Protease-Activated Receptor 4 Uses Anionic Residues To Interact with α-Thrombin in the Absence or Presence of Protease-Activated Receptor 1[†]

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ABSTRACT: Thrombin activates protease-activated receptor 1 (PAR1) faster than protease-activated receptor 4 (PAR4) due to a hirudin-like sequence in the exodomain of PAR1 that binds thrombin's exosite I. However, recombinant exodomain studies indicate that PAR4 does have extended contacts with α-thrombin that influence PAR4's kinetics of cleavage. In this report, the role of an anionic cluster (Asp^{57}, Asp^{59}, Glu^{62}, and Asp^{65}) in the exodomain of PAR4 is examined for its influence on cleavage and activation of PAR4 on cells in the absence or presence of PAR1. α-Thrombin induces wild-type PAR4 (PAR4-wt) calcium flux with an EC_{50} of 110 nM, whereas mutation of the four anionic residues (PAR4-AAAA) increases the EC_{50} to 641 nM. In contrast, PAR4-wt and PAR4-AAAA are activated by γ-thrombin with similar EC_{50} values (588 and 449 nM, respectively; $p = 0.48$), suggesting a role for α-thrombin's exosite I in PAR4 activation. Coexpression of PAR1 lowered the EC_{50} of cleavage for PAR4-wt from 321 to 26 nM and for PAR4-AAAA from 1.5 μM to 360 nM. Individual point mutations at Asp^{57}, Asp^{59}, Glu^{62}, and Asp^{65} show that PAR4-D57A is activated by α-thrombin with the same EC_{50} as PAR4-wt (140 nM) whereas PAR4-D59A is the same as PAR4-AAAA (699 nM). Glu^{62} and Asp^{65} contribute to α-thrombin recognition, but to a lesser extent. This report shows that PAR4 uses its anionic cluster to interact with α-thrombin and that this interaction is important even in the presence of PAR1.

Thrombin is the terminal enzyme of the clotting cascade. In addition to proteolyzing fibrinogen to fibrin, thrombin activates a variety of cell types via protease-activated receptors. The protease-activated receptor (PAR) family of G-protein-coupled receptors consists of four members (PAR1−4). PARs are activated by proteolysis of the N-terminal exodomain which exposes the tethered ligand (*1*). PAR1,[1] -3, and -4 are primarily activated by thrombin, whereas PAR2 is activated by trypsin or tryptase (*2*). Human platelets express PAR1 and -4 (*3*). PAR1 is activated by low concentrations of thrombin, whereas PAR4 requires high concentrations. PAR4 is associated with a prolonged stimulus as measured by intracellular Ca^{2+} mobilization and may be required for stable clot formation (*4, 5*). Further, PAR4 cooperates with the P2Y12 receptor to mediate platelet aggregation (*6*). In contrast, PAR1-mediated platelet aggregation was independent of P2Y12 signaling. PAR1 signals through phosphoinositide 3-kinase (PI3K) and phosphatidic acid formed by phospholipase D (PLD) (*7, 8*). PAR4 signaling was not disrupted by inhibitors of PI3K or PLD. Therefore, these data suggest that PAR1 and PAR4 stimulate different signaling pathways in platelets and likely cooperate to mediate platelets' complete response to thrombin. In addition to platelet function, PAR4 participates in leukocyte rolling and may influence neutrophilic response in systemic inflammation contributing to multiorgan failure (*9, 10*). Collectively, PAR4 has physiological roles independent of that of PAR1.

PAR1, like many thrombin substrates, interacts with thrombin's exosite I for specificity and to lower the activation energy of cleavage at the active site (*11*−*14*). The exosite I interaction is mediated by a hirudin-like sequence in the PAR1 exodomain (*15*). In contrast, PAR4 does not have a hirudin-like sequence (*16*). On the basis of studies with peptides and recombinant exodomains, the primary sites of interaction of PAR4 with α-thrombin are at the thrombin cleavage site. In particular, amino acids Leu^{43} at P5, Pro^{44} at P4, and Pro^{46} at P2 are important α-thrombin interaction sites (*17*−*19*). However, individual point mutations at Leu^{43}, Pro^{44}, and Pro^{46} did not influence thrombin binding (i.e., did not influence the K_m) but did reduce the rate of cleavage, indicating that Leu^{43}, Pro^{44}, and Pro^{46} are important for orienting PAR4 in the active site of thrombin for efficient cleavage (*17*). These data suggest that, like PAR1, PAR4 has extended contacts with α-thrombin that minimize the influence of the individual amino acids at the cleavage site (*17*). Earlier work by Jacques and Kuliopulos using purified exodomains demonstrates that mutations of the anionic cluster in the PAR4 exodomain (Asp^{57}, Asp^{59}, Glu^{62}, and Asp^{65}) increased the K_m of α-thrombin binding 4-fold from 56 to 208 μM (*18*). Further experiments demonstrate that the anionic cluster stabilizes the interaction with α-thrombin by slowing the dissociation rate (*18*).

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[1] Abbreviations: PAR1, protease-activated receptor 1; PAR4, protease-activated receptor 4.

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Table 1: Amino Acid Sequences of the PAR4 Exodomain and Mutants[a]

	sequence
PAR4-wt	T7-G_{18}GT...P_{41}SILPAPR*GYPGQVCANDSDTLELPDSSRALLLGWVPT...
PAR4-AAAA	T7-G_{18}GT...P_{41}SILPAPR*GYPGQVCAN**A**S**A**TL**A**LP**A**SSRALLLGWVPT...
PAR4-D57A	T7-G_{18}GT...P_{41}SILPAPR*GYPGQVCAN**A**SDTLELPDSSRALLLGWVPT...
PAR4-S58A	T7-G_{18}GT...P_{41}SILPAPR*GYPGQVCAND**A**DTLELPDSSRALLLGWVPT...
PAR4-D59A	T7-G_{18}GT...P_{41}SILPAPR*GYPGQVCANDS**A**TLELPDSSRALLLGWVPT...
PAR4-T60A	T7-G_{18}GT...P_{41}SILPAPR*GYPGQVCANDSD**A**LELPDSSRALLLGWVPT...
PAR4-E62A	T7-G_{18}GT...P_{41}SILPAPR*GYPGQVCANDSDTL**A**LPDSSRALLLGWVPT...
PAR4-D65A	T7-G_{18}GT...P_{41}SILPAPR*GYPGQVCANDSDTLELP**A**SSRALLLGWVPT...

