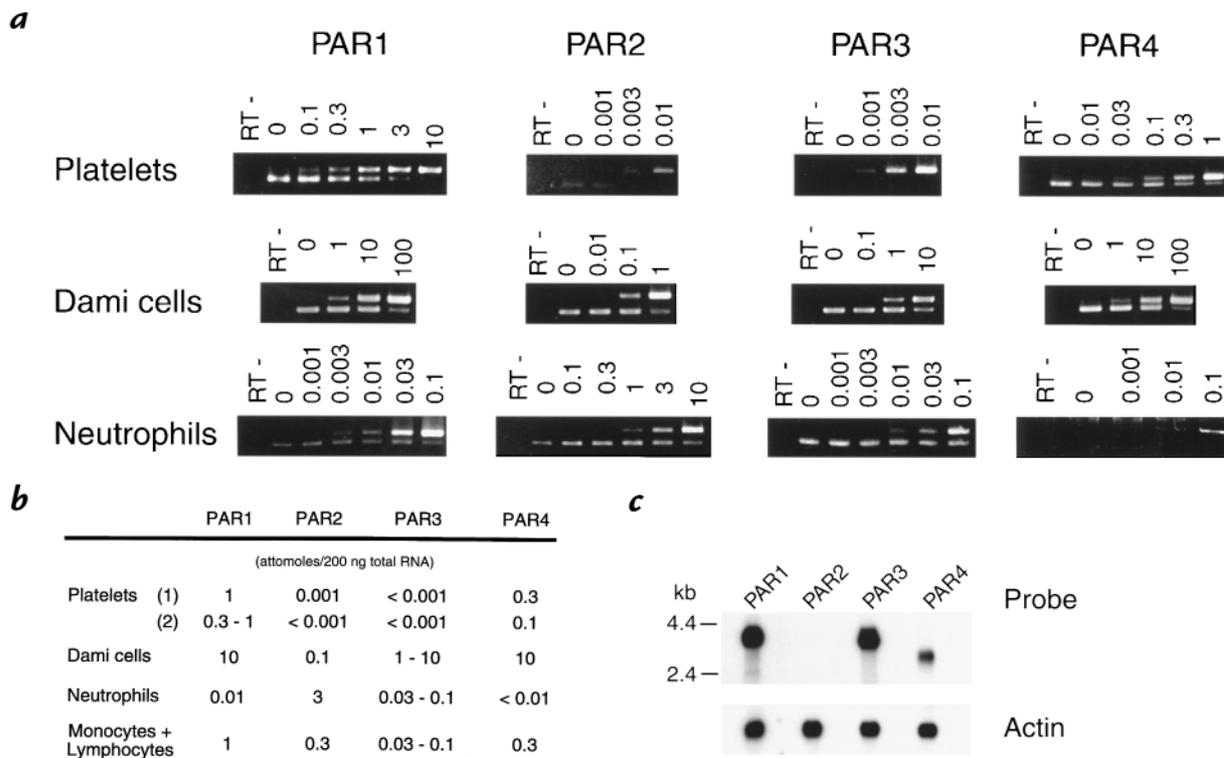


Figure 1

Expression of mRNAs encoding PAR1, PAR2, PAR3, and PAR4 in platelets, Dami cells, and neutrophils. (a) Competitive RT-PCR of total RNA from platelets, Dami cells, and neutrophils. Total cellular RNA (200 ng) mixed with the indicated quantity of competitor RNA (measured in attomoles) was reverse-transcribed and amplified. Products were digested with a restriction endonuclease to distinguish the products of competitor RNA (*uncleavable upper band*) vs. native cellular mRNA (*lower band*). RT indicates mock RT-PCR of total cellular RNA and the highest amount of competitor RNA with no reverse transcriptase added. Each sample was analyzed at least twice. Note that the single band seen in the platelet PAR3 RT-PCR is due to amplification of competitor RNA. (b) Quantitation of PAR mRNAs in platelet, Dami cell, neutrophil, and monocyte/lymphocyte preparations. Results indicate the range of values obtained from at least two experiments like that shown in a. 1 amol/200 ng corresponds to an mRNA relative abundance of roughly 1:3,000. The expression of PAR mRNA in the platelets of two unrelated individuals is shown. (c) Northern blot analysis of PAR gene expression in Dami cells. Blots were hybridized separately with coding region probes for PAR1, PAR2, PAR3, or PAR4, as well as with probe for β -actin mRNA as a control for lane loading. Note concordance with PCR data in b. PAR, protease-activated receptor; RT, reverse transcription.

Platelets: 31, 36, 36, 36; neutrophils: 36, 27, 31, 36; monocytes/lymphocytes: 31, 31, 33, 36; and Dami cells: 30, 32, 32, 33.

Primers used for RT and PCR of each receptor and the restriction endonuclease used to digest each PCR product. Nucleotide numbering is such that 1 equals the A of the start ATG.

PAR1, GenBank accession no. M62424: Primer for RT: TAG ACG TAC CTC TGG CAC TC (1148-1129). Sense-strand primer for PCR: CAG TTT GGG TCT GAA TTG TGT CG. Antisense primer for PCR: TGC ACG AGC TTA TGC TGC TGA C. Resulting PCR product: 505-1096. Mutated site: *AgeI* at position 596.

PAR2, GenBank accession no. U34038: Primer for RT: CTG CTC AGG CAA AAC ATC (699-682). Sense-strand primer for PCR: TGG ATG AGT TTT CTG CAT CTG TCC. Antisense primer for PCR: CGT GAT GTT CAG GGC AGG AAT G. Resulting PCR product: 182-672. Mutated site: *SfiI* at position 342.

PAR3 GenBank accession no. U92972: Primer for RT: TGA TGT CTG GCT GAA CAA G (727-709). Sense-strand primer for PCR: TCC CCT TTT CTG CCT TGG AAG. Antisense primer for PCR: AAA CTG TTG CCC ACA CCA GTC CAC. Resulting PCR product: 152-664. Mutated site: *NcoI* at position 251.

PAR4, GenBank accession no. AF080214: Primer for RT: TGA GTA GCT GGG ATT ACA G (1519-1501). Sense-strand primer for PCR: AAC CTC TAT GGT GCC TAC GTG C. Antisense primer for PCR: CCA AGC CCA GCT AAT TTT TG. Resulting PCR product: 949-1490. Mutated site: *BamHI* at position 1005.

After PCR amplification, 10 μ l of reaction product was digested overnight with the appropriate restriction endonuclease and analyzed by 1.5% agarose gel electrophoresis. The products of reactions that included only native mRNA were completely cleaved by the appropriate restriction endonuclease, while the products of reactions that included only competitor RNA remained undigested (Fig. 1 and data not shown). By adding varying amounts of competitor RNA to total cellular RNA before RT-PCR and determining the competitor RNA concentration at which the intensity of the competitor RNA-derived product (uncleaved band) matched that of the endogenous mRNA-derived product (cleaved band), the quantity of each PAR mRNA in the original sample was estimated.

