Cathepsin G Activates Protease-activated Receptor-4 in Human Platelets*

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Of the four known protease-activated receptors (PARs), PAR1 and PAR4 are expressed by human platelets and mediate thrombin signaling. Whether these receptors are redundant, interact, or play at least partially distinct roles is unknown. It is possible that PAR1 and/or PAR4 might confer responsiveness to proteases other than thrombin. The neutrophil granule protease, cathepsin G, is known to cause platelet secretion and aggregation. We now report that this action of cathepsin G is mediated by PAR4. Cathepsin G triggered calcium mobilization in PAR4-transfected fibroblasts, PAR4-expressing Xenopus oocytes, and washed human platelets. An antibody raised against the PAR4 thrombin cleavage site blocked platelet activation by cathepsin G but not other agonists. Desensitization with a PAR4 activating peptide had a similar effect. By contrast, inhibition of PAR1 function had no effect on platelet responses to cathepsin G. When neutrophils were present, the neutrophil agonist fMet-Leu-Phe triggered calcium signaling in Fura-2-loaded platelets. Strikingly, this neutrophil-dependent platelet activation was blocked by the PAR4 antibody. These data show that PAR4 mediates platelet responses to cathepsin G and support the hypothesis that cathepsin G might mediate neutrophil-platelet interactions at sites of vascular injury or inflammation.

Proteases regulate cellular behaviors in part via a family of G protein-coupled protease-activated receptors (PARs) for which the thrombin receptor, PAR1, is the prototype. PAR1 is activated by an unusual mechanism in which thrombin cleaves the receptor amino-terminal exodomain to expose a new amino terminus that then serves as a tethered ligand (1, 2). The synthetic peptide SFFLRN, which mimics the first six amino acids of this newly unmasked amino terminus, can activate PAR1 independent of receptor cleavage and thrombin. The cognate P1–P6 peptides of other PARs have been useful as agonists for probing the role of these receptors in various cellular responses (3–5).

Four distinct PARs are now known. PAR1, PAR3, and the recently characterized PAR4 can be activated by thrombin (1, 5–7). PAR2 is activated by trypsin and trypsin-like enzymes (4). The importance of platelet activation in myocardial infarction and other thrombotic diseases has prompted considerable focus on identifying the PARs that mediate platelet activation by thrombin. Available data suggest that thrombin signaling in human platelets is mediated largely by hPAR1 and hPAR4 (8). PAR1 is necessary for platelet activation at low concentrations of thrombin, and PAR4 can mediate signaling at higher concentrations. The presence of two receptors raises the question of whether PAR1 and PAR4 are redundant, interact, or serve at least partially distinct functions in human platelets. In particular, it is possible that PAR4 might confer responsiveness to proteases other than thrombin.

Platelet PARs may be exposed to a variety of proteases at sites of inflammation or coagulation, and leukocytes are one potential source. For example, neutrophils and platelets are both concentrated at sites of inflammation and thrombus formation. Moreover, aggregates of cells containing both neutrophils and platelets have been found in experimental models of inflammation or thrombosis, suggesting that interactions between these cell types may be physiologically relevant (9). Secreted platelet products act on neutrophils to stimulate adhesion, migration, and/or degranulation (10). A role for neutrophil-derived factors acting on platelets is less well established, but interestingly, the neutrophil protease cathepsin G and elastase have been shown to act on platelets (9, 11, 12). In particular, cathepsin G has been found to cause platelet secretion and aggregation ex vivo (11, 13, 14). This activity was shown to be dependent upon the active site of cathepsin G, giving rise to the hypothesis that cathepsin G signaling was mediated by a PAR. Studies of PAR1 as a candidate cathepsin G receptor suggested that this receptor did not account for cathepsin G signaling in platelets (15, 16).

In the present study we demonstrate that cathepsin G activates PAR4 and that activation of human platelets by cathepsin G is mediated by this receptor. Additionally, we provide evidence that cathepsin G and PAR4 can mediate neutrophil-dependent platelet activation ex vivo.