[a] Sequences are shown with standard one-letter abbreviations. The T7 epitope (MASMTGGQQMG) was added to the amino terminus. The asterisks indicate the thrombin cleavage site. Point mutations are indicated as bold and underlined. For space reasons, amino acids 21−40 of PAR4 are not shown.

The interaction of α-thrombin with PAR4 expressed on cells is less clear. PAR4 is cleaved with low efficiency when expressed on cells in the absence of PAR1 (*18*). However, in the presence of PAR1, as is the case on human platelets, PAR4 is activated more efficiently (*20*). PAR1 enhances PAR4 cleavage by remaining tightly bound to α-thrombin's exosite I at PAR1's hirudin-like sequence. The PAR1-bound α-thrombin cleaves an adjacent PAR4 molecule (*20*). A similar mechanism exists in mouse platelets with PAR3 and PAR4 (*21*). Therefore, it is unclear how PAR4 interacts with α-thrombin at exosite I in this model. Initial studies with human PAR4 demonstrate that α-thrombin and γ-thrombin activate PAR4 with the same efficiency (*16*). Since γ-thrombin is a proteolytic fragment of α-thrombin that does not have a functional exosite I (*22*), these data argue against PAR4 interacting with α-thrombin's exosite I. However, the assessment of γ-thrombin cleaving human PAR4 was based on an end point assay that measured inositol phosphate production which may not reveal kinetic differences.

This study indicates that the anionic cluster in the PAR4 exodomain (Asp^{57}, Asp^{59}, Glu^{62}, and Asp^{65}) contributes to PAR4's recognition and cleavage by α-thrombin. In contrast, γ-thrombin cleaved wild-type PAR4 and PAR4 with mutations in the anionic cluster equally well. Further, the anionic cluster contributes to the interaction of PAR4 with α-thrombin in the presence of PAR1. Finally, the contribution of each of the residues in the anionic cluster was determined. These studies define an additional interaction site on PAR4 for α-thrombin in the presence of PAR1 and provide additional targets for the development of PAR activation antagonists.

MATERIALS AND METHODS

Antibodies and Reagents. Human α-thrombin (specific activity of 3593 NIH units/mg) and γ-thrombin (specific activity of <1% fibrinogen clotting activity) were purchased from Haematological Technologies (Essex Junction, VT). PAR1 and PAR4 antibodies to the thrombin cleavage site were raised in goats and have been previously described (*23, 24*). The PAR1 monoclonal antibody, ATAP2, was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The anti-T7 monoclonal antibody was purchased from EMD Biosciences (La Jolla, CA). Alexa488-conjugated secondary antibodies to goat or mouse were purchased from Molecular Probes (Eugene, OR) for flow cytometry. PAR1 activating peptide (TFLLRN) and PAR4 activating peptide (AYPGKF) were synthesized at NeoMPS (San Diego, CA). Bradykinin was purchased from Bachem (Torrence, CA). All other reagents were from Sigma (St. Louis, MO) unless otherwise specified.

Cell Lines, Plasmids, and Transfection. HeLa cells were from American type Culture Collection (Bethesda, MD) and were cultured in DMEM supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). The cDNA encoding human PAR1 (provided by L. Brass, University of Pennsylvania, Philadelphia, PA) or human PAR4 (UMR cDNA Resource Center, www.cdna.org) was modified by inserting a FLAG (DYKDDDK) or T7 (MASMTGGQQMG) epitope tag at the amino terminus (see below for details) and subsequently cloned into the pLK-neo or pLK-pac vector for expression in HeLa cells. Vectors pLKneo and pLKpac were kindly provided by N. Fasel (University of Lausanne, Lausanne, Switzerland) and K. R. Johnson (University of Nebraska Medical Center, Omaha, NE), respectively (*25, 26*). These vectors are dexamethasone inducible. For all studies, expression of PARs in HeLa cells was induced with 10^{-7} M dexamethasone for 24 h.

The T7 epitope was inserted between the signal sequence and the mature PAR4 N-terminus using overlapping PCR as previously described (*24*). The primers used for inserting the T7 epitope were 5′-atggcatcaatgacaggaggtcaacagatggcggcggcacccagaccccagcgtc-3′ and 5′-gcccatctgttgacctcctggtcattgatgccatagacaggctgaaccccagcaccagg-3′. For PAR1, the FLAG epitope was inserted after the signal peptide using a similar strategy with primers 5′-atggattataagatgacgatgacgatgccgcaggccagaatcaaaagca-3′ and 5′-atcgtcatcgtcataattataatccatccgggtgcgggcagacaacagcgg-3′. PAR4-wt-T7 and PAR1-wt-FLAG were used as templates to introduce point mutations by overlapping PCR (Table 1). The primers for point mutations were 5′-gccaatgccagtgccaccctggcgctcccggggccagctc-3′ and 5′-gagctggccgggagcgccagggtggcactgccattggc-3′ (PAR4-AAAA), 5′-gccaatgccatggacaccctg-3′ and 5′-ctcactggcattggcacagac-3′ (PAR4-D57A), 5′-gtgccaatgacgctgacaccctg-3′ and 5′-ctccagggtgtcagcgtcattgg-3′ (PAR4-S58A), 5′-gacagtgacaccctggagctc-3′ and 5′-caggggtggcactgtcattggc-3′ (PAR4-D59A), 5′-gccaatgacagtgacgccctggagctc-3′ and 5′-gtccgggagctccagggcgtcactgtc-3′ (PAR4-T60A), 5′-accctggcgctcccggacgctc-3′ and 5′-cgggagcgccagggtgtcact-3′ (PAR4-E62A), 5′-ctcccggccagctcacgggc-3′ and 5′-gagctggccgggagctccagg-3′ (PAR4-D65A), or 5′-agatccccggtcagctcttctcag-3′ and 5′-ctgagaaagagctgaccggggatct-3′ (PAR1-F43A). All constructs were verified by DNA sequencing.

