Interaction of Thrombin with PAR1 and PAR4 at the Thrombin Cleavage Site†

Marvin T. Nieman* and Alvin H. Schmaier

Division of Hematology/Oncology, Case Western Reserve University, Cleveland, Ohio 44106-7284, and Division of Hematology/Oncology, Department of Internal Medicine, The University of Michigan, Ann Arbor, Michigan 48109-0640

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ABSTRACT: Investigations determined the critical amino acids for α-thrombin's interaction with protease-activated receptors 1 and 4 (PAR1 and PAR4, respectively) at the thrombin cleavage site. Recombinant PAR1 wild-type (wt) exodomain was cleaved by α-thrombin with a K_m of 28 μM, a k_{cat} of 340 s^{-1}, and a k_{cat}/K_m of 1.2×10^7. When the P4 or P2 position was mutated to alanine, PAR1-L38A or PAR1-P40A, respectively, the K_m was unchanged, 29 or 23 μM, respectively; however, the k_{cat} and k_{cat}/K_m were reduced in each case. In contrast, when Asp^{39} at P3 was mutated to alanine, PAR1-D39A, K_m and k_{cat} were both reduced ∼3-fold, making the k_{cat}/K_m the same as that of PAR1-wt exodomain. Recombinant PAR4-wt exodomain was cleaved by α-thrombin with a K_m of 61 μM, a k_{cat} of 17 s^{-1}, and a k_{cat}/K_m of 2.8×10^5. When the P5 or P4 position was mutated to alanine, PAR4-L43A or PAR4-P44A, respectively, there was no change in the K_m (69 or 56 μM, respectively); however, the k_{cat} was lowered in each case (9.7 or 7.7 s^{-1}, respectively). Mutation of the P2 position (PAR4-P46A) also had no effect on the K_m but markedly lowered the k_{cat} and k_{cat}/K_m ∼35-fold. PAR1-wt exodomain and P4 and P3 mutants were noncompetitive inhibitors of α-thrombin hydrolyzing Sar-Pro-Arg-pNA. However, PAR1-P40A displayed a mixed type of inhibition. Mutation of P4, P3, or P2 had no effect on the K_i. All PAR4 exodomains were competitive inhibitors of α-thrombin. Mutation of P5, P4, or P2 had no effect on the K_i. These investigations show that Leu at P4 in PAR1 or P5 in PAR4 critically influences the kinetics of α-thrombin binding and cleavage of PAR1 and PAR4 exodomains. It also implies that factors other than the hirudin-like binding region on PAR1 exodomain predominate in influencing PAR1 cleavage on cells.

The serine protease thrombin is the terminal enzyme of the hemostatic system. During clot formation, thrombin converts fibrinogen to fibrin, activates factor XIII that cross-links fibrin in the clot, and activates factors XI, VIII, and V to generate more thrombin (*1*). Further, thrombin activates protein C which negatively regulates thrombin generation by inactivating thrombin-activated factors VIII and V (*1*). Thrombin is also a major activator of platelets and does so by binding and cleaving protease-activated receptors 1 and 4 (PAR1[1] and -4, respectively). The protease-activated receptor family consists of a novel class of G-protein-coupled receptors (GPCRs) that are activated by the proteolysis of the N-terminal exodomain (*2*). Upon proteolysis, the newly formed N-terminus acts as a tethered ligand that activates the receptor and initiates multiple signaling cascades via heterotrimeric G-proteins (*3, 4*). There are four PARs (PAR1−4). PAR1, -3, and -4 are activated by thrombin, whereas PAR2 is activated by trypsin or tryptase (*5*). Other serine proteases like FVIIa, FXa, plasmin, activated protein C, cathepsin G, and MMP1 also activate PARs, although less efficiently (*6*−*10*).

The exodomain of PAR1 has two binding sites for thrombin (*11*). In addition to binding thrombin's active site, PAR1 has a hirudin-like sequence ($K^{51}YEPF^{55}$) that binds exosite I of thrombin, inducing allosteric effects on thrombin by lowering the energy required for PAR1 cleavage (*12*−*14*). The importance of the hirudin-like sequence for more efficient PAR1 activation has been confirmed with thrombin exosite and PAR1 exodomain mutations (*13*−*16*). Much less focus has been given to thrombin's interaction with PAR1 at its cleavage site. It has been proposed that the optimal sequence for thrombin substrates is Leu or Ile at P4 and Pro at P2 (*17*). PAR1 has the optimal residues in the P4 and P2 positions ($L^{38}DPR^{41}$). The P4 to P1 positions of PAR1 are the same as those of protein C which, in the absence of thrombomodulin, is a poor thrombin substrate due to the Asp^{167} at P3 interacting with thrombin's Glu^{192} (*18*). Mutation of Asp^{167} to Gly in protein C or Glu^{192} to Gln in thrombin results in an increased efficiency of cleavage of protein C by thrombin (*18, 19*). Similarly, mutation of the hirudin-like sequence in PAR1 results in a shift in the EC_{50} of thrombin activation from 0.1 to 20 nM when PAR1 is expressed on COS7 cells (*20*). This phenotype was partially rescued by mutating Asp^{39} at P3 to Gly (*20*).

PAR4 does not contain a hirudin-like sequence; as a result, it interacts with thrombin primarily at the active site. It has

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* To whom correspondence should be addressed. Present address: Division of Hematology/Oncology, Case Western Reserve University, Cleveland, OH 44106-7284. Telephone: (216) 368-4975. Fax: (216) 368-1166. E-mail: nieman@case.edu.

[1] Abbreviation: PAR, protease-activated receptor.