EXPERIMENTAL PROCEDURES

Materials—Cathepsin G was obtained from Athens Research & Technology (Athens, GA). Thrombin was obtained from Enzyme Research Laboratories (South Bend, IN). Hirudin was purchased from Sigma, and PPACK was from Calbiochem (San Diego, CA).

Blood Cells—Human platelets were isolated from donor blood collected in 0.2 volume of citrate buffer. The first 1 ml drawn was discarded. Blood was centrifuged at 200 × g for 15 min to separate platelet-rich plasma, buffy coat, and erythrocytes. Platelets were then washed in buffer (134 mM NaCl, 12 mM NaHCO3, 2.9 mM KCl, 0.34 mM Na2HPO4, 1 mM MgCl2, 10 mM Hepes, 5 mM glucose, 0.3% bovine serum albumin) containing prostaglandin E1 (1 μM) and EDTA (10 μM) and then resuspended in buffer alone prior to use. Neutrophils were isolated from buffy coat of the same donor blood as platelets. The buffy coat was layered over 63 and 72% Percoll solutions and centrifuged for 30 min at 1,500 × g for 45 min to separate neutrophils and platelets. These products were collected and washed three times in 1 × HEPES buffer (20 mM HEPES, 150 mM NaCl, 10 mM CaCl2, pH 7.4).

This paper is available on line at http://www.jbc.org

* This work was supported by National Institutes of Health Grant HL44907 and the Dizzi Research Center at UCSF. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PAR(s), protease-activated receptor(s); hPAR, human PAR; fMLP, N-formylmethionyl-leucyl-phenylalanine.
substrate Spectrozyme-TH (American Diagnostica). The apparent phenylalanyl-thiobenzyl ester (SynPep) and the chromogenic thrombin chromogenic cathepsin G substrate concentrations (18). Enzyme activity was monitored by cleavage of the injected into cRNAs encoding human PAR1, PAR3, and PAR4 and mouse PAR2 were radiolabeled with 45Ca, and calcium mobilization in response to 10 nM thrombin or 100 nM cathepsin G was assayed as described (1). Data are expressed as the mean ± S.E. of duplicate determinations. This experiment was replicated twice.

Calcium Measurement—Agonist-dependent calcium mobilization in platelets and cell lines was measured after loading with 4 μg/ml Fura-2/AM (Molecular Probes) for 30 min at 37 °C using a Hitachi F2000 fluorometer. For mixed cell experiments neutrophils (4 × 10^6/ml) were suspended together in a cuvette in the presence of 0.1 mM EGTA and 2.5 μg/ml cytochalasin B just prior to measurement.

Aggregation—Agonist-induced platelet aggregation was measured using a Chrono-Log lumiaggregometer. Platelets suspended in the buffer described above under stirring conditions in the presence of 1 mM CaCl_2 were introduced to agonist, and change in light transmission was measured.

Oocyte Experiments—FLAG epitope-tagged wild type and mutant PAR receptors were expressed in Xenopus oocytes following microinjection of appropriate cRNA. Responses to agonist stimulation were assessed as 45Ca release from radiolabeled oocytes as described previously (1). Relative expression levels of receptor expressed on the oocyte surface were determined using binding of M1 monoclonal antibodies.

Cell Culture—Lung fibroblasts derived from PAR1 null mice (17) and Rat1 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% bovine calf serum at 37 °C in a humidified CO_2 incubator.

Determination of Ecotin K_I Values—K_I values were determined by titration of cathepsin G (6 nM) or thrombin (8.5 nM) with various ecotin concentrations (18). Enzyme activity was monitored by cleavage of the chromogenic cathepsin G substrate N-succinyl-alanyl-alanyl-prolyl-phenylalanin-thiobenzyl ester (SypEep) and the chromogenic thrombin substrate Spectrozyme-TH (American Diagnostics). The apparent K_I for cathepsin G was determined by non-linear regression analysis of the data fit to an equation derived for kinetics of reversible tight binding inhibitors (19, 20). The apparent K_I for thrombin was determined by linear regression analysis of the data fit to an equation derived for Michaelis-Menten kinetics of competitive inhibitors.