HeLa cells were transfected using Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Stable cells lines were selected in 1 mg/mL G418 (Invitrogen) or 1 μg/mL puromycin (Sigma) and sorted for PAR expression by fluorescence-activated cell

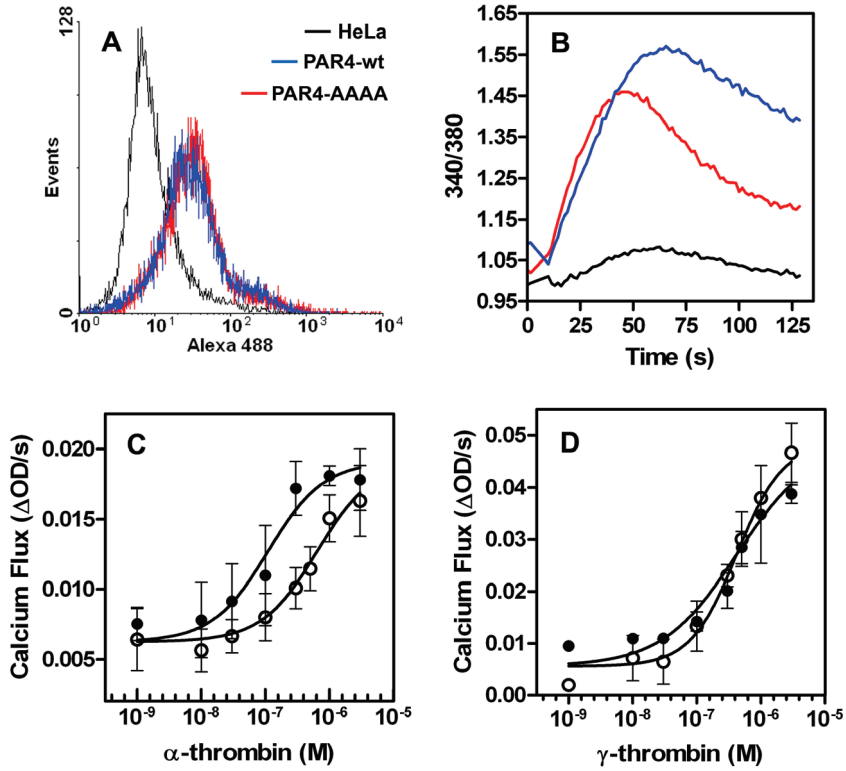

FIGURE 1: Anionic residues in the PAR4 exodomain are important for PAR4 activation by α-thrombin when expressed on cells. (A) PAR4-wt (blue) or PAR4-AAAA (red) was expressed in HeLa cells (black) that do not express PAR4, and expression levels were determined by flow cytometry with a monoclonal antibody to the T7 epitope. (B) Calcium mobilization tracings in mock transfected Hela cells (black) stimulated with α-thrombin (1000 nM) or PAR4-wt transfected HeLa cells (300 nM α-thrombin) (blue) or PAR4-AAAA transfected cells (1000 nM α-thrombin) (red). (C and D) HeLa cells expressing PAR4-wt (●) or PAR4-AAAA (○) were stimulated with 1−3000 nM α-thrombin (C) or γ-thrombin (D). Initial rates of calcium mobilization were determined at early time points where the signal was linear. Data were fit to sigmoidal dose−response curve with nonlinear least-squares global analysis to determine the EC_{50}. The error bars represent the standard deviation of four to six independent experiments, and the apparent absence of error bars indicates a small standard deviation.

sorting (FACS) on a BD Biosciences FACSAria instrument at the flow cytometry core facility of the Comprehensive Cancer Center of Case Western Reserve University. The expression of PAR1, PAR4, or mutants in the resulting cells was verified by flow cytometry to ensure that similar expression levels were obtained for each of the PAR mutants.

Calcium Mobilization. Calcium mobilization studies were carried out as previously described (*27*). Briefly, cells were removed from plates with versene and labeled with fura2-AM (Molecular Probes) in Hepes-Tyrodes buffer supplemented with magnesium and calcium. Cells (2.5×10^5) were placed in 96-well plates, stimulated with agonist, and read in a NOVOstar plate reader (BMG Labtech, Durham, NC). Initial changes in calcium flux were determined at early time points where the signal was linear.

Cleavage Assays. Cells were removed from plates with versene, washed twice with PBS, and resuspended in PBS (2.5×10^5 cells/250 μL). Reactions were initiated by adding α-thrombin (0.3−3000 nM) and mixtures incubated for 30 min at 37 °C. Reactions were stopped with a molar excess of hirudin and mixtures washed immediately with PBS containing 1% BSA. A monoclonal antibody to the T7 epitope (EMD Biosciences) was incubated for 30 min followed by the anti-mouse antibody conjugated to Alexa488 (Molecular Probes). The mean fluorescence was determined by flow cytometry on a Beckman Coulter-XL instrument at the flow cytometry core facility of the Comprehensive Cancer Center of Case Western Reserve University. The EC_{50} was determined by measuring the change in mean fluorescence as a result of the loss of the T7 epitope.

Data Analysis. Initial changes in calcium flux at each agonist concentration were fit to a sigmoidal dose−response curve using nonlinear least-squares regression analysis with Prism (Graphpad, San Diego, CA) (*28*). In the calculations, all data from all experiments were analyzed simultaneously (global analysis) (*28, 29*). *p* values were determined using an *F*-test of the globally fit data with Prism (Graphpad), and *p* values of <0.05 were considered statistically different.

RESULTS

To determine the influence of the anionic cluster (Asp^{57}, Asp^{59}, Glu^{62}, and Asp^{65}) in the PAR4 exodomain on PAR4 activation by α-thrombin, wild-type PAR4 (PAR4-wt) or a panel of PAR4 mutants of the anionic cluster were expressed in HeLa cells (Table 1 and Figure 1A). HeLa cells do not express endogenous PAR1 or PAR4 as determined by flow cytometry (data not shown). PAR4-wt or a mutant in which all four residues in the anionic cluster were mutated to alanine [PAR4-AAAA (Table 1)] was expressed in HeLa cells. These transfected cell lines exhibited equal levels of expression of PAR4-wt and PAR4-AAAA as determined by flow cytometry using a T7 monoclonal antibody (Figure 1A). HeLa cells do not mobilize calcium in response to 1 μM α-thrombin (Figure 1B), PAR1 activation peptide, TFLLRN (data not shown), or PAR4 activation peptide, AYPGKF (data not shown). HeLa cells expressing PAR4-wt or PAR4-AAAA

Table 2: Thrombin-Induced Calcium Mobilization of PAR4 Expressed on HeLa Cells[a]

	α-thrombin		γ-thrombin		
	EC_{50} (nM)	95% confidence interval	EC_{50} (nM)	95% confidence interval	*x*-fold change
PAR4-wt	110	34–352	588	144–2390	5.4*
PAR4-AAAA	642*	241–1710	449	174–1160	–1.4
PAR1-wt	2.06	1.26–3.37	ND		
PAR4-wt with PAR1-F43A	4.5*	1.5–13.7	ND		
PAR4-AAAA with PAR1-F43A	56*	9.0–349	569	66–1830	1.0*
PAR4-D57A	122	76–195	709	561–896	5.8*
PAR4-S58A	180	68–480	ND		
PAR4-D59A	699*	214–2280	479	295–779	–1.5
PAR4-T60A	110	30–412	ND		
PAR4-E62A	324*	197–531	571	251–1080	1.8
PAR4-D65A	336*	195–581	566	251–1280	1.8

[a] Calcium mobilization was assessed by fura2 fluorescence in HeLa cells expressing wild-type or mutant PAR4 alone or in the presence of PAR1-F43A, a nonsignaling PAR1 mutant, stimulated with α- or γ-thrombin (1−3000 nM). EC_{50} values were compared with an *F*-test and considered statistically different from those of cells expressing PAR4-wt alone at $p < 0.05$ (indicated with asterisks). The values of the *x*-fold change in EC_{50} from α-thrombin and γ-thrombin were similarly compared, and significant differences are indicated with asterisks.

mobilize calcium when stimulated with 300 or 1000 nM α-thrombin, respectively (Figure 1B).