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Table 1: PAR Exodomain Mutagenesis Primers

primer	sequence[a]
PAR1-L38A[b]	ACAAATGCC**GCA**GATCCCCGGTCATTT
PAR1-L38A[c]	AAATGACCGGGGATC**TGC**GGTGGCATTTGT
PAR1-D39A[b]	AATGCCACCTTA**GCA**CCCCGGTCATTTCTT
PAR1-D39A[c]	AAGAAATGACCGGGG**TGC**TAAGGTGGCAAT
PAR1-P40A[b]	GCCACCTTAGAT**GCA**CGGTCATTTCTTCT
PAR1-P40A[c]	GAGAAGAAATGACCG**TGC**ATCTAAGGTGGC
PAR4-L43A[b]	ACGCCCTCAATC**GCA**CCTGCCCCCGCGGC
PAR4-L43A[c]	GCCGCGGGGGCAGG**TGC**GATTGAGGGCGT

[a] Mutagenized codon in bold. [b] Sense primer. [c] Antisense primer.

Table 2: Mutant PAR1 and PAR4 Exodomains

exodomain	sequence[a]	mass[b]
PAR1-wt	...NATLDPRSFLL...	8493
PAR1-L38A	...NAT**A**DPRSFLL...	8451
PAR1-D39A	...NATL**A**PRSFLL...	8449
PAR1-P40A	...NATLDA**R**SFLL...	8467
PAR4-wt	...SILPAPRGYGK...	6435
PAR4-L43A	...SI**A**PAPRGYGK...	6394
PAR4-P44A	...SIL**A**APRGYGK...	6405
PAR4-P46A	...SILPAA**R**GYGK...	6405

[a] Mutations are shown in bold. The P1 arginine is underlined. [b] Mass as determined by mass spectrometry.

been proposed that PAR4 interacts with thrombin via two proline residues at P4 and P2, Pro[44] and Pro[46], respectively. There is also a role for Leu[43] at P5 for PAR4 cleavage. Ayala et al. (*13*) showed by molecular modeling with PAR4 peptides that Leu[43] interacts with thrombin residues Leu[99], Ile[174], and Trp[215]. Cleary et al. (*21*) demonstrated in NMR studies with PAR4 peptides that Leu[43] exhibits minor proton line broadening, suggesting Leu[43] binds to thrombin but to a lesser extent than the Pro[44]−Arg[47]region. Further, Leu[43] can interact with Pro[44] and Pro[46] in two distinct conformations in the presence of thrombin, suggesting that there is flexibility where Leu[43] interacts with thrombin (*21*). They demonstrate that the NMR structure of a PAR4 peptide (L[43]-PAP[46]) had a three-dimensional structure similar to that of a PAR1 peptide that was cocrystallized with thrombin (*21*, *22*). These combined studies indicate that more needs to be known about how thrombin interacts with the substrate cleavage site of the exodomains of PAR1 and PAR4.

In an effort to define how thrombin interacts with the exodomains of PAR1 and PAR4 at the cleavage site, we sought to identify the amino acids on PAR1 and PAR4 that are important for thrombin's interaction in the context of the full exodomain. The current report identifies the amino acids in the P4 (Leu[38]) and P2 (Pro[40]) positions of PAR1 and in the P5 (Leu[43]), P4 (Pro[44]), and P2 (Pro[46]) positions of PAR4 are important for efficient cleavage by thrombin since Ala substitution of each adversely affects the k_{cat}. Parenthetically, none of these point mutations introduced into PAR1 or PAR4 influence the K_m of thrombin cleavage. This investigation is the first functional evidence that Leu[38] at P4 for PAR1 and Leu[43] at P5 for PAR4 have an important role for thrombin binding and cleavage. These data suggest that the Leu and Pro in these positions on these receptors cooperate to form a similar three-dimensional structure that is recognized by thrombin.

MATERIALS AND METHODS

Cloning and Mutagenesis of PAR1 and PAR4 Exodomains. Recombinant PAR1-wt, PAR4-wt, PAR4-P44A, and PAR4-P46A exodomains have been previously described (*23*). Additional mutations were introduced into PAR1 or PAR4 exodomain by overlapping PCR with the same protocol. The mutagenizing primers for PAR1-L38A, PAR1-D39A, PAR1-P40A, and PAR4-L43A are shown in Table 1. All constructs were verified by DNA sequencing and inserted into pET31b (Novagen, San Diego, CA) for expression in *Escherichia coli*.

Recombinant Protein Production. Purified recombinant exodomain of PAR1, PAR1 mutants, PAR4, or PAR4 mutants was prepared from 2 L cultures of *E. coli* BLR-(DE3) as previously desribed (*23*). The M_r of each expressed PAR exodomain was verified by MALDI-TOF mass spectrometry (Pro-TOF 2000 MALDI mass spectrometer, Perkin-Elmer, Wellesley, MA) in the Center for Proteomics and Mass Spectrometry, Case Western Reserve University (Table 2). The concentration of purified recombinant exodomain was determined by UV absorption (ϵ_{280} = 9970 M^{-1} cm^{-1} for PAR1-wt, PAR1-L38A, PAR1-D39A, or PAR1-P40A, or ϵ_{280} = 8480 M^{-1} cm^{-1} for PAR4-wt, PAR4-L43A, PAR4-P44A, or PAR1-P46A).

Cleavage of Soluble Recombinant PAR Exodomain. Cleavage of purified PAR1-wt, PAR4-wt, or mutant exodomain (3−400 μM) in reaction buffer [10 mM Tris-HCl and 150 mM NaCl (pH 8.0)] was initiated by the addition of α-thrombin (0.5−50 nM, specific activity of 3725 units/mg; Haematological Technologies, Essex Junction, VT). The optimal range of substrate concentration varied depending on the PAR exodomain mutant that was being tested. Reactions were quenched at given time intervals by removing 100 μL aliquots and placing them into tubes that contained 50 μL of acetic acid. The reactions were monitored with RP-HPLC (Waters Delta 600, Waters Corp., Milford, MA) on a Waters Atlantis PAA column. The rate of product formation was determined by calculating the peak areas for the N-terminal and C-terminal cleavage products and comparing them to a standard curve. The standard curve was generated with purified cleavage products from completely cleaved exodomains prepared by treating exodomain with a molar excess of α-thrombin. Initial rates were determined at early time points where the progress curves were linear. The data were fit to the Henri−Michaelis−Menten equation (eq 1) using Sigmaplot (Systat, Inc.), and the kinetic constants were determined (see below).