Northern blot analysis. Poly(A)⁺ RNA (2 μ g) derived from Dami cells was electrophoresed, transferred to nitrocellulose membranes, and hybridized under high-stringency conditions. PAR1 mRNA was detected with a 400-bp *PstI/PvuII* cDNA probe; PAR2 mRNA was detected with a 260-bp *SfiI/BstEII* cDNA probe; PAR3 mRNA was detected with a 610-bp *KpnI/NsiI* cDNA probe; PAR4 mRNA was detected using a 450-bp *SacI/PstI* cDNA.

Generation and characterization of PAR polyclonal antibodies. The synthetic peptides GGDDSTPSILPAPRGYPGQVC (PAR4 amino acids 34-55), AKPTLPIKTFRGAAPPNSFEFFPFSALEGC (PAR3 amino acids 31-58 plus carboxyl glycine-cysteine) and NATLD-PRSFLLRNPNDKYEPFWEDEEGC (PAR1 amino acids 35-61 plus carboxyl glycine-cysteine) were conjugated to keyhole limpet hemocyanin and used to generate polyclonal antisera in rabbits. IgG was purified by protein-A affinity chromatography to generate the PAR4, PAR3, and PAR1 IgG preparations used in this study. Binding of these IgGs and PAR4 preimmune IgG to each receptor was tested on COS cells transiently expressing FLAG epitope-tagged receptors using an enzyme-linked immunosor-

bent assay (ELISA) (16, 20). cDNA for an epitope-tagged human PAR4 analogous to FLAG epitope-tagged PAR1 was constructed as described previously (15, 20) such that the FLAG epitope was fused to amino acid 22 in PAR4 to yield the following sequence: ... DYKDDDDVE/TPSVYD ... (where the slash indicates the junction with PAR4 sequence).

Flow cytometry. Washed platelets (17) and Dami cells were fixed with paraformaldehyde, incubated with PAR1 or PAR3 IgG at 10 μ g/ml or PAR4 IgG at 100 μ g/ml, washed, incubated with FITC-conjugated goat anti-rabbit IgG, washed, and then analyzed in a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, California, USA). Some fixed platelet samples were exposed to 30 nM thrombin at 37°C before incubation with primary antibody.

Functional studies in *Xenopus* oocytes. FLAG epitope-tagged PAR4 cDNA was subcloned into pFROG (3) to permit *in vitro* transcription of PAR4 cRNA. Signaling studies were performed in *Xenopus* oocytes microinjected with 2.0 ng of PAR4 cRNA or 25 ng of PAR1 cRNA per oocyte (3, 21).

Platelet aggregation and secretion. Aggregation and secretion were measured using washed human platelets (17). For desensitization studies, platelets resuspended from the first platelet pellet were incubated with SFLLRN (100 μ M) or GYPGKF (500 μ M) in the presence of prostaglandin E₁ (PGE₁) at room temperature for 30 min without stirring then washed by centrifugation (17).

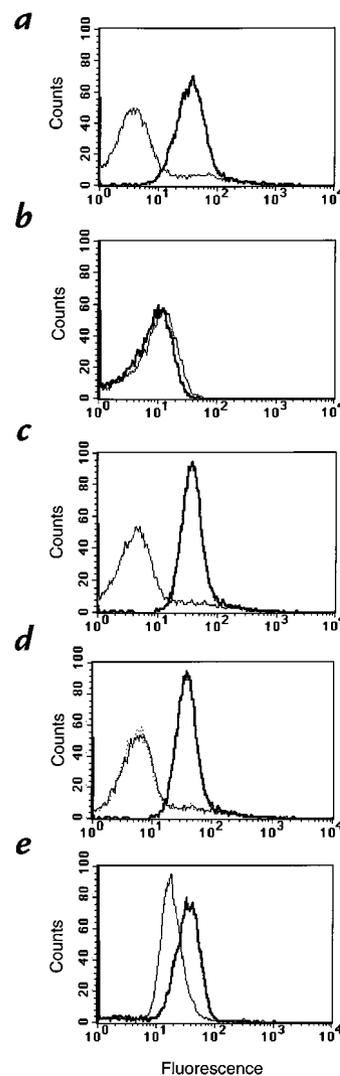


Figure 2

Flow cytometric analysis of platelets for surface expression of PAR1, PAR3, and PAR4. Fixed platelets were incubated with preimmune IgG (narrow lines) or PAR1 IgG (a), PAR3 IgG (b), or PAR4 IgG (c) (wide lines) and then analyzed as described in Methods. (d) Platelets were incubated with PAR4 IgG in the absence (wide line) or presence (thin line) of the peptide antigen (1 μ M) used to generate the PAR4 antiserum, or after treatment with 20 nM thrombin for 10 min at 37°C (dotted line). Each curve represents an analysis of 10,000 events. This experiment was repeated twice with separate donors with equivalent results. (e) Flow cytometric analysis of Dami cells as a positive control for detection of PAR3. Fixed Dami cells were incubated with preimmune IgG (narrow line) or PAR3 IgG (wide line) and then analyzed as above. Dami cells were also positive for PAR1 and PAR4 (not shown).

For functional studies with PAR1 or PAR4 antibody, washed platelets were incubated with antibody or preimmune IgG for 60 min before measurement of secretion and aggregation. PAR1 antagonist was added to stirring platelets 1–2 min before the addition of thrombin or other agonists.

Measurement of receptor cleavage by thrombin. Rat-1 fibroblasts were stably transfected with FLAG epitope-tagged PAR1 and PAR4 expression vectors (22), and cleavage of surface receptors was followed as described previously (20).

Measurement of PAR1 and PAR4 signaling. A *Par1*^{-/-} mouse lung fibroblast cell line that showed no thrombin signaling (14, 23) was used to generate stable cell lines expressing FLAG epitope-tagged PAR1 and PAR4. Increases in cytoplasmic calcium in response to thrombin were measured using the calcium-sensitive dye Fura-2 as described previously (14).

Results

Expression of PAR mRNAs in platelets and other blood cells. To validate an RT-PCR assay for PAR mRNAs, Dami cells, a human cell line that expresses some megakaryocyte markers (19), were analyzed. Competitive RT-PCR of Dami cell RNA (Fig. 1, *a* and *b*) revealed the presence of PAR1, PAR3, and PAR4 mRNA; PAR2 mRNA was also detected but at only 1% the level of PAR1 mRNA. Northern analysis was positive for PAR1, PAR3, and PAR4 but not PAR2 (Fig. 1*c*). At the protein level, PAR1, PAR3, and PAR4 were detected on the surface of Dami cells by flow cytometry (Fig. 2, and data not shown). Thus, results from RT-PCR were generally concordant with Northern and protein analysis.

