RESEARCH COMMUNICATION

A trypsin-like platelet protease propagates protease-activated receptor-1 cleavage and platelet activation

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Protease-activated receptor-1 (PAR-1) is a G-protein-linked receptor on platelets and perivascular cells activated by α -thrombin and the PAR-1-activating peptide, SFLLRN. α -Thrombin activates PAR-1 by cleaving it at R⁴¹–S⁴² to release the 41-residue peptide TR(1–41). Unexpectedly, platelet activation with SFLLRN was also associated with PAR-1 cleavage and the release of TR(1–41). Both PAR-1 cleavage and platelet activation

resulting from SFLLRN addition to platelets were markedly inhibited by the serine protease inhibitor 4,2-(aminoethyl)-benzene sulphonylfluoride·HCl (pefabloc SC) and soybean trypsin inhibitor, but not by inhibitors of calpain, cysteine proteases or metalloproteases. Thus, a trypsin-like platelet protease propagates SFLLRN-dependent PAR-1 cleavage and platelet activation.

INTRODUCTION

Originally called the thrombin receptor, protease-activated receptor 1 (PAR-1) is the prototype of the 7-transmembrane family of cell receptors activated by proteases [1–7]. α -Thrombin activates PAR-1 by cleaving it at R⁴¹–S⁴² to effect the release of a 41-residue peptide [abbreviated to TR(1–41) in this study] from platelets, and production of truncated PAR-1 still anchored to the platelets [1–6,8]. The first 6 (SFLLRN) to 14 (SFLLRNPNDKYEPF) residues of the truncated PAR-1 bind to a region within the second transmembrane domain of PAR-1 to activate platelets and other PAR-1-bearing cells [9–11]. Both α -thrombin and SFLLRN were recently reported to internalize PAR-1 on activated platelets [12], as has been shown for other PAR-1-bearing cells incubated with α -thrombin [13–15]. This study demonstrates that a trypsin-like platelet protease cleaves PAR-1 to optimize propagation of platelet activation initiated with SFLLRN or α -thrombin. Cleavage of PAR-1 by this platelet-derived protease may explain the reported internalization of PAR-1 by platelets activated by α -thrombin and SFLLRN [12].

MATERIALS AND METHODS

Soybean trypsin inhibitor (SBTI), SFLLRN and reagents for the buffers used were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). D-FPRCH₂Cl, GPRP and another SBTI preparation were obtained from Calbiochem (San Diego, CA, U.S.A.). All the other protease inhibitors used were obtained from Boehringer Mannheim (Laval, Canada). Recombinant hirudin and recombinant tissue-factor pathway inhibitor (TFPI) were gifts from Dr. R. B. Wallis (Ciba Geigy, Horsham, U.K.) and Dr. T. C. Wun (Monsanto, St. Louis, MO, U.S.A.) respectively.

Blood was collected from healthy volunteers who had taken no medication for at least 7 days and washed platelet suspensions were isolated and resuspended in modified Tyrode's buffer [16]. Washed platelets (1×10^{12} /l) supplemented with 5 mM GPRP (to prevent aggregation of the platelets by preventing polymerization of fibrin) were incubated with 10 nM α -thrombin (isolated by the procedures of Fenton et al. [17]) or 10 μ M SFLLRN for 10 s at 37 °C, followed immediately by the addition of 1 μ M hirudin. The platelet suspensions then received 4 vols. of 10 g/l paraformaldehyde (for flow cytometry as detailed below) or were centrifuged at 5000 *g* for 5 min and the supernatants recovered to quantify the TR(1–41) released from platelet PAR-1 using the procedures reported by Liu et al. [8]. In other experiments (Table 1), separate platelet suspensions were incubated with each of the protease inhibitors listed (at the concentrations noted) for 15 min at 37 °C before adding 10 nM α -thrombin or 10 μ M SFLLRN for 10 s.

Platelet activation was quantified by flow cytometry as detailed previously [8]. Briefly, the platelets were fixed in 1% paraformaldehyde for 10 min at ~23 °C. The paraformaldehyde was washed away with FACSFlow fluid (Becton Dickinson) by centrifugation at 1175 *g* for 15 min, and the platelets were resuspended in FACSFlow sheath fluid containing 1 g/l of BSA. The platelets were next incubated with phycoerythrin-conjugated monoclonal antibody to CD62 for 30 min at ~23 °C in the dark. After washing and resuspension in the FACSFlow sheath fluid, the percentage of 10000 platelets expressing CD62, and the associated fluorescence intensity, were determined using a FACScan argon ion flow cytometer operating at 488 nm and 15 mW power, and analysed using Lysis II software (Becton Dickinson). The instrument was set up to measure size, granularity and platelet fluorescence. All parameters were collected using a four-decade logarithm amplification.

Abbreviations used: PAR-1, protease-activated receptor 1; SBTI, soybean trypsin inhibitor; TR(1–41), residues 1–41 of PAR-1; pefabloc SC, 4,2-(aminoethyl)benzene sulphonyl fluoride·HCl; TFPI, tissue-factor pathway inhibitor; phosphoramidon, *N*- α -rhamnopyranosyl-oxyhydroxyl phosphoryl-Leu-Leu-Tyr; the one-letter symbols for amino acids are used throughout the paper.