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

In other experiments, the catalytic efficiency (k_{cat}/K_m) was determined at substrate concentrations much lower than the K_m, where eq 2 is valid. s is catalytic efficiency, e_T enzyme concentration, and t time (*13*).

$$[S] = [S]_0^{(-se_Tt)} \quad (2)$$

Inhibition of Thrombin Hydrolysis of Sar-Pro-Arg-pNA. Reactions were initiated by adding α-thrombin (0.5 nM) to Sar-Pro-Arg-pNA (35−1400 μM) in the absence or presence of PAR1-wt, PAR4-wt, or mutant exodomain (5−200 μM) in Tris-HCl and 150 mM NaCl (pH 8.0). For some experi-

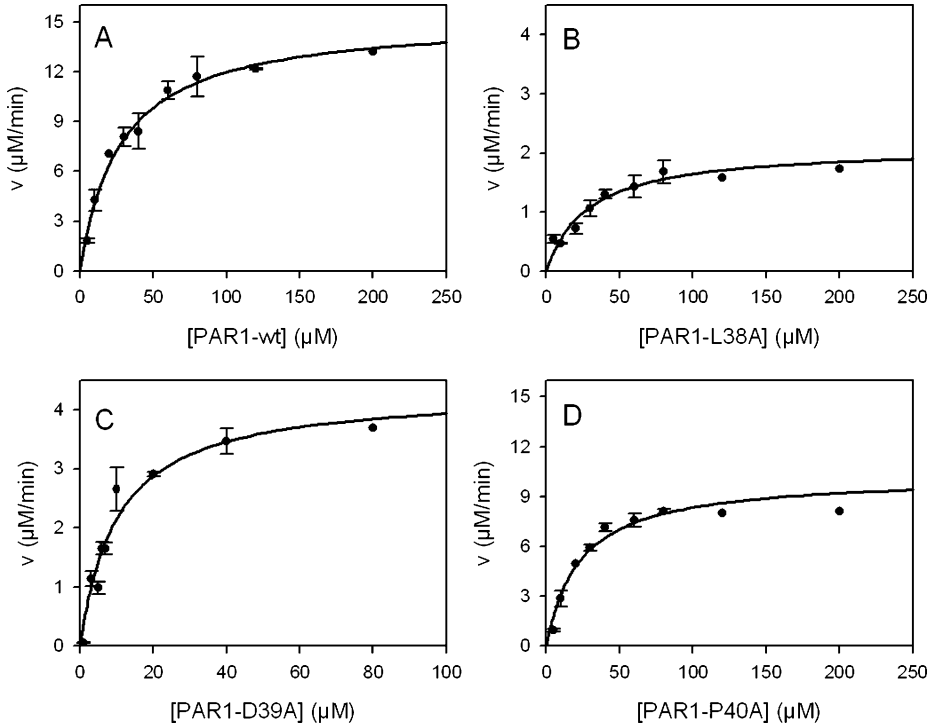

FIGURE 1: Cleavage of PAR1-wt or mutant exodomains with α-thrombin. Reactions with purified exodomains (3−200 μM) in reaction buffer [10 mM Tris-HCl and 150 mM NaCl (pH 8.0)] were initiated by the addition of α-thrombin (0.75 nM for panels A, C, and D or 0.5 nM for panel B). Acid-quenched reactions were resolved via HPLC, and initial rates were determined by calculating the peak areas at early time points where the progress curves were linear and compared to a standard curve generated by completely cleaved exodomain. Curve A is α-thrombin cleavage of PAR1-wt exodomain, curve B that of PAR1-D39A, curve C that of PAR1-L38A, and curve D that of PAR1-P40A. The data were fit to the Henri−Michaelis−Menten equation with nonlinear least-squares analysis to determine the kinetic constants. Error bars represent the standard deviation, and the apparent absence of error bars indicates a small standard deviation. Note the change in the scaling for the *y*-axis in panels B and C.

ments, thrombin was preincubated with 10 μM hirugen (hirudin C-terminal fragment 53−64, American Diagnostica, Inc., Stamford, CT) for 2 min prior to initiation of the reaction. The K_m for Sar-Pro-Arg-pNA hydrolysis by α-thrombin was determined to be 75 μM. Therefore, the range of concentrations used in these experiments (0.5 to ∼20 times the K_m) was adequate for characterizing the inhibition by the PAR exodomains. Initial rates were determined by taking absorbance readings every 5 s with a Beckman DU-64 kinetic spectrophotomer at early time points where the progress curves were linear. Absorbance readings were converted to concentration terms using an ε_{405} of 9887 M^{-1} cm^{-1} (*24*). The type of inhibition, K_i, and, where applicable, α (i.e., mixed inhibition, where α is the factor by which the K_i changes when the substrate is simultaneously bound to the enzyme) were determined as described in the next section.

Data Analysis. Initial velocity data were fit to the Henri−Michaelis−Menten equation (eq 1) by nonlinear least-squares regression analysis with SigmaPlot kinetic analysis software (Systat, Inc.) (*25*). In the calculations, all data from all experiments were analyzed simultaneously (*25*, *26*). Kinetic constants were derived from these analyses and are reported with a ±95% confidence interval. The K_i values for PAR exodomain inhibition of thrombin cleavage of Sar-Pro-Arg-pNA were determined by fitting initial velocity data to eight models of enzyme inhibition also using a global analysis nonlinear least-squares regression analysis with the SigmaPlot enzyme kinetics module (Systat, Inc.) (*26*). The best model was chosen on the basis of the Akaike information criteria (AIC) (*27*). The K_i for each PAR exodomain is reported with a ±95% confidence interval. Double-reciprocal plots are shown only for graphical representation of the model and were not used to determine the K_i or type of inhibition. *P* values were determined using a two-tailed *t*-test with Prism (Graphpad, San Diego, CA).

RESULTS

Investigations aimed at identifying the amino acids at the α-thrombin cleavage site of PAR1 and PAR4 that are important for α-thrombin to bind and cleave were performed. Toward this end, a series of PAR1 and PAR4 exodomain alanine substitution mutants were generated (Table 2), and the efficiency with which α-thrombin binds and cleaves was determined. The homogeneity of each recombinant exodomain was assured between preparations by mass spectrometry (Table 2).