Competitive RT-PCR of platelet RNA revealed PAR1 mRNA to be present at approximately 1 amol/200 ng total RNA. Assuming mRNA is 1% of total platelet RNA and an average mRNA size of 2 kb, PAR1 mRNA represents 1 in 3,000 platelet mRNAs. PAR4 mRNA was also detected at 10%–30% of PAR1 mRNA levels. By contrast, PAR3 mRNA was undetectable. PAR3 competitor RNA added to

platelet RNA was detected at 0.001 amol/200 ng total RNA, suggesting that PAR3 mRNA was at least 1,000-fold less abundant than PAR1 mRNA in these samples. PAR2 mRNA was not detected in platelet RNA from one individual (no. 2), and only 0.001 amol/200ng was detected in the other (no. 1). The latter measurement may be due to trace contamination of the platelet preparation by neutrophils (see below), consistent with the observation that the specific PAR2 agonist peptide SLIGKV is unable to activate human platelets (data not shown).

The pattern of PAR mRNA expression in neutrophils and mononuclear cells was distinct from that seen in platelets, suggesting that contamination of platelet preparations by leukocytes did not significantly influence the PAR expression pattern detected in platelets. In particular, while virtually absent from platelets, substantial PAR2 mRNA was detected in both neutrophils and mononuclear cells. The relatively high PAR2 mRNA level in neutrophils is consistent with previous studies demonstrating neutrophil responses to PAR2-activating peptide (24). In contrast to platelets, PAR3 mRNA was consistently detected at low levels in mononuclear cells. PAR4 mRNA was also found in mononuclear cell preparations but not in neutrophils. These results demonstrate the presence of mRNA encoding PAR1 and PAR4, but not PAR2 or PAR3, in human platelets.

Expression of PAR proteins on the surface of human platelets. IgG was purified from rabbit antisera raised to peptides representing the NH₂-terminal exodomains of PAR1, PAR3, or PAR4. To assess ability to recognize native PARs and cross-reactivity, antibody binding to the surface of receptor-expressing COS cells was measured. Each IgG preparation bound to the surface of cells expressing the appropriate receptor without significant cross-reactivity (data not shown).

Figure 3

Effects of PAR1- and PAR4-activating peptides. (*a*) Specificity and potency. Peptide-triggered ⁴⁵Ca release was measured in *Xenopus* oocytes expressing human PAR1 and human PAR4 tagged at their NH₂-termini with a FLAG epitope. Data are mean ± SEM (*n* = 3) and are expressed as fold increase over baseline for each receptor. Surface expression of PAR1 measured with anti-FLAG monoclonal antibody was 1.3 times that of PAR4. This experiment was replicated twice. (*b–d*) Activation of human platelets with the PAR1-activating peptide SFLLRN and the PAR4-activating peptides GYPGKF and GYPGQV. (*b*) Platelets were exposed to either SFLLRN (10 μM) or GYPGKF (500 μM) or GYPGQV (1 mM) at time 0, and aggregation was measured as change in light transmission. (*c*) SFLLRN-desensitized platelets (see Methods) were exposed to either SFLLRN (500 μM) or GYPGKF (500 μM) at time 0, and aggregation was measured as change in light transmission. (*d*) GYPGKF-desensitized platelets (see Methods) were exposed to either SFLLRN (500 μM) or GYPGKF (500 μM) at time 0, and aggregation was measured as change in light transmission. The experiments in *b*, *c*, and *d* were repeated three times.

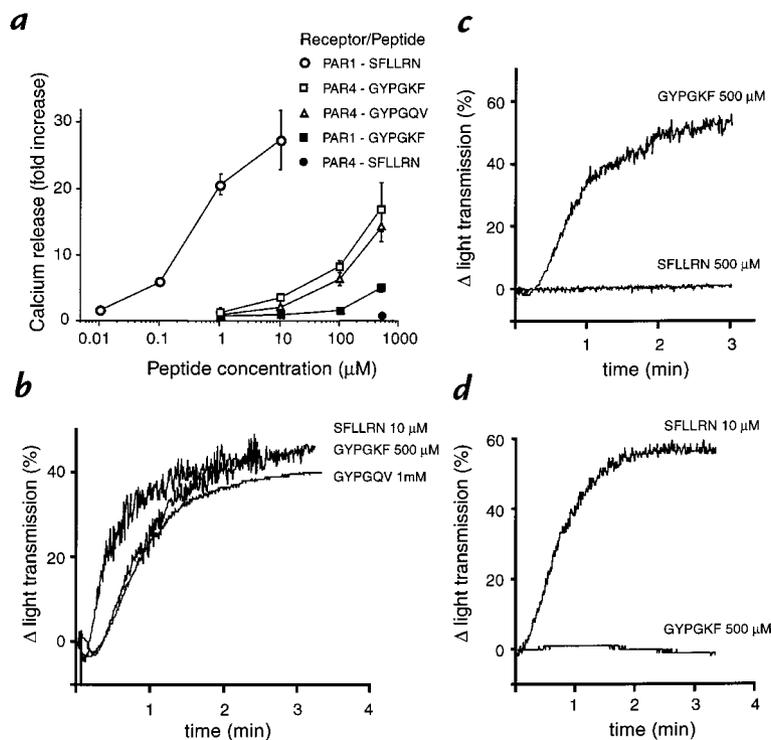
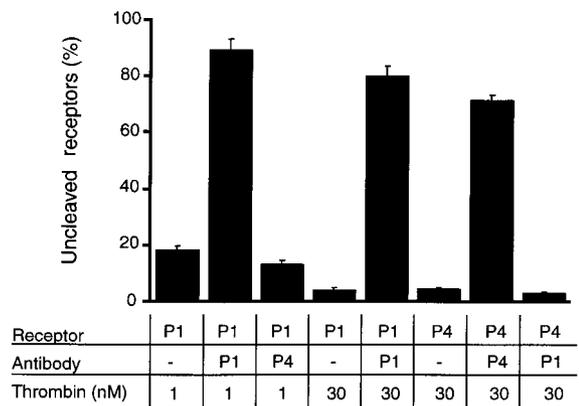


Figure 4

Inhibition of thrombin cleavage of receptor NH₂-terminus by anti-PAR1 and anti-PAR4 antibodies. Rat-1 cells expressing PAR1 and PAR4 bearing the FLAG epitope at their extreme NH₂-termini were fixed and then incubated with PAR1 IgG (P1; 100 μg/ml), PAR4 IgG (P4; 1 mg/ml), or buffer alone for 60 min before exposure to either 1 or 30 nM thrombin for 10 min at 37°C. Receptor cleavage was measured as loss of binding sites for M1 monoclonal antibody to the FLAG epitope, which was NH₂-terminal to the thrombin cleavage site in both receptors, so as to be lost from the cells upon receptor cleavage. Data (mean ± SEM; n = 3) are expressed as percent of control cells exposed to buffer alone. This experiment was repeated twice.