Experiments next determined the efficiency of receptor activation as indicated by calcium mobilization. PAR4-wt was activated by α-thrombin with an EC_{50} of 110 nM [Table 2 and Figure 1C (●)]. In contrast, when all four of the anionic residues were mutated to alanine (PAR4-AAAA), the EC_{50} for α-thrombin activation was increased to 642 nM [Table 2 and Figure 1C (○)], 6-fold higher than that of the wild-type receptor ($p = 0.0009$). To determine if α-thrombin's exosite I contributed to the difference in activation, the efficiency of γ-thrombin-induced activation of PAR4-wt and PAR4-AAAA was determined. In contrast to α-thrombin, PAR4-wt and PAR4-AAAA were activated by γ-thrombin with the same EC_{50} (588 nM for PAR4-wt vs 449 nM for PAR4-AAAA; $p = 0.48$) (Table 2 and Figure 1D). These data suggested that the anionic cluster in the PAR4 exodomain may have contributed to its activation by α-thrombin via exosite I interactions.

To evaluate the ability of other G-protein-coupled receptors to couple to G-proteins in the presence of the exogenously expressed PARs, HeLa cells and HeLa cells expressing PAR4-wt or PAR1-wt were examined to determine their response to bradykinin. The EC_{50} of bradykinin stimulating calcium mobilization in HeLa cells (90.6 nM) was not affected by the expression of PAR1 (67.5 nM; $p = 0.70$) or PAR4 (71.7 nM; $p = 0.89$) in these cells (Figure 2).

To independently verify that these mutations influenced the rate of receptor cleavage by α-thrombin, experiments next determined the efficiency of PAR4-wt and PAR4-AAAA cleavage by α-thrombin on cells as measured by the disappearance of the T7 antibody epitope using flow cytometry. PAR4-wt was cleaved by α-thrombin with an EC_{50} of 321 nM [Table 3 and Figure 3A (●)]. These data are comparable to published results for PAR4-wt expressed on COS-7 cells (*18*). In contrast, PAR4-AAAA was cleaved by α-thrombin with an EC_{50} of 1.54 μM, ~10-fold higher than that of PAR4-wt [Table 3 and Figure 3B (●)].

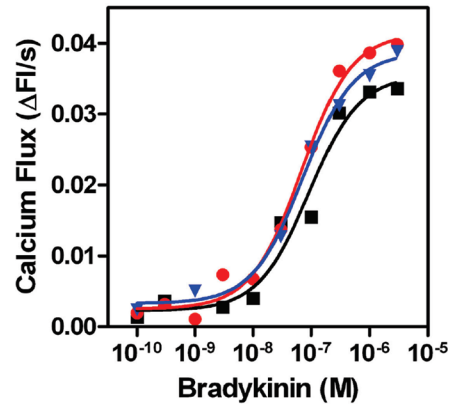

FIGURE 2: Exogenous expression of PAR1 or PAR4 in HeLa cells does not influence bradykinin-induced calcium mobilization. HeLa cells (■) or HeLa cells expressing PAR1-wt (●) or PAR4-wt (▼) were stimulated with 0.1−1000 nM bradykinin. Initial rates of calcium mobilization were determined at early time points where the signal was linear. Data were fit to a sigmoidal dose−response curve with nonlinear least-squares global analysis to determine the EC_{50}.

Table 3: Cleavage of PAR4 Expressed on HeLa Cells by α-Thrombin[a]

	α-thrombin		
	EC_{50} (nM)	95% confidence interval	*x*-fold change
PAR4-wt	321	63–1630	
PAR4-AAAA	1540*	800–2930	
PAR4-wt with PAR1-wt	26*	9.5–71	–12.3*
PAR4-AAAA with PAR1-wt	360	242–534	–4.3*

[a] Cells were incubated with α-thrombin (0.3−3000 nM) for 30 min, and the loss of the T7 epitope was determined by flow cytometry. EC_{50} values were compared with an *F*-test and considered to be statistically different from that of PAR4-wt at $p < 0.05$ (indicated with asterisks). The values of the *x*-fold change in EC_{50} for cells coexpressing PAR1-wt were similarly compared, and significant changes are indicated with asterisks.

On human platelets, PAR1 serves as a cofactor for PAR4 to lower the concentration of α-thrombin required for PAR4 cleavage (*20*). Experiments determined the influence of PAR1 coexpression on the efficiency of PAR4-wt and PAR4-AAAA cleavage by α-thrombin on HeLa cells. Coexpression of PAR1 lowered the EC_{50} of PAR4-wt cleavage 12.3-fold to 26 nM ($p = 0.041$) [Table 3 and Figure 3A (○)]. Similarly, coexpression of PAR1 with PAR4-AAAA lowered the EC_{50} 4.3-fold to 360 nM ($p = 0.017$) [Table 3 and Figure 3B (○)]. Therefore, even in the presence of PAR1, the anionic cluster contributes to PAR4 recognition by α-thrombin in a manner independent of PAR1.