Initial studies directly determined the influence of α-thrombin on the cleavage of PAR1-wt exodomain. The K_m (28 μM), k_{cat} (340 s^{-1}), and k_{cat}/K_m (1.2×10^7 s^{-1} M^{-1}) values for α-thrombin-cleaved PAR1-wt were consistent with previously published data (Figure 1A and Table 3) (*13*, *14*). As an independent confirmation of this approach, we determined the k_{cat}/K_m (1.1×10^7 s^{-1} M^{-1}) at low substrate concentrations where eq 2 is valid (*13*). The K_m of the P4 mutant (PAR1-L38A) was unchanged from that of PAR1-wt (Figure 1B and Table 3). However, the k_{cat} was decreased 7-fold to 47 s^{-1} ($p = 0.013$). The K_m for PAR1-D39A was 10.1 μM ($p = 0.02$), 3-fold lower than that of PAR1-wt (Table 3 and Figure 1C). However, the k_{cat} for PAR1-D39A was also lowered 3-fold ($p = 0.0002$) (Table 3). The mutation of Asp^{39} at P3 influenced both k_{cat} and K_m, yielding

Table 3: Kinetic Constants for α-Thrombin Cleaving Soluble PAR Exodomains[a]

	K_m (μM)[b]	k_{cat} (s^{-1})[b]	k_{cat}/K_m (M^{-1} s^{-1})[c]	k_{cat}/K_m (M^{-1} s^{-1})[d]
PAR1-wt	28 ± 8.8	340 ± 36	1.2×10^7	1.1×10^7
PAR1-L38A	29 ± 11	47 ± 9.2[e]	1.7×10^{6}[e]	3.5×10^{6}[e]
PAR1-D39A	10 ± 3.4[e]	140 ± 20[e]	1.4×10^7	1.3×10^7
PAR1-P40A	23 ± 7.8	230 ± 26[e]	9.9×10^{6}[e]	9.1×10^{6}[e]
PAR4-wt	61 ± 24	17 ± 2.3	2.8×10^5	4.1×10^5
PAR4-L43A	69 ± 28	9.7 ± 1.6[e]	1.4×10^{5}[e]	2.1×10^{5}[e]
PAR4-P44A	56 ± 22	7.7 ± 1.1[e]	1.5×10^{5}[e]	2.5×10^{5}[e]
PAR4-P46A	72 ± 32	0.64 ± 0.08[e]	8.9×10^{3}[e]	7.9×10^{3}[e]

[a] In 10 mM Tris-HCl and 150 mM NaCl at pH 8.0 and 37 °C. [b] With a ±95% confidence interval. [c] Determined by direct measurement of k_{cat} and K_m. [d] Determined by the equation $[S] = [S]_0 \exp(-se_T t)$ at $[S] \ll K_m$. [e] $p < 0.05$ compared to that of the wild-type sequence.

a catalytic efficency similar to that of PAR1-wt, which indicates nonproductive binding of this mutant to the active site of thrombin (*28*). Alternatively, the K_m for the P2 mutant (PAR1-P40A) was unchanged from that of PAR1-wt, but the k_{cat} was lowered to 230 s^{-1} ($p = 0.03$) (Figure 1D and Table 3). Overall, the lack of an effect on the K_m of most of the PAR1 mutants was likely due to the hirudin-like sequence of the PAR1 exodomain ($K^{51}YEPF^{55}$) which bound to thrombin's exosite I in a manner independent of the active site. As a result, thrombin's dependence on the amino acids at the cleavage site was weakened for its binding to PAR1. The k_{cat}/K_m for each of the PAR1 mutants was confirmed at low substrate concentrations where eq 2 is valid as described above for PAR1-wt. In each case, the k_{cat}/K_m agreed with the data presented in Table 3.

Additional investigations identified the amino acids that were important for the α-thrombin−PAR4 interaction at the α-thrombin cleavage site as described above for PAR1. Initial studies characterized the interaction of α-thrombin with the PAR4-wt exodomain. The k_{cat} and K_m for PAR4-wt were 61 μM and 17 s^{-1}, respectively (Table 3 and Figure 2A). The mutations introduced at positions P5 (PAR4-L43A), P4 (PAR4-P44A), and P2 (PAR4-P46A) had no effect on the K_m compared to that of PAR4-wt [69 ($p = 0.65$), 56 ($p = 0.57$), and 72 μM ($p = 0.56$), respectively] (Figure 2A−D and Table 3). However, the k_{cat} was reduced (9.7 s^{-1}, $p = 0.001$) for PAR4-L43A, indicating that Leu^{43} at P5 is important for efficient PAR4 cleavage by thrombin (Figure 2B and Table 3). PAR4-P44A also had a reduced k_{cat} (7.7 s^{-1}, $p = 0.001$), confirming a role for Pro^{44} in the PAR4 cleavage by thrombin (Figure 2C and Table 3). The k_{cat} (0.64 s^{-1}) was also significantly reduced for PAR4-P46A (Figure 2D and Table 3). Twenty-fold more α-thrombin was required for cleavage of PAR4-P46A which was reflected in the 35-fold decrease in the k_{cat} compared to that of PAR4-wt. Further, the k_{cat}/K_m for each PAR4 mutant exodomain was confirmed at low substrate concentrations, where eq 2 is valid as described above for PAR1 (Table 3). Taken together, these data indicated that mutating the P5, P4, or P2 position of PAR4 exodomain did not influence binding of PAR4 to thrombin as assessed by the K_m; however, each of these positions was important for efficient cleavage of PAR4 by α-thrombin as reflected in the decrease in k_{cat}.