These IgG preparations were then used for flow cytometric analysis of human platelets (Fig. 2). Significant surface binding was detected with PAR1 IgG vs. preimmune IgG (Fig. 2a), consistent with previous studies (9, 11, 25, 26). A similar increase in platelet surface binding was detected with PAR4 IgG vs. PAR4 preimmune IgG (Fig. 2c). Preincubation of PAR4 IgG with the peptide antigen to which it was raised abolished this increase (Fig. 2d). Moreover, the epitope to which the PAR4 antiserum was raised spans PAR4's thrombin cleavage site, and treatment of platelets with thrombin indeed abolished PAR4 IgG binding (Fig. 2d). These data strongly suggest that PAR1 and PAR4 are expressed on the surface of human platelets.

PAR3 immune IgG showed no specific binding to human platelets (Fig. 2b). To determine whether this antibody preparation could detect PAR3 expressed at "natural" levels, this experiment was repeated with Dami cells (Fig. 2e, and data not shown), which had been shown by Northern blot to express PAR3 mRNA (Fig. 1). A significant increase in antibody binding was seen with PAR3 antibody vs. non-immune IgG, consistent with RT-PCR and Northern blot analysis (Fig. 1). This suggests that the absence of detectable PAR3 protein on the surface of human platelets is not due to insensitivity of the assay. Taken together, these data confirm the presence of PAR1 and PAR4, but not PAR3, on the surface of human platelets.

Activation of human platelets by PAR1- and PAR4-activating peptides. Synthetic peptides that mimic the tethered ligands of PAR1 and PAR2 function as agonists for their respective receptors (3, 7, 8) and have been used as pharmacological tools to probe the function of these receptors in various cell types. Unfortunately, the cognate peptide for PAR3 appears to be insufficiently avid to function as a free ligand (15). We and others (17, 18) recently showed that a peptide mimicking the tethered ligand for PAR4 can function as an agonist for that receptor, albeit at a concentration higher than that seen with the PAR1 and PAR2 peptides and their cognate receptors. To determine the specificity of the PAR1 and PAR4 tethered ligand peptides, we first assessed their ability to trigger calcium mobilization in *Xenopus* oocytes heterologously expressing PAR1 and PAR4, our most sensitive assay system (Fig. 3a). No responses were detected in oocytes expressing neither receptor (not shown). Both the human PAR4 peptide GYPGQV and the mouse PAR4 peptide GYPGKF activated oocytes expressing human PAR4, but

with an EC₅₀ roughly two orders of magnitude higher than that of SFLLRN for PAR1 activation (Fig. 3a and ref. 17). SFLLRN showed no activity at PAR4. At 500 μM, the PAR4 peptide GYPGKF did show minimal activity at PAR1. However, because PAR1 is overexpressed such that the sensitivity for detection of PAR1 activation in the oocyte assay is 10- to 100-fold greater than in platelets, it is likely that PAR1 activation at 500 μM GYPGKF is unimportant in the platelet studies described below.

The PAR1 peptide SFLLRN and the PAR4 peptides GYPGKF and GYPGQV all activated human platelets (Fig. 3b). The PAR4 peptides were considerably less potent than the PAR1 peptide for activating human platelets; GYPGKF was slightly more potent than GYPGQV (Fig. 3b, and data not shown). The potencies of these peptides for platelet activation thus correlated with their relative potencies for activation of their respective receptors in the oocyte system (Fig. 3).

Incubation of PGE₁-treated platelets with SFLLRN rendered them refractory to subsequent stimulation by SFLLRN but did not affect responsiveness to GYPGKF (Fig. 3c). Conversely, incubation with GYPGKF rendered platelets refractory to subsequent stimulation by GYPGKF but did not affect responsiveness to SFLLRN (Fig. 3d). These results suggest that activation of either PAR1 or PAR4 with their cognate peptide agonists is sufficient to activate human platelets. Taken together, the results presented above show that PAR1 and PAR4 function in human platelets.

PAR1 and PAR4 antibodies inhibit thrombin cleavage of their respective receptors. To determine the necessary roles of PAR1 and PAR4 in platelet activation by thrombin, we developed blocking antibodies. The previously described PAR1 antibody raised against PAR1's hirudin-like domain (9) is predicted to inhibit thrombin cleavage of PAR1's NH₂-terminal exodomain by disrupting binding to thrombin's anion-binding exosite. Because no analogous hirudin-like domain was apparent in the sequence of PAR4's NH₂-terminal exodomain, antiserum was raised to a peptide that represented sequence spanning PAR4's thrombin cleavage site. This antiserum specifically recognized PAR4 (Fig. 2, and data not shown). To test the ability of the PAR1 and PAR4 antibodies to block cleavage of PAR1 and PAR4, Rat-1 fibroblasts expressing FLAG epitope-tagged PAR1 and PAR4 were preincubated with antibody. Receptor cleavage was then measured as loss of FLAG epitope from the cell surface upon expo-