In cells expressing PAR1 and -4, both receptors are activated by thrombin. To eliminate signaling from PAR1, a signaling deficient mutant (PAR1-F43A) was expressed in HeLa cells (Figure 4A). PAR1-F43A binds α-thrombin; however, the mutation in the tethered ligand region renders this mutant incapable of signaling (Figure 4B) (*20*). In contrast, there was a robust response when these cells were stimulated with the PAR1 agonist peptide, TFLLRN, indicating that PAR1-F43A is expressed on the surface of the cell in the proper conformation to allow signaling (Figure 4B). Since PAR1-F43A does not signal in response to α-thrombin, when PAR4-wt or PAR4-AAAA is coexpressed with PAR1-F43A the signal generated in response to thrombin is

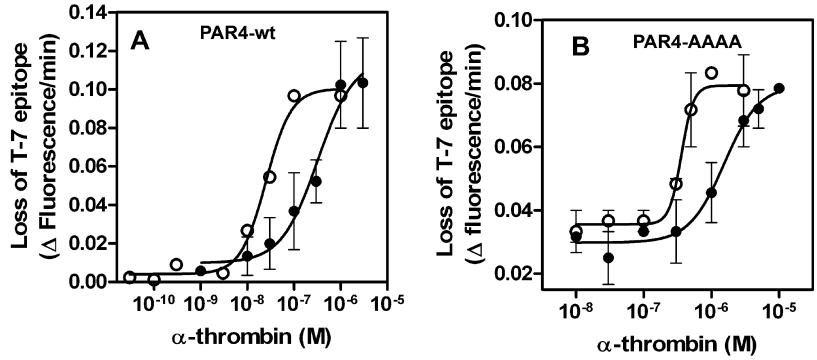

FIGURE 3: Anionic residues in the PAR4 exodomain are important for PAR4 cleavage on cells by α-thrombin. (A) HeLa cells expressing PAR4-wt (●) or PAR4-wt and PAR1-wt (○) were incubated with α-thrombin (0.3−3000 nM) for 30 min, and the reactions were stopped with a molar excess of hirudin. The loss of the T7 epitope was measured with flow cytometry. Data were fit to a sigmoidal dose−response curve with nonlinear least-squares global analysis to determine the EC_{50}. The error bars represent the standard deviation of four independent experiments, and the apparent absence of error bars indicates a small standard deviation. (B) HeLa cells expressing PAR4-AAAA (●) or PAR4-AAAA and PAR1-wt (○) were treated and analyzed as described for panel A.

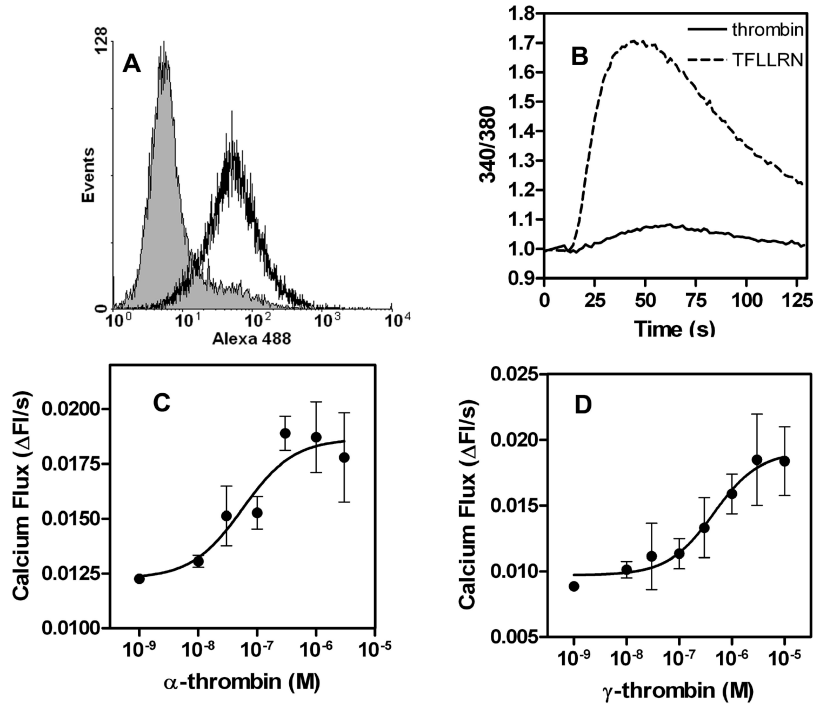

FIGURE 4: PAR1 coexpression enhances PAR4-AAAA calcium mobilization. (A) PAR1-F43A (open) was expressed in HeLa cells (shaded) that do not express PAR1, and expression levels were determined by flow cytometry with a PAR1 antibody. (B) Calcium mobilization tracings of HeLa cells expressing the signaling deficient PAR1 mutant (PAR1-F43A) stimulated with α-thrombin (1000 nM, solid line) or the PAR1 agonist, TFLLRN (100 μM, dashed line). (C and D) HeLa cells coexpressing PAR1-F43A and PAR4-AAAA were stimulated with 1−3000 nM α-thrombin (C) or γ-thrombin (D). Initial rates of calcium mobilization were determined at early time points where the signal was linear. Data were fit to a sigmoidal dose−response curve with nonlinear least-squares global analysis to determine the EC_{50}. The error bars represent the standard deviation of four to six independent experiments, and the apparent absence of error bars indicates a small standard deviation.

exclusively from PAR4-wt or PAR4-AAAA activation. The EC_{50} of α-thrombin-induced calcium mobilization of PAR4-AAAA activation in the presence of PAR1-F43A was 55.8 nM, ~10-fold lower than in the absence of PAR1 (Table 2 and Figure 4C). Similarly, coexpression of PAR1-F43A lowered the EC_{50} of PAR4-wt activation by α-thrombin to 4.5 nM (Table 2). In contrast, PAR1-F43A coexpression did not influence PAR4-AAAA activation by γ-thrombin (EC_{50} = 569 nM vs a value of 449 nM for PAR4-AAAA expressed alone) (Table 2 and Figure 4D).

To determine the contribution of the individual anionic amino acid residues to PAR4's activation by α-thrombin, individual point mutations were introduced into PAR4 (Table 1). These transfected cell lines had equal expression of each of the PAR4 mutants as determined by flow cytometry using a T7 monoclonal antibody (Figure 5A). The EC_{50} for α-thrombin-induced calcium mobilization was measured for each mutant and compared to that of PAR4-wt (Table 2 and Figure 5B). When Asp^{57} was mutated to alanine (PAR4-D57A), the EC_{50} was 122 nM which was not different from that of PAR4-wt ($p = 0.74$). In contrast, when Asp^{59} was mutated to alanine (PAR4-D59A), the EC_{50} was increased 6.4-fold over that of PAR4-wt to 699 nM ($p = 0.0006$). Mutation of Glu^{62} (PAR4-E62A) and Asp^{65} (PAR4-D65A) increased the EC_{50} 3-fold to 324 nM ($p = 0.0087$) and 336 nM ($p = 0.045$), respectively (Table 2 and Figure 5B). Interestingly, a point mutation at Asp^{59} was activated by α-thrombin with the same EC_{50} as when all four residues