The ability of PAR1 or PAR4 exodomains to inhibit thrombin hydrolyzing a chromogenic substrate has been used in previous studies to obtain important information regarding the nature of the thrombin−PAR interactions (*12*, *14*, *29*). A separate set of experiments determined the ability of PAR1-wt, PAR4-wt, or mutant exodomains to inhibit α-thrombin hydrolysis of a small chromogenic substrate (Sar-Pro-Arg-pNA). Previous studies have demonstrated that PAR1-wt was a noncompetitive inhibitor of α-thrombin due to the high-affinity exosite I binding site on the PAR1 exodomain ($K^{51}YEPF^{55}$) (*14*). In our studies, PAR1-wt also was a pure noncompetitive inhibitor of thrombin hydrolyzing Sar-Pro-Arg-pNA with a K_i of 26 μM ($\alpha = 1$) (Figure 3A and Table 4). To confirm the role of the exosite I binding region and verify that PAR1 exodomain was a noncompetitive inhibitor, thrombin was preincubated with 10 μM hirugen. In the presence of hirugen, PAR1-wt was unable to inhibit hydrolysis of Sar-Pro-Arg-pNA at concentrations up to 100 μM (Table 4). The P4 and P3 mutant PAR1 exodomains also were pure noncompetitive inhibitors with a K_i of 38 μM for PAR1-L38A and a K_i of 7.1 μM for PAR1-D39A (Figure 3B,C and Table 4). However, the P2 mutant (PAR1-P40A) exhibited a mixed type of inhibition with a K_i of 26 μM and an α of 3.8 (Figure 3D and Table 4). The change from noncompetitive to a mixed type of inhibition may be due to a change in the orientation of the exodomain at the active site of thrombin. However, the difference between the two models was small for mixed inhibition versus pure noncompetitive inhibition ($R^2 = 0.991$ vs 0.989 and AIC = −213.8 vs −204.7, respectively). Further, within error, α was equal to 1 which would signify pure noncompetitive inhibition (Table 4). Therefore, one cannot rule out the possibility that the PAR1-P40A mutant is also a pure noncompetitive inhibitor ($\alpha = 1$).

Unlike PAR1, PAR4 does not have a hirudin-like sequence. As a result, the primary site of interaction with α-thrombin is at the active site. Therefore, due to the single point of interaction with α-thrombin, one would predict that the PAR4 exodomain would be a competitive inhibitor of α-thrombin. PAR4-wt was a competitive inhibitor of thrombin hydrolyzing Sar-Pro-Arg-pNA with a K_i of 64 μM (Figure 4A and Table 4). PAR4-L43A, PAR4-P44A, and PAR4-P46A were also competitive inhibitors, and like the K_m, the K_i was unchanged from those of PAR4-wt [56 ($p = 0.62$), 41 ($p = 0.24$), and 68 μM ($p = 0.95$), respectively] (Figure 4B−D and Table 4). In the presence of hirugen, the PAR4-wt exodomain remained a competitive inhibitor; however, the K_i shifted from 64 to 110 μM ($p = 0.035$) (Table 4).

DISCUSSION

Using a library of PAR1 and PAR4 exodomains with single-amino acid alanine substitutions, we have examined their interaction with α-thrombin. These studies determined that P4 (Leu^{38}) and P2 (Pro^{40}) are important for the efficient cleavage of PAR1 by thrombin as mutations at these positions decrease the k_{cat}. For PAR4, mutations at P5, P4, or P2 also did not influence the K_m of cleavage of PAR4 exodomain by α-thrombin. However, alanine substitutions at P5 (Leu^{43}) and P4 (Pro^{44}) reduced the k_{cat} of this reaction to the same extent. The alanine substitution of Pro^{46} at P2 results in a more dramatic decrease in k_{cat}. Cleary et al. demonstrated that Leu^{43} at P5 interacts with Pro^{44} or Pro^{46} to stabilize the secondary structure of a PAR4 peptide (*21*). Ayala et al. (*13*) have suggested that Leu^{43} also interacts

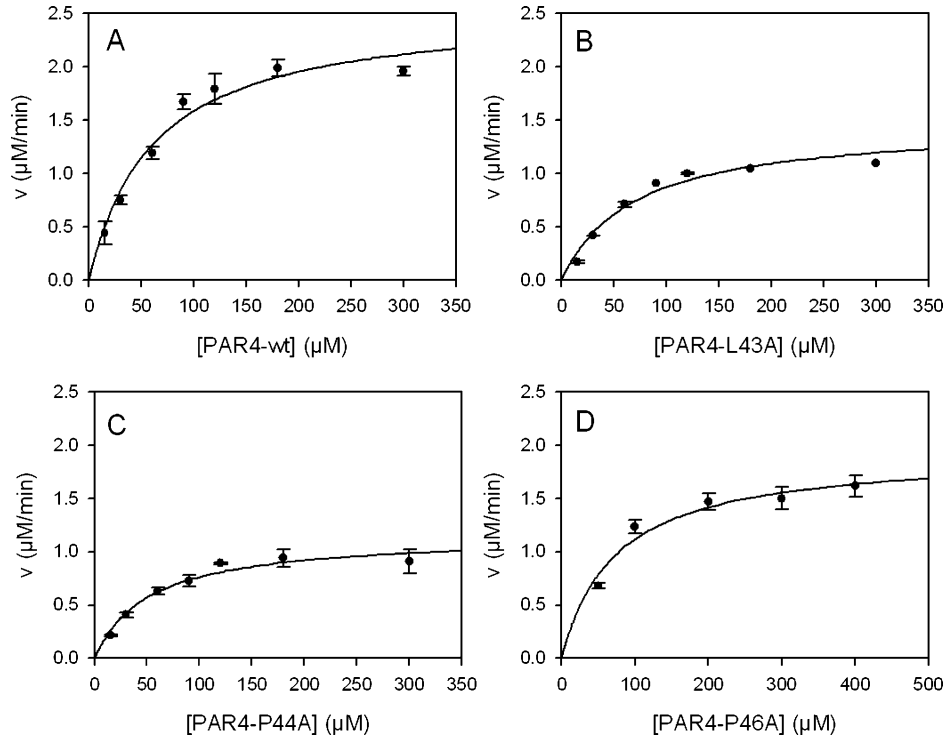

FIGURE 2: Cleavage of PAR4-wt or mutant exodomains with α-thrombin. Reactions with purified exodomains (20−400 μM) in reaction buffer [10 mM Tris-HCl and 150 mM NaCl (pH 8.0)] were initiated by the addition of α-thrombin (5.0 nM for panels A−C or 50 nM for panel D). Acid-quenched reactions were resolved via HPLC, and initial rates were determined by calculating the peak areas at early time points where the progress curves were linear and compared to a standard curve generated by a completely cleaved exodomain. Curve A is α-thrombin cleavage of PAR4-wt exodomain, curve B that of PAR4-L43A, curve C that of PAR4-P44A, and curve D that of PAR4-P46A. The data were fit to the Henri−Michaelis−Menten equation with nonlinear least-squares analysis to determine the kinetic constants. Error bars represent the standard deviation, and the apparent absence of error bars indicates a small standard deviation.