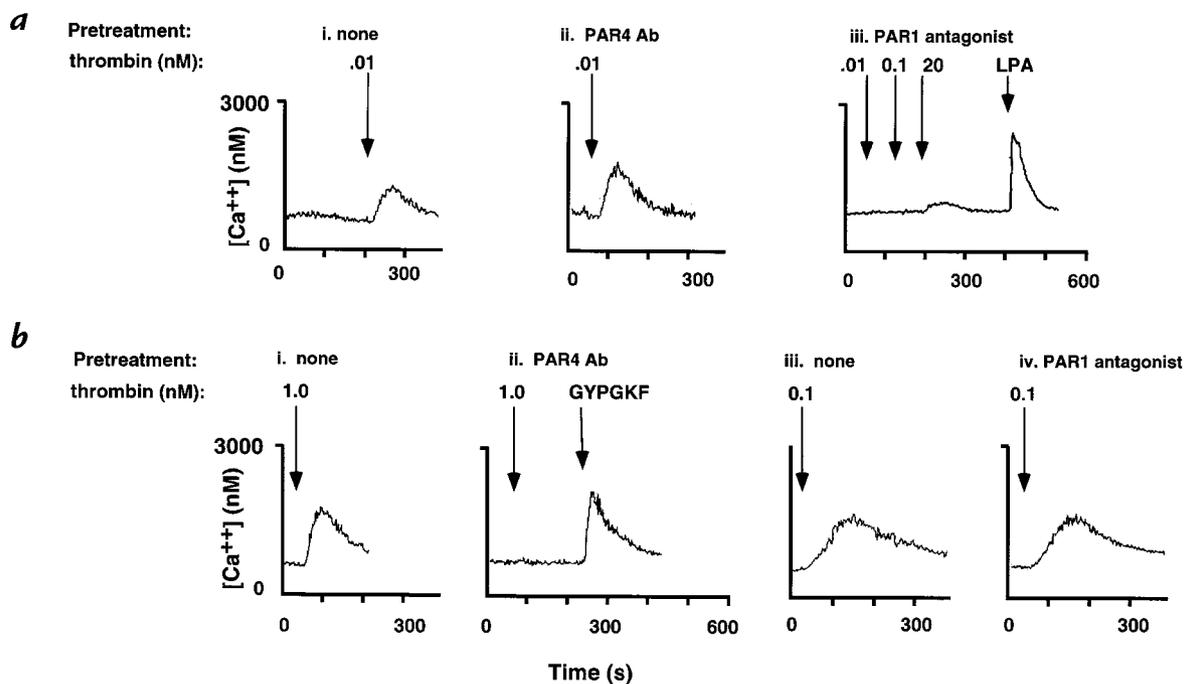


Figure 5

Inhibition of PAR1 and PAR4 signaling by PAR1- and PAR4-blocking antibodies and PAR1 antagonist. Fibroblast cell lines in which thrombin signaling was mediated solely by PAR1 (**a**) or by PAR4 (**b**) were incubated with buffer alone (*none*), PAR4 IgG (*PAR4 Ab*; 1 mg/ml), or the PAR1 antagonist BMS200261 (100 μ M) for 30 min at 37°C. Cells were then exposed to thrombin (0.01, 0.1, 1.0, or 20 nM as indicated), GYPGKF (500 μ M), or lysophosphatidic acid (*LPA*; 5 μ M). Receptor-triggered increases in cytoplasmic calcium were measured fluorometrically using the calcium sensitive dye Fura-2. This experiment was repeated three times with similar results. *Ab*, antibody.

sure to thrombin (20) (Fig. 4). PAR1 cleavage was markedly inhibited by PAR1 antibody but not by PAR4 antibody. Conversely, PAR4 cleavage was markedly inhibited by PAR4 antibody, but not by PAR1 antibody. These data predicted that the PAR1 and PAR4 antibodies should selectively attenuate thrombin signaling via PAR1 and PAR4, respectively.

Inhibition of thrombin signaling by PAR1 and PAR4 antibodies, and by a PAR1 antagonist. A fibroblast cell line derived from PAR1-deficient mice (23) was used to generate lines stably expressing human PAR1 and PAR4. Because no thrombin responses were detectable in untransfected PAR1-deficient fibroblasts, signaling in the transfected cells could be attributed to the transfected receptor. In the PAR1-expressing cell line, increases in cytoplasmic calcium were reliably elicited by thrombin at concentrations as low as 10 pM (Fig. 5a). PAR4 IgG had no inhibitory effect, even on these threshold responses (Fig. 5). As demonstrated previously (9), PAR1 IgG markedly attenuated such signaling, and nonimmune antibody was without effect (data not shown).

The PAR1 antagonist BMS200261 (27) attenuated PAR1 signaling even at high thrombin concentrations (Fig. 5). Responsiveness to lysophosphatidic acid was unaffected by the antagonist, as was PAR4 signaling (Fig. 5, and data not shown), suggesting that the inhibitory effect of BMS200261 was specific.

In the PAR4-expressing cell line, increases in cytoplasmic calcium were reliably triggered at 1 nM thrombin (Fig. 5b). PAR4 IgG blocked such responses but had no effect on responses to GYPGKF, consistent with the anti-

body's acting by preventing receptor cleavage by thrombin. PAR4 preimmune IgG, PAR1 IgG, and PAR1 antagonist (100 μ M) failed to inhibit PAR4 signaling even at threshold thrombin concentrations (Fig. 5b, and data not shown). Taken together, these results established specific tools for blocking PAR1 or PAR4. PAR1 and PAR4 could each be blocked with a specific IgG. PAR1 could also be specifically blocked with BMS200261 or by homologous desensitization with SFLLRN. This presented an opportunity to assess the roles of PAR1 and PAR4 in platelet activation by thrombin.

Inhibition of thrombin-induced platelet aggregation by blocking PAR1 and PAR4 signaling. The contribution of PAR1 and PAR4 signaling to thrombin activation of human platelets was tested using the strategies outlined above. By itself, PAR4 IgG had no effect on platelet aggregation, even at low (1 nM) thrombin (Fig. 6). By contrast, PAR1 IgG or BMS200261 markedly inhibited platelet aggregation in response to 1nM thrombin, as did prior desensitization of platelets with the PAR1 agonist SFLLRN (Fig. 6). None of these maneuvers inhibited platelet aggregation in response to GYPGKF or submaximal concentrations of adenosine diphosphate (ADP) (Fig. 6 and data not shown). These data suggest that PAR1 is the major mediator of platelet activation at low concentrations of thrombin, consistent with previous studies (9, 25).

In contrast to the case at 1 nM thrombin, at 30 nM thrombin, inhibition of PAR1 signaling by either PAR1 IgG, antagonist, or SFLLRN desensitization was largely ineffective, only slowing aggregation slightly, such that shape change became detectable (see 0- to 30-second portions of

the aggregation curves in Fig. 6, *b-d*). Inhibition of PAR4 signaling with PAR4 IgG was similarly ineffective (Fig. 6*b*).

Strikingly, when signaling via PAR1 and PAR4 was blocked simultaneously, aggregation in response to even high concentrations of thrombin was virtually abolished (Fig. 6). Such synergy was seen regardless of the means by which PAR1 was blocked (desensitization, PAR1 IgG, or antagonist) (Fig. 6). PAR4 preimmune IgG had no effect in such experiments (data not shown), and platelet activation by ADP and by GYPGKF, which bypasses the effect of the PAR4 antibody, were not inhibited (Fig. 6, and data not shown). These data strongly suggest that both PAR1 and PAR4 contribute to platelet activation at high (30 nM) concentrations of thrombin and that inhibition of both receptors is required to ablate thrombin-triggered platelet aggregation.

Inhibition of thrombin-induced platelet secretion by blocking PAR1 and PAR4 signaling. A more quantitative measure of platelet activation is the amount and rate of adenosine triphosphate (ATP) release due to the secretion of platelet granule contents. We therefore measured the effect of BSM200261, PAR4 IgG, or both, on peak ATP release and the time to half-maximal ATP release in response to 30 nM thrombin (Fig. 7). PAR1 antagonism with BSM200261 decreased maximal ATP secretion to approximately one-third of control levels and prolonged the time to half-maximal secretion by approximately threefold. PAR1 IgG alone had a similar effect (not shown). PAR4 antibody alone had no effect on the tempo of ATP secretion and only a small effect, if any, on peak response. Strikingly, the combination of BSM200261 and PAR4 IgG ablated ATP secretion in response to 30 nM thrombin. Even after 10 minutes, no secretion was detected. Similar data were obtained when PAR1 IgG was combined with PAR4 IgG. These maneuvers did not block secretion in response to GYPGKF (Fig. 7), and preimmune and non-immune IgG were without effect (data not shown). These results support the hypothesis that PAR1 and PAR4 account for platelet secretion and aggregation in response to thrombin at concentrations as high as 30 nM. They also suggest that PAR1 is necessary for rapid platelet activation by thrombin even at high thrombin concentrations.