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Table 1 Effects of protease inhibitors on α -thrombin-dependent and SFLLRN-dependent platelet-PAR-1 cleavage and platelet activation

A summary of the protease inhibitors and their respective concentrations incubated for 15 min (at 37 °C) with washed platelets resuspended in a modified Tyrode's buffer containing 1 g/l glucose, 3.5 g/l BSA, 1 mM MgCl₂, 2 mM CaCl₂, 5 mM Hepes and apyrase, adjusted to pH 7.35 and incubated with one of two agonists, namely 10 nM α -thrombin or 10 μ M SFLLRN (without stirring), for 10 s at 37 °C, followed by 1 μ M hirudin. The concentrations of TR(1–41) released and platelet activation achieved were determined by ELISA and flow cytometry respectively. The values reported are the means for six to nine determinations. The S.E.M.s (not shown) varied between 5 and 15% for both PAR-1 cleavage and platelet activation.

Protease inhibitor	Target proteases	Percentage inhibition of PAR-1 cleavage and platelet activation by:			
		α -Thrombin		SFLLRN	
		Cleavage	Activation	Cleavage	Activation
4 mM Pefabloc SC	Serine proteases	89	100	86	100
1 mM SBTI	Trypsin-like proteases	92	94	74	40
1 μ M Hirudin	α -Thrombin	100	100	None	None
1 μ M FPRCH ₂ C1	α -Thrombin	100	100	None	None
10 mM Calpain inhibitor 1*	Calpain	None	None	None	None
1 μ M TFPI	Factor Xa	None	None	None	None
10 μ M Leupeptin	Serine/cysteine proteases	None	None	None	None
0.3 μ M Aprotinin	Plasmin	None	None	None	None
0.6 mM Phosphoramidon	Metalloproteases	None	None	None	None
10 μ M Phenanthroline	Metalloproteases	None	None	None	None
74 μ M Antipain	Cathepsin A and B	None	None	None	None
130 μ M Bestatin	Aminopeptidases	None	None	None	None
100 μ M Chymostatin	Chymotrypsin	None	None	None	None
28 μ M E-64†	Cysteine proteases	None	None	None	None
1 μ M Pepstatin	Aspartyl proteases	None	None	None	None

* Calpain inhibitor 1 is *N*-acetyl-Leu-Leu-Nle.

† E-64 is *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane.

RESULTS AND DISCUSSION

As expected, incubation of washed platelets with 10 μ M SFLLRN for 10 s at 37 °C resulted in $92 \pm 3\%$ platelet activation (Figure 1), measured as platelets expressing CD62 and monitored by flow cytometry. Surprisingly, the supernatants recovered from SFLLRN-activated platelets isolated from 18 healthy subjects also contained material immunoreactive with anti-TR(1–41) Ig and equivalent to 0.89 ± 0.21 nM TR(1–41), as quantified by an ELISA for this activation peptide. Incubation of washed platelets from the same 18 individuals with 10 nM human α -thrombin for 10 s (followed by 1 μ M hirudin to inactivate the thrombin) caused the activation of over 80% of the platelets and the release of 0.94 ± 0.12 nM TR(1–41) into the supernatants (range 0.19–1.3 nM) (Figure 1). Platelets fully activated by incubation with SFLLRN released additional TR(1–41) when they were incubated with 1.0 nM α -thrombin (Figure 1).

Detection of TR(1–41) in the supernatants of SFLLRN-treated platelets suggested that: (i) a platelet-derived protease(s) cleaved PAR-1 during platelet activation; (ii) PAR-1 was extruded from platelets; or (iii) microparticle formation accounted for the TR(1–41) measured in the platelet supernatants. However, less than 5% of the total particles counted were microparticles, as has also been reported by others using similar experimental conditions [12]. To determine whether PAR-1 was extruded from platelets, or whether a platelet-derived protease cleaved PAR-1 resident on SFLLRN-treated platelets to effect TR(1–41) release, the platelet suspensions were also preincubated separately with each of the protease inhibitors listed in Table 1 for 15 min before agonist addition. Only 4,2-(aminoethyl)-benzene sulphonylfluoride (pefabloc SC) and SBTI inhibited TR(1–41) release and platelet activation (Table 1).

Figure 1 Activation of washed platelets (top) and cleavage of platelet PAR-1 (bottom) resulting after 10 nM thrombin or 10 μ M SFLLRN was added to the platelets for 10 s

Washed platelets in modified Tyrode's buffer (column A), or washed platelets in the same buffer treated with 10 nM human α -thrombin for 10 s (column B), 10 μ M SFLLRN for 10 s (column C) or 10 μ M SFLLRN for 10 s followed by 1 nM human α -thrombin for 10 s (column D). After the addition of 1 μ M hirudin, the platelets were processed to determine their activation by flow cytometry and PAR-1 cleavage by ELISA. The results reported are the means $\pm$ S.E.M. for 18 determinations.