with thrombin on the basis of molecular modeling data. These investigations show for the first time with full exodomains that Leu at P4 (PAR1) or P5 (PAR4) critically influences the kinetics of α-thrombin cleavage of PAR1 and PAR4 exodomains.

Alanine substitution at P4 and P2 of PAR1 exodomain had no effect on the K_m of α-thrombin cleavage, but the k_{cat} was reduced in both cases (Table 3). Each of these PAR1 mutants has an intact hirudin-like sequence ($K^{51}YEPF^{55}$) that binds α-thrombin's exosite I. This high-affinity interaction allows each of the mutants to bind to α-thrombin with the same affinity. In contrast, the mutations at P4 or P2 affect how the cleavage site fits into the active site of α-thrombin, adversely affecting the rate of the reaction. Interestingly, the Leu at P4 had a more dramatic effect than the Pro at P2. Structural data indicate that the P4 position of L-amino acid polypeptide substrates would point to the hydrophobic binding site adjacent to the S1 pocket termed the aryl binding site (*30*). Our data indicate that this interaction is critical for the proper orientation of the PAR1 cleavage site into the active site of thrombin for efficient cleavage. In contrast, mutating Asp^{39} at P3 lowered the K_m 3-fold versus that of PAR1-wt exodomain (Table 3). However, the catalytic efficiency was the same as that of PAR1-wt due to a concomitant decrease in k_{cat}. The simultaneous decrease in K_m and k_{cat} indicates nonproductive binding (*28*). These data argue that Asp at P3 is important for orienting the PAR1 exodomain in the active site of thrombin. The increased binding efficiency of this mutant may interfere with turnover of this reaction. When PAR1 peptides were cocrystallized with thrombin, structures in which the active site of thrombin interacted with $L^{38}DPR^{41}$ or the exosite I region interacted with $K^{51}YEPF^{55}$ were determined; however, none of these structures had exosite I and the active site simultaneously filled (*22*). The hirudin-like sequence ($K^{51}YEPF^{55}$) induces a change in conformation when the Ala^{190}−Gly^{197} region of thrombin with the Glu^{192} side chain becomes disordered, helping to accommodate the negatively charged Asp at P3 for PAR1. The crystals in which the active site is filled by the $L^{38}DPR^{41}$ sequence show that Leu^{38} occupies the aryl binding pocket formed by Ile^{174} and Trp^{215} as predicted by Bode et al. (*30*). The intervening sequence between $L^{38}DPR^{41}$ and $K^{51}YEPF^{55}$ of PAR1 ($F^{43}LLRNP^{48}$) is disordered with no electron density (*22*). Using the data from the two sets of crystals in Mathews et al. (PDB entries 1NRS and 1NRN), Huntington has proposed models for a single PAR1 peptide interacting with thrombin's active site and exosite I simultaneously (*31*).

In the cleavage experiments, a single α-thrombin binds to a single PAR1 exodomain. In contrast, for the K_i experiments, α-thrombin binds to a PAR1 exodomain (at exosite I) and Sar-Pro-Arg-pNA (at the active site) simultaneously. As a result, the PAR1-wt, PAR1-L38A, and PAR1-D39A exodomains are noncompetitive inhibitors of α-thrombin cleaving Sar-Pro-Arg-pNA. As in the cleavage experiments, the P3 mutant (PAR1-D39A) also binds α-thrombin more tightly in this assay. In contrast, the PAR1-P40A exodomain exhibited a mixed type of inhibition. These data may indicate that mutating the P2 position alters the fit of the exodomain in the active site of thrombin. However, as indicated in the Results, the difference between the two models was small for mixed inhibition versus pure noncom-