Discussion

In this study, we addressed the roles of PAR1, PAR3, and PAR4 in activation of human platelets by thrombin. We showed that PAR1 and PAR4 are functionally expressed in human platelets and that these receptors account for most if not all thrombin signaling in these cells. We further demonstrate that PAR1 mediates platelet responses at low concentrations of thrombin and is necessary for the most rapid and robust platelet responses, even at high concentrations of thrombin, consistent with previous studies (9, 10). In contrast to PAR1, PAR4 mediates platelet activation only at high thrombin concentrations and PAR4 signaling appears unnecessary for platelet activation when PAR1 function is intact.

The observation that specific inhibition of PAR1 and PAR4 ablate thrombin signaling in human platelets suggests thrombin binding to GPIIb α (28) is not sufficient to trigger platelet activation; whether such binding plays any adjunctive role is not known. Our results also sug-

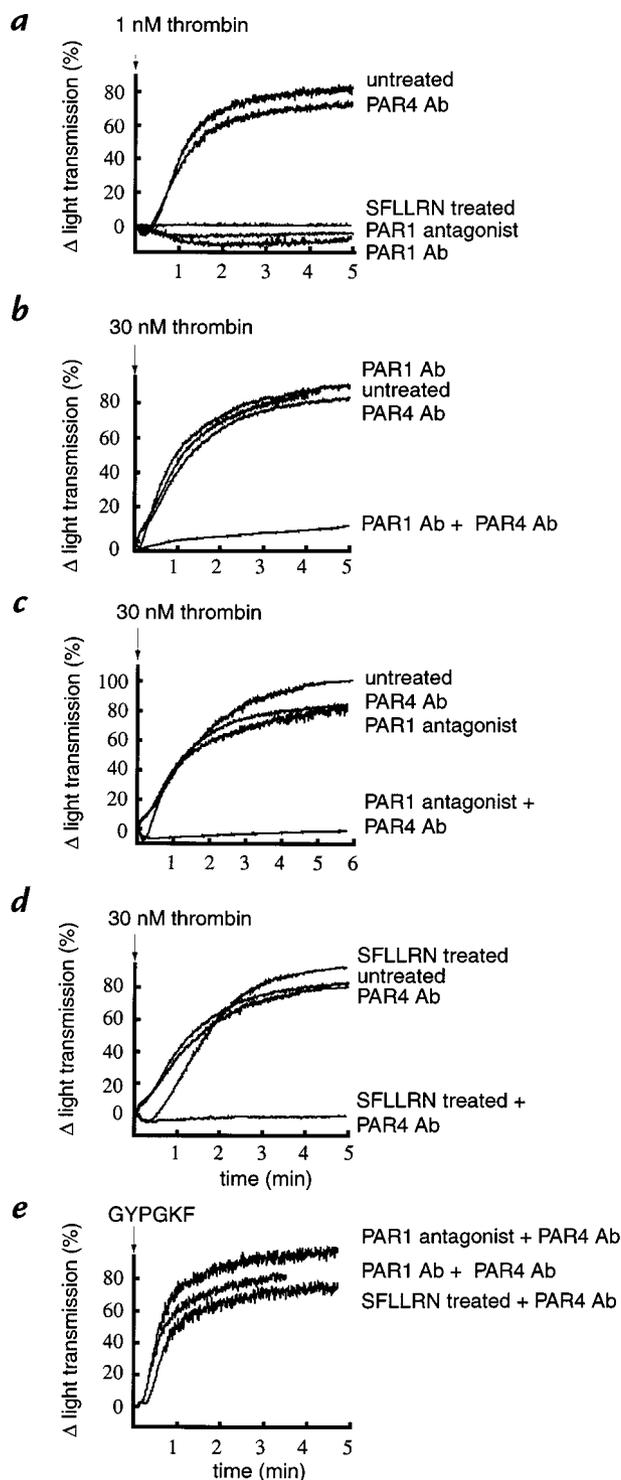


Figure 6

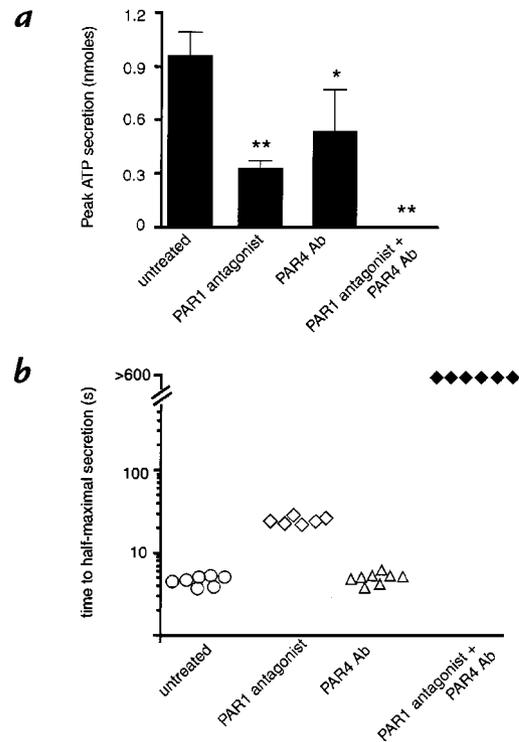
The effects of inhibition of PAR1 and/or PAR4 on aggregation of human platelets in response to low (1 nM) and high (30 nM) concentrations of thrombin. Platelets were pretreated with buffer alone, PAR1 IgG (10 μ g/ml), PAR4 IgG (1 mg/ml), or PAR1 antagonist (100 μ M), or were desensitized to SFLLRN as indicated and then exposed to 1 nM thrombin (*a*), 30 nM thrombin (*b-d*), or 500 μ M GYPGKF (*e*) at time 0. Aggregation was measured as increase in light transmission. Preimmune or nonimmune IgG were without effect (not shown). This experiment was performed using triplicate samples (*a, c, e*) or four times (*b, d*). Representative tracings are shown.