RESULTS AND DISCUSSION

Cathepsin G is a neutral serine protease found in neutrophil azurophilic granules and is released upon stimulation of cells by chemotactic agents such as N-formylmethionyl-leucyl-phenylalanine (fMLP) or platelet-activating factor (21). The protease is known to hydrolyze matrix components such as laminin and fibronectin after methionine, leucine, phenylalanine, lysine, or arginine residues. The ability of this protease to activate platelets in a manner dependent upon protease activity prompted us to explore the possibility that cathepsin G might activate one or more of the known PARs. For sensitivity, we first sought responses in Xenopus oocytes overexpressing the various PARs. Cathepsin G purified from human neutrophils triggered consistent calcium signaling in Xenopus oocytes expressing human PAR1 or PAR4 (Fig. 1). This response was not the result of thrombin contamination in the cathepsin G preparation; thrombin inhibitors such as hirudin or PPACK did not prevent receptor activation by cathepsin G but did block activation by thrombin (Fig. 2). By contrast, the macromolecular serine protease inhibitor, ecotin (18), blocked cathepsin G-induced calcium responses but had no effect on thrombin. The apparent K_I for inhibition of cathepsin G by wild type ecotin was determined to be 15 ± 6 μM, whereas the K_I for the inhibition of thrombin was 1.1 ± 0.2 μM. This gives ecotin a specificity index of 73,000 in favor of blocking cathepsin G and not thrombin.

Cells expressing a mutant PAR1 in which alanine was substituted for phenylalanine at position 2 of the agonist peptide failed to respond to either thrombin or cathepsin G but did respond to SFLLRN (Fig. 3). Similarly, a PAR4 mutant with a glycine to proline mutation at position 1 of the agonist peptide rendered the receptor uncleavable by thrombin or cathepsin G but still responsive to the PAR4-activating peptide AYPGKF. Thus activation of PAR1 and PAR4 by cathepsin G and thrombin almost certainly occurs by the same mechanism, unmasking of the respective tethered ligands by cleavage at the Arg^{41}-Ser^{42} peptide bond in PAR1 and the Arg^{77}-Gly^{78} peptide bond in PAR4.

Cathepsin G also activated PAR1 and PAR4 stably expressed in mammalian cells. A previously described fibroblast cell line derived from PAR1 knockout mice was transfected to stably express FLAG epitope-tagged hPAR1 or hPAR4 (17). Cathepsin G (100 nM) triggered increases in cytoplasmic calcium in PAR1-transfected cells (Fig. 4). Cells transfected with PAR4 required 500 nM cathepsin G to produce a similar response. No response was detected in untransfected cells. Similar results were observed in transfected Rat1 fibroblasts expressing PAR1 and PAR4 (data not shown). Thus, cathepsin G can activate PAR1 and PAR4 in mammalian cell expression systems. These re-
results raised the possibility that PAR1 and/or PAR4 might mediate cathepsin G activation of human platelets.

Cathepsin G (500 nM) triggered both aggregation and robust increases in cytoplasmic calcium in washed human platelets (Figs. 5 and 6). The roles of PAR1 and PAR4 in this response were first examined by desensitization studies. Platelets preincubated with the PAR1-activating peptide SFLLRN (100 μM) for 30 min failed to respond to subsequent challenge with SFLLRN but did respond to cathepsin G (Fig. 5E). By contrast, platelets preincubated with the PAR4-activating peptide AYPGKF (100 μM) were refractory to stimulation with cathepsin G but did respond robustly to SFLLRN (Fig. 5F). These desensitization studies suggest that signaling via PAR4 but not PAR1 is required for cathepsin G responses in human platelets.