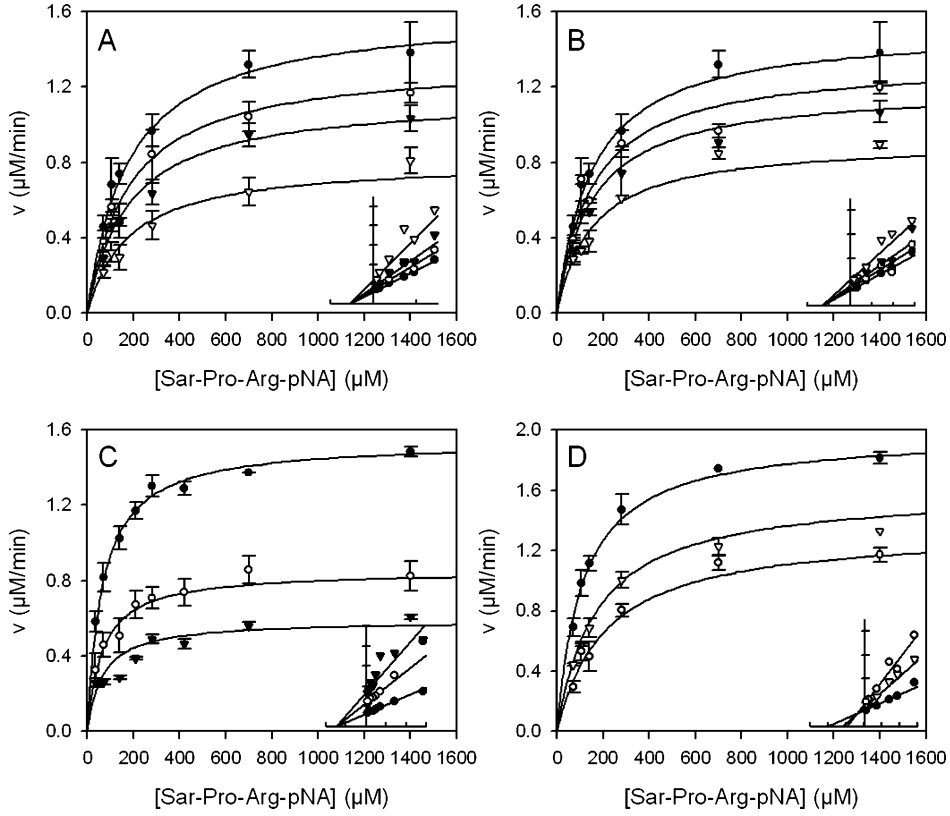

FIGURE 3: α-Thrombin hydrolysis of Sar-Pro-Arg-pNA in the absence or presence of PAR1-wt or mutant exodomain. α-Thrombin (0.5 nM) was added to Sar-Pro-Arg-pNA (70−1400 μM) in the absence (●) or presence of 5 (○), 10 (▼), 25 (▽), or 50 μM (gray circles) PAR1-wt exodomain (A), PAR1-L38A (B), PAR1-D39A (C), or PAR1-P40A (D). Initial velocity data were fit to eight models of enzyme inhibition using a global analysis nonlinear least-squares regression analysis to determine the K_i and the type of inhibition (see Materials and Methods). Double-reciprocal plots (insets) are shown only for graphical representation of the model and were not used to determine the K_i or type of inhibition.

Table 4: Inhibition of α-Thrombin Hydrolysis of Sar-Pro-Arg-pNA by PAR Exodomain[a]

	K_i (μM)[b]	inhibition type[c]	R^2 [d]
PAR1-wt	26 ± 7.7	NC	0.97
PAR1-L38A	38 ± 9.9	NC	0.98
PAR1-D39A	7.1 ± 1.0[e]	NC	0.99
PAR1-P40A	26 ± 12	M	0.99
	α = 3.8 ± 2.8		
PAR4-wt	64 ± 23	C	0.99
PAR4-L43A	56 ± 16	C	0.98
PAR4-P44A	41 ± 15	C	0.98
PAR4-P46A	68 ± 25	C	0.99
PAR1-wt[f]	(100)[g]	NI	–
PAR4-wt[f]	110 ± 15[e]	C	0.98

[a] In 10 mM Tris-HCl and 150 mM NaCl at pH 8.0 and 25 °C. [b] With a ±95% confidence interval. [c] NC, noncompetitive; C, competitive; M, mixed; NI, no inhibition. [d] Goodness of fit to inhibition model. [e] $p < 0.05$ compared to the wild-type sequence. [f] In the presence of 10 μM hirugen. [g] No inhibition at the concentration in parentheses.

petitive inhibition. Therefore, one cannot rule out the possibility that this interaction is also that of a pure noncompetitive inhibitor. The importance of the exosite I binding region on PAR1 for thrombin interaction was further confirmed by blocking this interaction with hirugen. In the presence of hirugen, PAR1-wt exodomain was unable to inhibit hydrolysis of the chromogenic substrate. Jacques et al. demonstrated that PAR1 exodomain mutants that have the hirudin-like sequence ($K^{51}EYPF^{55}$) deleted or mutated to alanine were also unable to inhibit thrombin hydrolyzing a chromogenic substrate, further emphasizing the role of this region (*29*). In sum, these combined data indicate that the exosite I interactions are the primary determinants of PAR1 binding and explain why mutations at the cleavage site do not affect binding but have an influence on the k_{cat}. The influence of exosite interactions has also been described for prothrombin activation by prothrombinase in which point mutations at cleavage sites do not influence overall binding due to exosite interactions but do influence the rate of cleavage (*32*).

Jacques and Kuliopulos prepared chromogenic substrates based on the PAR1, PAR3, and PAR4 cleavage sites from mouse and human sequences of these receptors and after cleavage studies concluded that α-thrombin interacts with PAR4 through the prolines at P4 and P2 (*29*). We directly tested the role of these residues interacting with α-thrombin using full exodomains, and our data support these conclusions. However, our study also demonstrates that Leu^{43} at P5 influences the rate of PAR4 cleavage by thrombin to the same extent as Pro^{44} at P4. NMR studies with the PAR4 peptide $S^{38}TPSILPAPR^{47}$ demonstrate that Leu^{43} interacts with Pro^{44} or Pro^{46} and has a minor role in interacting with thrombin. Our data with entire exodomains suggest that Leu^{43} is as important as Pro^{44} at P4 for efficient cleavage of PAR4 by thrombin. Thus, the hydrophobic properties of Leu^{43} at P5 may be required for the proper three-dimensional arrangement of the amino acids at the α-thrombin cleavage site. Jacques et al. also demonstrated that the addition of an acetyl group to the N-terminus lowered the K_m from 270 to 61 μM and increased the k_{cat} from 49 to 81 s^{-1}, with a net