Figure 7

The effects of inhibition of PAR1 and/or PAR4 on platelet ATP secretion in response to thrombin. **(a)** Peak ATP secretion. Platelets were pretreated with buffer alone, PAR4 IgG (1 mg/ml), PAR1 antagonist BMS200261 (100 μ M), or PAR1 antagonist plus PAR4 IgG as indicated, and then stimulated with 30 nM thrombin. Peak ATP concentration in the 10 min after addition of thrombin was measured by lumiaggregometry. Preimmune IgG had no effect (not shown). Data are mean \pm SD ($n = 5-7$) of peak secretion measured; similar results were obtained with platelets from two individuals. Data were analyzed by two-way ANOVA and t test with a Bonferroni correction for multiple comparisons. * $P \leq 0.06$, ** $P < 0.001$ compared with untreated group. Note that no secretion was detected during the 10 min after addition of 30 nM thrombin to platelets treated with PAR1 antagonist plus PAR4 IgG. **(b)** Time to half-maximal secretion. Time to reach 50% of the peak ATP secretion response elicited by 30 nM thrombin in each group **(a)** was measured. Platelets were pretreated with buffer alone (*open circles*), PAR1 antagonist (*open diamonds*), PAR4 IgG (*open triangles*), or PAR1 antagonist plus PAR4 Ab (*closed diamonds*) as in **a**, and then stimulated with 30 nM thrombin. Points displayed as >600 s indicate no measurable secretion within 10 min after addition of thrombin. PAR4 preimmune IgG had no effect inhibitory effect in such experiments, even in the presence of PAR1 antagonist (not shown).

gest that PAR3 has no important role in activation of human platelets by thrombin. Indeed, PAR3 mRNA and protein were not detected in human platelets but were readily detected in Dami cells. Such negative data regarding PAR3 expression in platelets must of course be interpreted with caution. Our failure to detect PAR3 mRNA and protein in human platelets is concordant with our functional data but at variance with a recent report of PAR3 expression in human platelets detected by RT-PCR and flow cytometry (26). In the latter study, the RT-PCR was not quantitative, and potential cross-reactivity of the PAR3 antiserum was not discussed, possibly accounting for our different results. Alternatively, platelets from only a handful of individuals have been studied, and it is possible that regulation or individual differences in PAR3 expression account for our differing results. Regardless, we know of no data that implicate PAR3 function in activation of human platelets by thrombin.

It is interesting to compare and contrast thrombin signaling in human and mouse platelets. This study shows that human platelets utilize both PAR1 and PAR4, with no apparent role for PAR3. By contrast, mouse platelets utilize PAR3 and PAR4 (17), with no apparent role for PAR1 (14). A definitive answer to whether additional receptors play a role in the mouse awaits generation of PAR3/PAR4 double knockout mice. In human platelets, PAR1 is necessary for responses to low concentrations of thrombin; in mouse platelets, PAR3 plays this role (17). Thus, despite the use of distinct receptors, platelets in both species use a dual thrombin receptor system in which a high-affinity receptor (PAR1 in human, PAR3 in mouse) mediates responses to low concentrations of thrombin and a low-affinity receptor (PAR4) mediates signaling at high concentrations. Interestingly, both PAR1 and PAR3 have obvious hirudin-like domains (3, 29). In PAR1, this domain binds thrombin's fibrinogen binding exosite and is critical for PAR1's efficient cleavage and activation by thrombin (3, 5, 29-32). PAR4 has no such domain, perhaps accounting for its slower cleavage by thrombin and right-shifted concentration response curve (17, 18).



The biological significance of having dual thrombin receptors in platelets remains uncertain. PAR3-deficient mice showed no spontaneous bleeding and had normal bleeding times; thus, in mice, the high-affinity receptor is not necessary for normal hemostasis when the low-affinity receptor is present. Whether different challenges will unmask a requirement for PAR3 in hemostasis or thrombosis is unknown, and whether combined deficiency in PAR3 and PAR4 will provoke a bleeding diathesis remains to be determined. It is possible that two receptors in platelets simply provide redundancy in an important system, but a variety of more interesting possibilities are apparent. It is possible that a capacity to respond to thrombin over a greater concentration range is important for reasons not yet understood. More broadly, PAR1, PAR3, and PAR4 might mediate responses to proteases or ligands other than thrombin or allow thrombin itself to activate distinct signaling pathways or to trigger signaling with varied tempos of activation or shutoff. The existence of multiple receptors also allows for distinct temporal and spatial expression. The finding of PAR3 expression in Dami cells (Figs. 1 and 2), but not in human platelets, is provocative in this regard. Dami cells were derived from a patient with megakaryoblastic leukemia and express a number of megakaryocyte markers (19). PAR3 is also expressed by HEL cells and K562 cells (data not shown, and ref. 26). These results raise the possibility that PAR3 might be expressed by hematopoietic cells in the erythroid/megakaryocyte lineage but extinguished in mature megakaryocytes and platelets. The role of PAR expression in hematopoiesis, if any, and whether PARs might serve as useful markers of differentiation, remains to be explored.

Because of the role of thrombin and platelet activation in myocardial infarction and other pathological processes, identifying and blocking the receptors by which thrombin

activates platelets has been an important goal. Iterations around PAR1's tethered ligand sequence SFLLRN have already led to the development of potent peptide-based antagonists (27). These antagonists blocked human platelet activation by SFLLRN itself and by low concentrations of thrombin but were ineffective at high concentrations of thrombin (27). This study strongly suggests that persistent platelet responses to high thrombin concentrations in the presence of a PAR1 antagonist were due to PAR4, which is not blocked by the antagonist. The PAR1 antagonist BMS200261 was in fact quite effective at blocking activation of PAR1 by high concentrations of thrombin (Fig. 5, and data not shown) but became effective at blocking platelet activation by high concentrations of thrombin only when PAR4 was blocked simultaneously.

The finding that PAR1 and PAR4 account for all, or virtually all, of the ability of human platelets to respond to thrombin should excite interest in the development of thrombin receptor antagonists as possible antithrombotic agents. Agents that inhibit signaling via the thromboxane and ADP receptors are effective antithrombotic drugs (33, 34). Given thrombin's remarkable potency as a platelet activator and its ability to activate even aspirin-treated platelets (35, 36), blockade of thrombin signaling in platelets might also prove to be a useful strategy for preventing thrombosis. Because inhibition of PAR1 alone markedly attenuated platelet responses at low concentrations of thrombin, PAR1 antagonism might be sufficient for an antithrombotic effect. In this scenario, PAR4 might ensure a minimal level of thrombin signaling and act as a safety buffer. Alternatively, it may be necessary to block both PAR1 and PAR4 to prevent thrombosis. Using genetically modified mice and inhibitor studies in other species, it will now be possible to determine if these strategies should be pursued.

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1. Davey, M., and Luscher, E. 1967. Actions of thrombin and other coagulant and proteolytic enzymes on blood platelets. *Nature*. **216**:857-858.
2. Berndt, M., and Phillips, D. 1981. Platelet membrane proteins: composition and receptor function. In *Platelets in biology and pathology*. J.L. Gordon, editor. Elsevier. Amsterdam, the Netherlands. 43-74.
3. Vu, T.-K.H., Hung, D.T., Wheaton, V.I., and Coughlin, S.R. 1991. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell*. **64**:1057-1068.
4. Rasmussen, U.B., et al. 1991. cDNA cloning and expression of a hamster alpha-thrombin receptor coupled to Ca²⁺ mobilization. *FEBS Lett*. **288**:123-128.
5. Vu, T.-K.H., Wheaton, V.I., Hung, D.T., and Coughlin, S.R. 1991. Domains specifying thrombin-receptor interaction. *Nature*. **353**:674-677.
6. Chen, J., Ishii, M., Wang, L., Ishii, K., and Coughlin, S.R. 1994. Thrombin receptor activation: confirmation of the intramolecular tethered ligand hypothesis and discovery of an alternative intermolecular liganding mode. *J. Biol. Chem*. **269**:16041-16045.
7. Vassallo, R.J., Kieber, E.T., Cichowski, K., and Brass, L.F. 1992. Structure-function relationships in the activation of platelet thrombin receptors by receptor-derived peptides. *J. Biol. Chem*. **267**:6081-6085.
8. Scarborough, R.M., et al. 1992. Tethered ligand agonist peptides. Structural requirements for thrombin receptor activation reveal mechanism of proteolytic unmasking of agonist function. *J. Biol. Chem*. **267**:13146-13149.