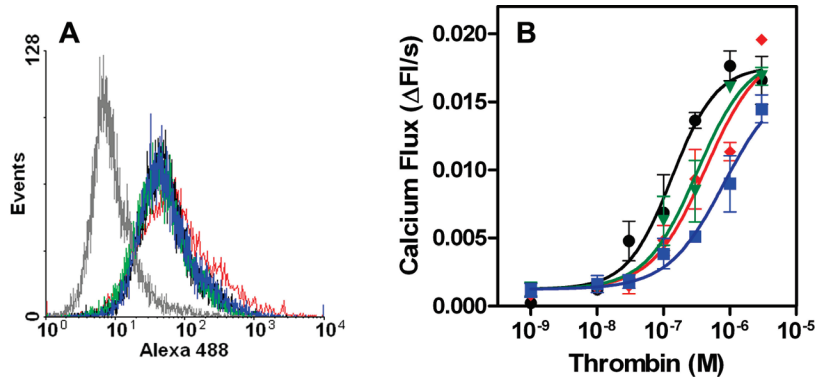

FIGURE 5: Influence of individual anionic residues on PAR4 activation by α-thrombin. (A) HeLa cells (gray) expressing PAR4-D57A (black), PAR4-D59A (blue), PAR4-E62A (red), or PAR4-D65A (green) were analyzed for expression levels with anti-T7 antibodies and flow cytometry. (B) HeLa cells expressing PAR4-D57A (●), PAR4-D59A (■), PAR4-E62A (◆), or PAR4-D65A (▼) were stimulated with 1−3000 nM α-thrombin. Initial rates of calcium mobilization were determined at early time points where the signal was linear. Data were fit to a sigmoidal dose−response curve with nonlinear least-squares global analysis to determine the EC_{50}. The error bars represent the standard deviation of four to six independent experiments, and the apparent absence of error bars indicates a small standard deviation.

were mutated ($p = 0.90$), and mutations at Glu^{62} and Asp^{65} resulted in an EC_{50} 2-fold lower than that when all four residues were mutated ($p = 0.018$ and 0.025, respectively). For each of the individual point mutations, activation by γ-thrombin was not different for PAR4-wt or PAR4-AAAA (Table 2).

Experiments next determined if noncharged residues in the anionic cluster contribute to PAR4 activation by α-thrombin. Since Asp^{59} was critical for PAR4 activation by α-thrombin, Ser^{58} and Thr^{60} were substituted with alanine [PAR4-S58A and PAR4-T60A, respectively (Table 1)]. The EC_{50} of α-thrombin-induced calcium mobilization was not changed compared to that of PAR4-wt in PAR4-S58A (180 nM; $p = 0.70$) or PAR4-T60A (110 nM; $p = 0.89$) (Table 2).

DISCUSSION

This report indicates that the anionic cluster in the PAR4 exodomain (Asp^{57}, Asp^{59}, Glu^{62}, and Asp^{65}) modulates α-thrombin activation of PAR4 both in the absence and in the presence of PAR1. These studies determine that the EC_{50} for PAR4 activation is 6-fold higher when all four residues of the anionic cluster are mutated to Ala. These mutations do not influence the EC_{50} of γ-thrombin-induced calcium mobilization, suggesting that this region may interact with α-thrombin's exosite I. In addition, the contribution of each of the residues in the anionic cluster is determined, and Asp^{59} has the greatest influence (Table 2). Leger et al. demonstrated (*20*) that PAR1 serves as a cofactor for PAR4 activation by interaction with α-thrombin's exosite I. Previous studies with purified exodomains determined that PAR4 has extended contacts with α-thrombin and that PAR4's anionic cluster may interact with α-thrombin's exosite I (*17, 18*). This investigation furthers these observations by showing for the first time that the complete PAR4 receptor utilizes the anionic cluster to interact with α-thrombin on cells and that this interaction is also important in the presence of PAR1.

To date, the interaction of thrombin with PAR4 has been primarily studied with peptides and purified exodomains (*17*−*19*). Initial studies using PAR4 peptides (corresponding to residues Ser^{38}−Glu^{62}) showed that PAR4 interacts primarily with thrombin's active site (*19*). Although this peptide contained two (Asp^{59} and Glu^{62}) of the three residues that contribute to PAR4's interaction with α-thrombin (Table 2 and Figure 4), Asp^{59} and Glu^{62} may have different conformations when placed at the extreme C-terminus of a peptide. Other studies using purified full-length exodomains show that PAR4 has extended contacts with thrombin (*17, 18*). Jacques and Kuliopulos demonstrate that PAR4 interacts with thrombin via two proline residues at P4 (Pro^{44}) and P2 (Pro^{46}) (*18*). These data support the NMR work of Cleary et al. (*19*). Additional studies with purified exodomains examine the role of individual amino acids at the thrombin cleavage site and demonstrate that Leu^{43} at P5 also influenced thrombin's interaction with PAR4 (*17*). Interestingly, none of these individual point mutations at P5 (Leu^{43}), P4 (Pro^{44}), or P2 (Pro^{46}) influences the K_m of α-thrombin binding. However, in each case, the k_{cat} is decreased, thereby lowering the catalytic efficiency (k_{cat}/K_m) of proteolysis by α-thrombin. Since the reaction efficiency is decreased without influencing the K_m, PAR4 may have extended contacts with thrombin that minimize the effects of individual amino acids at the cleavage site. This assessment is supported by earlier work that showed that the anionic cluster (Asp^{57}, Asp^{59}, Glu^{62}, and Asp^{65}) functions to decrease the dissociation rate of the PAR4 exodomain with thrombin, enhancing cleavage of PAR4 by α-thrombin (*18*). Although each of these studies suggests that PAR4 interacts with thrombin's exosite I, this interaction is weaker than that of PAR1 and does not induce allosteric changes in α-thrombin (*17, 18*). In contrast, PAR1 binds tightly to thrombin's exosite I via a hirudin-like sequence, and this interaction allosterically influences the conformation at α-thrombin's active site (*14, 15, 17, 30*). Although an initial report describing PAR4 suggests that it is cleaved with the same efficiency by γ-thrombin as α-thrombin, these studies determined the inositol phosphate production with a single γ-thrombin concentration (100 nM) at a fixed time point (2 h) which may not detect kinetic differences between PAR4 activation by α- and γ-thrombin (*16*). The data presented in this report demonstrate that the EC_{50} for activation of PAR4 is 6-fold lower for α-thrombin than for γ-thrombin (Table 2 and Figure 1A,C). This report also shows that Asp^{59}, Glu^{62}, and Asp^{65} of PAR4's anionic cluster contribute to PAR4's recognition by α-thrombin but Asp^{57} does not (Table 2 and Figure 5B).