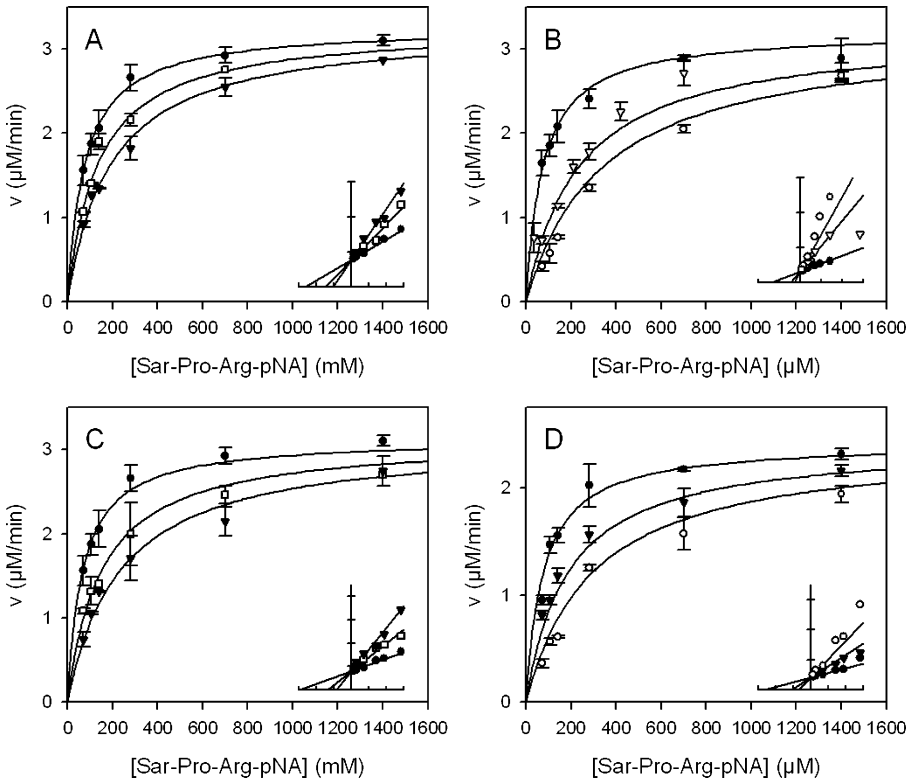

FIGURE 4: α-Thrombin hydrolysis of Sar-Pro-Arg-pNA in the absence or presence of PAR4-wt or mutant exodomain. α-Thrombin (0.5 nM) was added to Sar-Pro-Arg-pNA (70−1400 μM) in the absence (●) or presence of 50 (gray squares), 100 (▼), 120 (▽), or 200 μM (○) PAR4-wt (A), PAR4-L43A (B), PAR4-P44A (C), or PAR4-P46A (D). Initial velocity data were fit to eight models of enzyme inhibition using a global analysis nonlinear least-squares regression analysis to determine the K_i and the type of inhibition (see Materials and Methods). Double-reciprocal plots (insets) are shown only for graphical representation of the model and were not used to determine the K_i or type of inhibition.

effect of increasing the k_{cat}/K_m 7.2-fold (*29*). These authors suggest that the acetyl group serves as a surrogate for the hydrophobic residue at P5, resulting in a lower K_m and a higher k_{cat}. Our data support this hypothesis for P5 increasing the k_{cat}. Like PAR4-wt, each of the PAR4 mutants is a competitive inhibitor of thrombin hydrolyzing Sar-Pro-Arg-pNA, supporting the hypothesis that PAR4 interacts primarily with the active site of thrombin. The point mutations at P5, P4, and P2 do not influence the ability of PAR4 to bind thrombin; however, the amino acids at the cleavage site likely are coordinated for proper insertion into the active site of thrombin for efficient cleavage because these mutations influence the rate of the reaction. The presence of hirugen shifted the K_i of PAR4-wt from 64 to 110 μM (Table 4). These data are in agreement with a published report in which mutating the proposed region for exosite I interaction on PAR4 to alanine shifted the K_i to 113 μM compared to 35 μM for the wild type (*29*). Further, the K_m of thrombin cleavage of these mutants was increased from 56 to 208 μM (*29*). Therefore, PAR4 also has an extended interaction with thrombin. Our data in the current report indicate that PAR4's extended interaction with thrombin minimizes the influence of individual point mutations on overall binding. However, as with PAR1, these residues are important for productive binding in achieving efficient cleavage.

Finally, it is of interest that the presence of the exosite binding region on PAR1 only lowers the K_m of α-thrombin cleavage of PAR1 exodomain 2-fold. In contrast, 10-fold more thrombin is required to activate PAR4 than PAR1 on platelets which express both receptors. However, when PAR4 was expressed in COS-7 cells in the absence of PAR1, the EC_{50} was >400-fold higher than that for PAR1 (*29*). Therefore, there is a dramatic difference in the rate of PAR4 activation depending on the context in which it is expressed. A recent report has reconciled the difference of PAR4 activation on COS-7 cells and platelets by demonstrating that PAR1 is a cofactor for PAR4 activation (*33*). When PAR1 is coexpressed with PAR4, the PAR4 EC_{50} is lowered 10-fold. This cofactor mechanism is similar to PAR4 activation via PAR3 in mice (*34*). Thrombin activates PAR4 while its exosite I is bound to the hirudin-like region of PAR1 or PAR3. However, this mechanism does not completely explain the dramatic difference in PAR1 and PAR4 activation on cells compared to the 2-fold difference in the K_m of purified exodomain. GPIb has been shown to enhance PAR1 activation on platelets by interacting with exosite II of thrombin (*31*, *35*). However, GPIb is specific for platelets and is unlikely expressed on COS7. An alternative possibility is that the PAR4 exodomain is shorter than PAR1 (75 vs 62 amino acids). Further, the cleavage site of PAR4 is 32 amino acids from the transmembrane region compared to 60 amino acids for PAR1. Therefore, a potential explanation for the dramatically slower activation of PAR4 on the cell surface is that the PAR4 cleavage site is less accessible because it is closer to the plasma membrane. Further kinetic studies of the complete receptors on cells are needed to characterize the complete mechanism(s) of PAR activation.

These studies suggest that compounds directed to the thrombin cleavage site on PAR1 and/or PAR4 may function as selective thrombin receptor activation antagonists. The fact that a proline at the P2 or P4 position and a leucine at the P4 or P5 position influence thrombin's interaction with

these exodomains suggests that there may be a common three-dimensional structure that allows for one compound to inhibit thrombin activation of both receptors. Such a finding would explain how peptide RPPGF, the angiotensin-converting enzyme breakdown product of bradykinin, binds and blocks the proteolytic cleavage of PAR1 and -4 exodomains and inhibits thrombin-induced platelet activation of both human and mouse platelets (*23*, *36*). On the basis of the results of this report, Leu^{38} of PAR1 and Leu^{43} are potential targets for the development of thrombin receptor antagonists that act simultaneously on PAR1 and PAR4.

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