9. Hung, D.T., Vu, T.-K.H., Wheaton, V.I., Ishii, K., and Coughlin, S.R. 1992. Cloned platelet thrombin receptor is necessary for thrombin-induced platelet activation. *J. Clin. Invest*. **89**:1350-1353.
10. Brass, L.F., et al. 1992. Structure and function of the human platelet thrombin receptor. Studies using monoclonal antibodies directed against a defined domain within the receptor N terminus. *J. Biol. Chem*. **267**:13795-13798.
11. Molino, M., Bainton, D.F., Hoxie, J.A., Coughlin, S.R., and Brass, L.F. 1997. Thrombin receptors on human platelets. Initial localization and subsequent redistribution during platelet activation. *J. Biol. Chem*. **272**:6011-6017.
12. Derian, C.K., Santulli, R.J., Tomko, K.A., Haertlein, B.J., and Andrade-Gordon, P. 1995. Species differences in platelet responses to thrombin and SFLLRN. Receptor-mediated calcium mobilization and aggregation and regulation by protein kinases. *Thromb. Res*. **6**:505-519.
13. Connolly, T.M., et al. 1994. Species variability in platelet and other cellular responsiveness to thrombin receptor-derived peptides. *Thromb. Haemost.* **72**:627-633.
14. Connolly, A.J., Ishihara, H., Kahn, M.L., Farese, R.V., and Coughlin, S.R. 1996. Role of the thrombin receptor in development and evidence for a second receptor. *Nature*. **381**:516-519.
15. Ishihara, H., et al. 1997. Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature*. **386**:502-506.
16. Ishihara, H., Zeng, D., Connolly, A.J., Tam, C., and Coughlin, S.R. 1998. Antibodies to protease-activated receptor 3 inhibit activation of mouse platelets by thrombin. *Blood*. **91**:4152-4157.
17. Kahn, M.L., et al. 1998. A dual thrombin receptor system for platelet activation. *Nature*. **394**:690-694.
18. Xu, W.F., et al. 1998. Cloning and characterization of human protease-activated receptor 4. *Proc. Natl. Acad. Sci. USA*. **95**:6642-6646.
19. Greenberg, S.M., Rosenthal, D.S., Greeley, T.A., Tantravahi, R., and Handin, R.I. 1988. Characterization of a new megakaryocytic cell line: the Dami cell. *Blood*. **72**:1968-1977.
20. Ishii, K., Hein, L., Kobilka, B., and Coughlin, S.R. 1993. Kinetics of thrombin receptor cleavage on intact cells. Relation to signaling. *J. Biol. Chem*. **268**:9780-9786.
21. Williams, J.A., McChesney, D.J., Calayag, M.C., Lingappa, V.R., and Logsdon, C.D. 1988. Expression of receptors for cholecystokinin and other Ca²⁺-mobilizing hormones in *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA*. **85**:4939-4943.
22. Shapiro, M.J., Trejo, J., Zeng, D.W., and Coughlin, S.R. 1996. Role of the thrombin receptor's cytoplasmic tail in intracellular trafficking - distinct determinants for agonist-triggered versus tonic internalization and intracellular localization. *J. Biol. Chem*. **271**:32874-32880.
23. Trejo, J., Connolly, A.J., and Coughlin, S.R. 1996. The cloned thrombin receptor is necessary and sufficient for activation of mitogen-activated protein kinase and mitogenesis in mouse lung fibroblasts. Loss of responses in fibroblasts from receptor knockout mice. *J. Biol. Chem*. **271**:21536-21541.
24. Howells, G.L., et al. 1997. Proteinase-activated receptor-2: expression by human neutrophils. *J. Cell Sci*. **110**:881-887.
25. Brass, L.F., et al. 1992. Structure and function of the human platelet thrombin receptor. Studies using monoclonal antibodies directed against a defined domain within the receptor N terminus. *J. Biol. Chem*. **267**:13795-13798.
26. Schmidt, V.A., et al. 1998. The human proteinase-activated receptor-3 (PAR-3) gene. Identification within a Par gene cluster and characterization in vascular endothelial cells and platelets. *J. Biol. Chem*. **273**:15061-15068.
27. Bernatowicz, M.S., et al. 1996. Development of potent thrombin receptor antagonist peptides. *J. Med. Chem*. **39**:4879-4887.
28. Okamura, T., Hasitz, M., and Jamieson, G.A. 1978. Platelet glycolipin: interaction with thrombin and role as thrombin receptor on the platelet surface. *J. Biol. Chem*. **253**:3435-3443.
29. Ishihara, H., et al. 1997. Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature*. **386**:502-506.
30. Liu, L., Vu, T.-K.H., Esmon, C.T., and Coughlin, S.R. 1991. The region of the thrombin receptor resembling hirudin binds to thrombin and alters enzyme specificity. *J. Biol. Chem*. **266**:16977-16980.
31. Mathews, I.L., et al. 1994. Crystallographic structures of thrombin complexed with thrombin receptor peptides: existence of expected and novel binding modes. *Biochemistry*. **33**:3266-3279.
32. Ishii, K., Gerszten, R., Zheng, Y.-W., Turck, C.W., and Coughlin, S.R. 1995. Determinants of thrombin receptor cleavage: receptor domains involved, specificity, and role of the P3 aspartate. *J. Biol. Chem*. **270**:16435-16440.
33. Patrono, C. 1994. Aspirin as an antiplatelet drug. *N. Engl. J. Med*. **330**:1287-1294.
34. Balsano, F., et al. 1990. Antiplatelet treatment with ticlopidine in unstable angina. A controlled multicenter clinical trial. The Studio della Ticlopidina nell'Angina Instabile Group. *Circulation*. **82**:17-26.
35. Evans, G., Packham, M.A., Nishizawa, E.E., Mustard, J.F., and Murphy, E.A. 1968. The effect of acetylsalicylic acid on platelet function. *J. Exp. Med*. **128**:877-894.
36. Smith, J.B., and Willis, A.L. 1971. Aspirin selectively inhibits prostaglandin production in human platelets. *Nature New Biol*. **231**:235-237.