A recent report described a mechanism for PAR1 facilitating efficient cleavage of PAR4 by α-thrombin (*20*). This

mechanism is similar to PAR4 activation via PAR3 in mice (*21*). Thrombin activates PAR4 while its exosite I is bound to the hirudin-like region of PAR1 or PAR3. The work reported here shows that PAR1 enhances the cleavage of mutant PAR4 (PAR4-AAAA) to the same degree as PAR4-wt (~10-fold) (Table 2). Further, it is noteworthy that PAR4-AAAA is not cleaved at the same EC_{50} as PAR4-wt in the presence of PAR1 (Figure 3). If PAR1's interaction at α-thrombin's exosite I supersedes the interaction of PAR4 with this region, one would expect the EC_{50} of PAR4-wt and PAR4-AAAA cleavage to be the same in cells coexpressing PAR1. Instead, PAR1 lowers the EC_{50} to the same degree for PAR4-wt and PAR4-AAAA. This finding indicates that the anionic residues on PAR4 also contribute to PAR4 activation even in the presence of PAR1.

Crystallography studies provided a model for how murine PAR3 and PAR4 could simultaneously interact with murine thrombin. These studies used peptides derived from murine PAR3 ($Ser^{38}-Glu^{56}$) or PAR4 ($Lys^{51}-Ala^{81}$) for cocrystallization with mouse thrombin (*31*). The resulting structures with each peptide were modeled to show the PAR3 peptide binding to exosite I and the PAR4 peptide interacting at the active site. The contacts of the PAR4 peptide near the active site of α-thrombin support previously published interactions described for human recombinant PAR4 exodomains (*17*−*19*). However, in contrast to functional data with human PAR4 presented here and elsewhere (*17, 18*), the anionic residues of PAR4 are not interacting with α-thrombin. A potential explanation for this difference is that the anionic residues do not interact with α-thrombin but rather are required for the proper folding of the PAR4 exodomain. However, several lines of evidence argue against this explanation. First, kinetic studies with purified exodomains suggest that there are extended contacts with α-thrombin (*17, 18*). Second, mutating noncharged residues (Ser^{58} or Thr^{60}) within the anionic cluster does not influence the ability of these receptors to be activated by α-thrombin (Table 2). Third, the mutant PAR4 receptors are activated by γ-thrombin with the same efficiency as PAR4-wt (Table 2). If the anionic residues are important for the structure of the PAR4 exodomain, one would expect differences in γ-thrombin activation as well. Finally, the sequence of human and murine PAR4 is highly conserved in the anionic cluster region. In particular, the residues that are important in the murine crystal structure (Gly^{60} at P1′, Pro^{62} at P3′, and Cys^{66} at P7′) are identical. These residues coordinate to direct the C-terminus of the PAR4 exodomain toward the autolysis loop and away from exosite I. Therefore, PAR4 may be interacting with the region near the autolysis loop. Further, the crystal structure of γ-thrombin indicates that there are two regions that become disordered as a result of the cleavages that occur to α-thrombin during the formation of γ-thrombin (*22*). Cleavages at Arg^{75} and Arg^{77a} disrupt the eight residues between Ser^{72} and Glu^{80} which alters exosite I, and a cleavage at Lys^{149a} disorders the 14 residues from Gly^{142} and Pro^{152} in the autolysis loop. The murine PAR4 peptide cocrystallized with murine thrombin indicates that PAR4 may in fact be interacting with the region near the autolysis loop. However, PAR4-wt expressed on HeLa cells is activated by β-thrombin with the same efficiency as γ-thrombin (M. T. Nieman, unpublished data). β-Thrombin has cleavages at only Arg^{75} and Arg^{77a} which may not influence the autolysis loop (*22*). However, the crystal structure of β-thrombin has not been determined. Therefore, it is as yet unknown how the autolysis loop is altered during the generation of β-thrombin.

PAR antagonists are an attractive target for antithrombotic therapies. Inhibition of PAR activation or signaling provides promising therapies for cardiovascular disease. To date, much focus has been on targeting PAR1. However, PAR4 may provide additional safety in that PAR4 is required for stable clot formation (*4, 5*). Thus, antagonism of PAR4 may allow platelets to respond initially via PAR1, but the later stages of clot formation may be blocked by PAR4 inhibition. PAR1 is also expressed on a wide variety of tissues in addition to platelets, whereas PAR4 is mainly expressed on platelets. Thus, targeting PAR4 may more specifically target platelet activation. This study defines thrombin interaction sites on PAR4 that are important for its activation in the presence of PAR1, indicating a target for development of anti-PAR4 activity. Further, in the presence of PAR1, PAR4 is activated with a nearly equal EC_{50} as PAR1, furthering the importance of PAR4 activation on platelets. Antagonists targeted to the anionic region of PAR4 may increase the extent of platelet inhibition at reduced risk to bleeding.

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REFERENCES

1. Coughlin, S. R. (2000) Thrombin signalling and protease-activated receptors. *Nature 407*, 258–264.
2. Steinhoff, M., Buddenkotte, J., Shpacovitch, V., Rattenholl, A., Moormann, C., Vergnolle, N., Luger, T. A., and Hollenberg, M. D. (2005) Proteinase-activated receptors: Transducers of proteinase-mediated signaling in inflammation and immune response. *Endocr. Rev. 26*, 1–43.
3. Kahn, M. L., Nakanishi-Matsui, M., Shapiro, M. J., Ishihara, H., and Coughlin, S. R. (1999) Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *J. Clin. Invest. 103*, 879–887.
4. Mazharian, A., Roger, S., Berrou, E., Adam, F., Kauskot, A., Nurden, P., Jandrot-Perrus, M., and Bryckaert, M. (2007) Protease-activating receptor-4 induces full platelet spreading on a fibrinogen matrix: Involvement of ERK2 and p38 and Ca^{2+} mobilization. *J. Biol. Chem. 282*, 5478–5487.
5. Covic, L., Singh, C., Smith, H., and Kuliopulos, A. (2002) Role of the PAR4 thrombin receptor in stabilizing platelet-platelet aggregates as revealed by a patient with Hermansky-Pudlak syndrome. *Thromb. Haemostasis 87*, 722–727.
6. Holinstat, M., Voss, B., Bilodeau, M. L., McLaughlin, J. N., Cleator, J., and Hamm, H. E. (2006) PAR4, but not PAR1, signals human platelet aggregation via Ca^{2+} mobilization and synergistic P2Y12 receptor activation. *J. Biol. Chem. 281*, 26665–26674.
7. Holinstat, M., Voss, B., Bilodeau, M. L., and Hamm, H. E. (2007) Protease-activated receptors differentially regulate human platelet activation through a phosphatidic acid-dependent pathway. *Mol. Pharmacol. 71*, 686–694.
8. Voss, B., McLaughlin, J. N., Holinstat, M., Zent, R., and Hamm, H. E. (2007) PAR1, but not PAR4, activates human platelets through a Gi/o/phosphoinositide-3 kinase signaling axis. *Mol. Pharmacol. 71*, 1399–1406.
9. Slofstra, S. H., Bijlsma, M. F., Groot, A. P., Reitsma, P. H., Lindhout, T., ten Cate, H., and Spek, C. A. (2007) Protease-activated receptor-4 inhibition protects from multiorgan failure in a murine model of systemic inflammation. *Blood 110*, 3176–3182.
10. Vergnolle, N., Derian, C. K., D'Andrea, M. R., Steinhoff, M., and Andrade-Gordon, P. (2002) Characterization of thrombin-induced leukocyte rolling and adherence: A potential proinflammatory role for proteinase-activated receptor-4. *J. Immunol. 169*, 1467–1473.