Thus, a trypsin-like platelet protease cleaved PAR-1 during platelet activation by SFLLRN. As expected, the two potent α -thrombin inhibitors, hirudin and FPRCH₂Cl, abrogated PAR-1 cleavage and platelet activation by α -thrombin. However, neither hirudin nor FPRCH₂Cl inhibited SFLLRN-dependent PAR-1 cleavage and platelet activation (Table 1). Furthermore, addition of pefabloc SC or SBTI to platelets pre-incubated with SFLLRN or α -thrombin for 10 s did not inhibit PAR-1 cleavage or platelet activation. Thus, PAR-1 cleavage and platelet activation occurred during the first 10 s of incubating the platelets with either agonist.

Pefabloc SC, which also inactivated over 80% of α -thrombin in 10 s, markedly inhibited thrombin-mediated PAR-1 cleavage and abrogated platelet activation. Unexpectedly, however, 1 mM SBTI nearly abrogated both PAR-1 cleavage and platelet activation by α -thrombin. Since less than 10% of the α -thrombin was inactivated by 1 mM SBTI in 60 s, the platelet-derived protease, and not α -thrombin, appears to propagate platelet PAR-1 cleavage. Based on this near abrogation of α -thrombin-mediated PAR-1 cleavage and platelet activation by SBTI, the key roles of α -thrombin in platelet activation may be its occupancy of PAR-1 (at the hirudin-like domain of PAR-1) and its initiation of PAR-1 cleavage. Consistent with the above ideas, inhibition of α -thrombin-dependent PAR-1 cleavage and platelet activation caused by SBTI decreased to less than 50% when the incubation of SBTI-treated platelets with α -thrombin was increased to 60 s from 10 s. This observation suggests that α -thrombin cleaves PAR-1 rather inefficiently. Additionally, ATAP 138, a monoclonal antibody directed against the hirudin-like domain of PAR-1, which inhibits α -thrombin binding to platelets, increases by >10-fold the concentration of α -thrombin required to cleave PAR-1 and to activate platelets [8,18].

The following experiments confirmed that PAR-1 cleavage by a platelet-derived protease, and not PAR-1 extrusion, caused the release from platelets incubated with SFLLRN of product(s) immunoreactive with anti-TR(1-41) IgG. Specifically, the sizes of PAR-1 fragments released during platelet activation with SFLLRN and α -thrombin were compared by gel filtration on a 1 $\times$ 30 cm Sephadex-G-25 column. The eluting buffer consisted of 10g/l fatty acid-free BSA in the modified Tyrode's buffer and 0.5 ml fractions were collected. Fractions containing PAR-1 fragments immunoreactive with anti-TR(1-41) IgG were identified by the ELISA for TR(1-41). Gel filtration of the supernatants obtained after platelets were incubated with α -thrombin yielded a product with a symmetrical elution profile, which was identical with that of authentic TR(1-41), M_r 4400. In addition, a second immunoreactive material constituting less than 10% of anti-TR(1-41) immunoreactive products, and eluting later than TR(1-41), was also detected. The supernatants from platelets incubated with SFLLRN yielded a product, the elution profile of which was superimposable on that of authentic TR(1-41). Therefore, PAR-1 cleavage by a platelet protease, and not its extrusion, accounts for the release of TR(1-41)-related material into supernatants of platelets incubated with SFLLRN.

Inhibition of SFLLRN-dependent PAR-1 cleavage by SBTI suggests that a trypsin-like platelet protease aids the propagation of PAR-1 cleavage and platelet activation initiated with this agonist. This platelet protease also helps to propagate PAR-1 cleavage and platelet activation initiated with α -thrombin (Table 1). Inactivation of this platelet-derived protease (achieved by incubating the platelets with pefabloc SC for 15 min before agonist addition) yields platelets that have become unresponsive

to SFLLRN. Inactivation of the platelet-derived protease by SBTI also renders platelets less responsive to α -thrombin and SFLLRN. This protease is probably not platelet gelatinase A that can aggregate platelets [19], since neither 10 μ M phenanthroline nor 0.6 mM phosphoramidon (*N*- α -rhamnopyranosyloxyhydroxyl phosphoryl-Leu-Leu-Tyr) (both metalloprotease inhibitors) altered platelet PAR-1 cleavage or platelet activation by α -thrombin or SFLLRN (Table 1).

In preliminary experiments, incubation of washed platelets with 10 mg/l collagen for 60 s resulted in 0.21 ± 0.06 nM TR(1-41) being released, and this occurred prior to the collagen-treated platelets becoming significantly activated. We conclude, therefore, that cleavage of platelet PAR-1 by a platelet-derived protease with trypsin-like properties is an important, but previously unreported, mechanism for propagating platelet activation by α -thrombin and, to a lesser extent, by SFLLRN. Cleavage of PAR-1 by this platelet protease probably accounts for the reported internalization of PAR-1 by SFLLRN and α -thrombin [12].

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