A PAR4-blocking antibody and a PAR1-specific antagonist were also used to probe the roles of PAR1 and PAR4 in platelet responses to cathepsin G. Activation of PAR1 by thrombin or SFLLRN can be blocked with BMS200261 (22), a peptide-based antagonist that does not block PAR4 activation (8). Similarly, a polyclonal antibody raised to a receptor peptide that spans the thrombin cleavage site of PAR4 blocks cleavage and activation of the receptor by thrombin. Because the antiserum works by blocking cleavage, it does not block PAR4 activation by AYPGKF. Treatment of platelets with BMS200261 blocked calcium mobilization and aggregation in response to SFLLRN but not cathepsin G (Figs. 5B and 6). By contrast, presence of the antibody to PAR4 effectively blocked the cathepsin G-triggered platelet responses but not responses to AYPGKF or SFLLRN (Figs. 5C, D, and data not shown). Pre-immune rabbit IgG was without effect (Fig. 5D), and the specificity of the PAR4 antibody for blockade of PAR4 but not PAR1 was reported previously (8). These data strongly suggest that activation of PAR4 but not PAR1 is necessary for activation of platelets by cathepsin G.

These data are concordant with previous studies concluding...
that cathepsin G does not activate platelets via PAR1 (15, 16). Molino et al. (16) showed that cathepsin G could cleave PAR1 either productively at the Arg41-Ser42 peptide bond or non-
productively at the Phe55-Trp56 peptide bond in the PAR1 amino-terminal exodomain. The latter cleavage served to re-
move the PAR1 tethered ligand rendering it unresponsive to thrombin although still responsive to SFLLRN. In the context of the model that PAR1 and PAR4 mediate platelet activation by thrombin (8), this observation predicts that if PAR4 function were inhibited, cathepsin G might actually block platelet activation by thrombin. Accordingly, platelets were first desensitized with the PAR4 agonist AYPGKF and then challenged with thrombin or cathepsin G. As expected, thrombin induced a response, presumably via PAR1, whereas cathepsin G elicited no response (Fig. 5, G and H). After both desensitization with AYPGKF and exposure to cathepsin G, platelets became refrac-
tory to thrombin but still responded to SFLLRN (Fig. 5H). Taken together, these data are consistent with the model that cathepsin G cleaves PAR1 at two (or more) sites (16), with a small fraction of cleavage events occurring at the activating site but the majority occurring at the inhibitory site(s). Signal-
ings responses to cathepsin G in cultured cells that express high levels of PAR1 (Fig. 4) are perhaps due to cleavage of a small fraction, but still adequate absolute number, of receptors at the activating site. The absent PAR1-mediated thrombin signaling but retained responsiveness to SFLLRN in cathepsin-treated platelets is likely due to cleavage of the majority of receptors at the inhibitory site.

In cell suspensions containing both neutrophils and plate-
lets, the neutrophil agonist fMLP induces activation of plate-
lets, presumably by release of cathepsin G from neutrophil
azurophilic granules (23). This model predicts that, in such a mixed cell system, fMLP-triggered platelet activation should be PAR4-dependent. To test this prediction, washed human plate-
lets were loaded with Fura-2 and mixed with neutrophils iso-
lated from the same donor prior to measurement of calcium mobilization (Fig. 7). Stimulation with fMLP (100 nM) resulted in reproducible increases in cytoplasmic calcium in Fura-2-
loaded platelets. Addition of fMLP to platelets alone did not induce an observable signal (data not shown). Pretreatment of platelets with the PAR4-blocking antibody but not pre-immune IgG prevented platelet activation in the mixed cell experiment. These data strongly suggest that, in this system, neutrophil-
dependent platelet activation is mediated by the action of cathepsin G on platelet PAR4.

In summary, this study strongly suggests that PAR4 is the “cathepsin G receptor” on human platelets and that activation of PAR4 by cathepsin G is necessary for neutrophil-dependent platelet activation in the above studies. The importance of cathepsin G-induced platelet activation in vivo is unknown. The ratio of neutrophils to platelets required to achieve robust neutrophil-dependent platelet calcium mobilization was ap-
approximately 1/12, one-half to one log higher than that likely to be achieved in whole blood. However, endothelial cells that have been activated by thrombin or cytokines bind neutrophils and platelets, thereby concentrating both at sites of inflamma-
tion and thrombosis. In addition, shear stress alone can induce neutrophil-platelet aggregates. Such mechanisms presumably create microenvironments that favor neutrophil-platelet inter-
actions (see below). In this regard, minimal-free cathepsin G is detectable in solution after neutrophil stimulation, and like many other proteases including thrombin, cathepsin G is in-
hibited by circulating protease inhibitors (11, 24). However, a significant proportion of cathepsin G released by neutrophils remains bound to the extracellular membrane where it is cat-
alytically active and relatively resistant to inactivation by pro-
tease inhibitors (24–26). Thus platelet activation might be induced by neutrophil-bound, rather than fluid-phase, cathep-
sin G via direct cell-cell contact (27). Indeed, when P-selectin antibodies were used to block the formation of neutrophil-
platelet aggregates, neutrophil-dependent platelet activation became more sensitive to inhibition by protease inhibitors (27).