11. Vu, 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.
12. Liu, L. W., Vu, T. K., 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.
13. Ayala, Y. M., Cantwell, A. M., Rose, T., Bush, L. A., Arosio, D., and Di Cera, E. (2001) Molecular mapping of thrombin-receptor interactions. *Proteins 45*, 107–116.
14. Jacques, S. L., LeMasurier, M., Sheridan, P. J., Seeley, S. K., and Kuliopulos, A. (2000) Substrate-assisted catalysis of the PAR1 thrombin receptor. Enhancement of macromolecular association and cleavage. *J. Biol. Chem. 275*, 40671–40678.
15. Vu, T. K., Wheaton, V. I., Hung, D. T., Charo, I., and Coughlin, S. R. (1991) Domains specifying thrombin-receptor interaction. *Nature 353*, 674–677.
16. Xu, W. F., Andersen, H., Whitmore, T. E., Presnell, S. R., Yee, D. P., Ching, A., Gilbert, T., Davie, E. W., and Foster, D. C. (1998) Cloning and characterization of human protease-activated receptor 4. *Proc. Natl. Acad. Sci. U.S.A. 95*, 6642–6646.
17. Nieman, M. T., and Schmaier, A. H. (2007) Interaction of thrombin with PAR1 and PAR4 at the thrombin cleavage site. *Biochemistry 46*, 8603–8610.
18. Jacques, S. L., and Kuliopulos, A. (2003) Protease-activated receptor-4 uses dual prolines and an anionic retention motif for thrombin recognition and cleavage. *Biochem. J. 376*, 733–740.
19. Cleary, D. B., Trumbo, T. A., and Maurer, M. C. (2002) Protease-activated receptor 4-like peptides bind to thrombin through an optimized interaction with the enzyme active site surface. *Arch. Biochem. Biophys. 403*, 179–188.
20. Leger, A. J., Jacques, S. L., Badar, J., Kaneider, N. C., Derian, C. K., Andrade-Gordon, P., Covic, L., and Kuliopulos, A. (2006) Blocking the protease-activated receptor 1−4 heterodimer in platelet-mediated thrombosis. *Circulation 113*, 1244–1254.
21. Nakanishi-Matsui, M., Zheng, Y. W., Sulciner, D. J., Weiss, E. J., Ludeman, M. J., and Coughlin, S. R. (2000) PAR3 is a cofactor for PAR4 activation by thrombin. *Nature 404*, 609–613.
22. Rydel, T. J., Yin, M., Padmanabhan, K. P., Blankenship, D. T., Cardin, A. D., Correa, P. E., Fenton, J. W., and Tulinsky, A. (1994) Crystallographic structure of human γ-thrombin. *J. Biol. Chem. 269*, 22000–22006.
23. Hasan, A. A., Warnock, M., Nieman, M., Srikanth, S., Mahdi, F., Krishnan, R., Tulinsky, A., and Schmaier, A. H. (2003) Mechanisms of Arg-Pro-Pro-Gly-Phe inhibition of thrombin. *Am. J. Physiol. 285*, H183–H193.
24. Nieman, M. T., Pagan-Ramos, E., Warnock, M., Krijanovski, Y., Hasan, A. A., and Schmaier, A. H. (2005) Mapping the interaction of bradykinin 1−5 with the exodomain of human protease activated receptor 4. *FEBS Lett. 579*, 25–29.
25. Hirt, R. P., Poulain-Godefroy, O., Billotte, J., Kraehenbuhl, J. P., and Fasel, N. (1992) Highly inducible synthesis of heterologous proteins in epithelial cells carrying a glucocorticoid-responsive vector. *Gene 111*, 199–206.
26. Islam, S., Carey, T. E., Wolf, G. T., Wheelock, M. J., and Johnson, K. R. (1996) Expression of N-cadherin by human squamous carcinoma cells induces a scattered fibroblastic phenotype with disrupted cell-cell adhesion. *J. Cell Biol. 135*, 1643–1654.
27. Nieman, M. T., Warnock, M., Hasan, A. A., Mahdi, F., Lucchesi, B. R., Brown, N. J., Murphey, L. J., and Schmaier, A. H. (2004) The preparation and characterization of novel peptide antagonists to thrombin and factor VIIa and activation of protease-activated receptor 1. *J. Pharmacol. Exp. Ther. 311*, 492–501.
28. Johnson, M. L., and Faunt, L. M. (1992) Parameter estimation by least-squares methods. *Methods Enzymol. 210*, 1–37.
29. Beechem, J. M. (1992) Global analysis of biochemical and biophysical data. *Methods Enzymol. 210*, 37–54.
30. Myles, T., Le Bonniec, B. F., and Stone, S. R. (2001) The dual role of thrombin's anion-binding exosite-I in the recognition and cleavage of the protease-activated receptor 1. *Eur. J. Biochem. 268*, 70–77.
31. Bah, A., Chen, Z., Bush-Pelc, L. A., Mathews, F. S., and Di Cera, E. (2007) Crystal structures of murine thrombin in complex with the extracellular fragments of murine protease-activated receptors PAR3 and PAR4. *Proc. Natl. Acad. Sci. U.S.A. 104*, 11603–11608.

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