Thus microenvironments created at sites of thrombosis or in-
flammation may provide a setting where close proximity or direct contact of activated neutrophils and platelets allows cathepsin G-mediated activation of platelet PAR4 to occur.

Given that activation of platelet PAR4 by neutrophil cathepsin G in vivo is plausible, it is worth speculating on what roles this phenomenon might play. A link between inflammation and coagulation is well established and occurs at multiple levels. Pertinent to the present study, endothelial activation promotes both neutrophil and platelet adhesion and rolling via stimula-
tion of von Willebrand factor release and P-selectin expression on the endothelial surface (28–31). Thus endothelial activation provides a mechanism to concentrate platelets and neutrophils at the same site, perhaps promoting neutrophil-platelet inter-
action in the setting of graded local inflammatory responses. Interactions between marginated neutrophils and rolling plate-
lets might become especially prominent in the setting of pro-
found endothelial activation as occurs in the Shwartzman re-
action (32) and related systemic phenomena associated with sepsis and disseminated intravascular coagulation. In the local Shwartzman reaction, for example, a cytokine stimulus at an endotoxin-primed skin site triggers robust margination of neut-
rophils and microvascular thrombosis within 15 min. Whether interrupting platelet-neutrophil interactions would be benefi-
cial in such settings is unknown.

The foregoing discussion casts neutrophil-platelet interac-
tions as proinflammatory and/or prothrombotic. That is, acti-
vated neutrophils might induce platelet activation as a means of further promoting inflammatory cell recruitment by causing release of platelet cytokines (10), stimulating thrombin gener-
ation via platelet procoagulant activity (thereby further acti-
vating the endothelium locally), or perhaps inciting local thrombosis to isolate a site of infection. Similarly, activated neutrophils might bind and activate platelets as a means of promoting their own incorporation into nascent thrombi at sites of injury. Conversely, it is conceivable that activated neutrophils might bind and activate platelets as part of a shutoff mechanism that limits the systemic effects of local inflammation by preventing activated neutrophils from circulating. Experimental models of inflammation in mice may provide a means of testing these hypotheses.

Cathepsin G-deficient mice showed only a mildly augmented inflammatory response during wound healing but were otherwise without apparent phenotype, suggesting that cathepsin G activation of platelet PAR4 is not necessary for survival in an unstressed setting (33). Clearly, multiple ligand-receptor systems regulate inflammatory and thrombotic responses, and whether deficiencies in other pathways that serve redundant functions might unmask an important role for neutrophil cathepsin G is unknown. Moreover, as noted above, activation of platelets by neutrophil cathepsin G may become important only at sites where activated neutrophils and platelets are substantially concentrated, and the effect of cathepsin G deficiency in models that might engender interactions, such as the Shwartzman reaction, between activated neutrophils and platelets has not been reported. Further study of relevant models of inflammation or thrombosis in cathepsin G-deficient mice, PAR4-deficient mice, and perhaps mice in which these deficiencies are combined with others, will be useful for probing the significance of this interaction between neutrophils and platelets.

In addition to describing cathepsin G activation of PAR4 as a possible pathway for neutrophil-dependent platelet activation, this study makes the general point that PARs that were originally described as thrombin receptors may also confer responsiveness to proteases other than thrombin (1, 4–6). The differential importance of PAR1 versus PAR4 for thrombin versus cathepsin G signaling in human platelets supports the notion that these receptors may serve, at least in part, distinct roles. Thus PAR4 might serve not only to mediate thrombin responsiveness; it might expand the platelet’s repertoire by mediating responses to other proteases.

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