Activation of Prothrombin by a Novel Membrane-associated Protease

AN ALTERNATIVE PATHWAY FOR THROMBIN GENERATION INDEPENDENT OF THE COAGULATION CASCADE*

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We herein report that a novel membrane-associated protease capable of activating prothrombin is present in several mammalian cells. This protease can directly convert prothrombin to active thrombin and induces blood clotting both in vivo and in vitro but is apparently different from coagulation factor Xa, which has been thought to be the only physiological activator of prothrombin. This protease activity was initially found and was very high in 8C feline kidney fibroblast cells, and we characterized its enzymological features using this cell line. Activity was detected in neither the cytosolic fraction nor the culture medium but found in the membranes and identified on the surface of intact cells. The activation of prothrombin required Ca²⁺ ions, and the apparent Kₐ value for prothrombin was 0.2 μM. The activity was irreversibly inhibited by exposure to EDTA, but various inhibitors for serine proteases including antithrombin III were without effect. Based on these results, we propose that this novel enzyme, membrane-associated prothrombin activator, catalyzes an alternative pathway for generation of thrombin, which is independent of the blood coagulation cascade, and that the thrombin generated is involved in certain pathological states and/or in activation of cells that are spatially separated from the bloodstream.

The serine protease thrombin is a multifunctional enzyme that participates in diverse biological processes. It is essential in hemostasis, for both blood coagulation and platelet aggregation. It also regulates vascular tone, wound healing, and inflammatory reactions by stimulating cells in vascular walls and leukocytes (1, 2). In addition to these well-documented actions, thrombin's effects on cells other than the hematopoietic lineage or constituents of vascular walls, e.g., neuroblasts (3, 4) and osteoblasts (5), have been recognized (2). The receptor for thrombin responsible for these multiple actions on cells has recently been cloned, and a unique activation mechanism involving proteolysis and the unmasking of the agonist portion within the thrombin's effects on cells other than the hematopoietic lineage or constituents of vascular walls, e.g., neuroblasts (3, 4) and osteoblasts (5), have been recognized (2). The receptor for thrombin responsible for these multiple actions on cells has recently been cloned, and a unique activation mechanism involving proteolysis and the unmasking of the agonist portion within the surface of intact cells. The receptor molecule has been revealed (6). We are now gaining insight into the activation of cells by thrombin, but the way in which thrombin is delivered to these cells remains obscure.

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The zymogen prothrombin is converted to the two-chain active form α-thrombin by limited proteolysis upon activation. This activation process is believed to be solely catalyzed by the enzyme prothrombinase (factor Xa, anionic phospholipids, and Ca²⁺ ions) at the final stage of the complex cascade of blood coagulation and occurs solely in the plasma (for reviews, see Refs. 7–9). It had also been believed that prothrombin is synthesized only in the liver and circulates in the bloodstream. Recent results have shown, however, that it is also expressed in other tissue (10). Therefore, even when a cell is not in contact with blood, it may have prothrombin in the vicinity, but the prothrombin is unable to be converted to thrombin. Note that there is nothing impaired about the ability of these cells to interact with thrombin. A number of cell types that possess thrombin receptor (e.g., fibroblasts and neuronal cells) are spatially separated from the bloodstream, at least under physiological conditions. How do these cells encounter thrombin? Extravasation of thrombin from vessels seems a simple and probable explanation. Alternatively, one can postulate that the receptor is only operative under extraordinary conditions, such as severe tissue injury, or that there exists an as yet unidentified ligand for the receptor. Another attractive hypothesis is the existence of an alternative pathway for prothrombin activation that is independent of the coagulation cascade. We herein report a novel cell-associated protease that efficiently converts prothrombin to active thrombin. We propose that this protease catalyzes the postulated alternative pathway, providing a mechanism for the supply of thrombin to these cells.

EXPERIMENTAL PROCEDURES

Materials—The following proteins were prepared according to published methods: human prothrombin and factor X (11), human α-thrombin (12), human prothrombin 1-13, bovine factor VII (14), bovine factor VIIa (15), bovine prothrombin, factors IX and X and protein S (16), bovine factor Xa (17), bovine activated protein C (18), and bovine antithrombin III (19). All of these proteins were homogeneous as judged by SDS-PAGE.† Prothrombin was treated with p-amidophenylmethylsulfonyl fluoride prior to use in order to negate the effect of possible contamination by thrombin or factor Xa. Note that the α-thrombin to which we refer as the authentic standard and that obtained by incubation of prothrombin with activators is actually α-thrombin(des-1–13 residues Thr777–Arg800 + Ile801–Glu817 in human prothrombin). This is because human α-thrombin undergoes rapid autolysis, with removal of the N-terminal 18 residues in the A-chain, and true α-thrombin (residues Thr777–Arg800 + Ile801–Glu817) exists only as a labile intermediate.

† The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; Boc, t-butyloxycarbonyl; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; Dns-EGRck, 1,5-dansyl-Glu-Gly-Arg chloromethyl ketone; VPR-pNA, Boc-Val-Pro-Arg-p-nitroanilide; MDCG, Madin-Darby canine kidney.
Antisera against factor X were prepared by immunizing rabbits. Collection) were maintained in 150-mm plastic culture dishes (Corning) in Eagle's minimum essential medium supplemented with 10% inactivated fetal calf serum and 60 μg/ml kanamycin in a humidified atmosphere of 95% air and 5% CO2 at 37 °C, and cells were routinely reseeded every 3–4 days after trypsinization. Confluent cultures of cells (30 dishes) were rinsed three times with Tris-buffered saline (TBS; 50 mM Tris-HCl, 100 mM NaCl, pH 8.0), and cells were scraped off. Harvested cells were suspended in 30 ml of 50 mM Tris-Cl (pH 8.0), and homogenized with a motor-driven Teflon homogenizer (15 strokes). The resultant cell lysate was first centrifuged at 600 × g for 5 min to remove nuclei and unbroken cells and then at 5,000 × g for 10 min. The supernatant was further centrifuged at 100,000 × g for 60 min. All fractionation procedures were performed at 4 °C. The 5,000 × g and 100,000 × g fractions (rich in plasma membranes and microsomes, respectively) were suspended in TBS.

To assess the secretion of prothrombin activator, conditioned medium was prepared. A confluent culture of 8C cells in a 150-mm dish was rinsed once with Eagle's minimum essential medium without serum, and then 10 ml of fresh Eagle's minimum essential medium was added. After an overnight incubation, the medium was collected and subjected to the assay for activator.

Detergent-solubilized enzyme was prepared as follows. The plasma membranes suspended in TBS were homogenized in the presence of 0.3% CHAPS, incubated for 30 min on ice, and then centrifuged at 100,000 × g for 60 min at 4 °C. The resultant supernatant was used as the solubilized activator.

Other cell lines tested were from American Type Culture Collection, and maintained as recommended by ATCC.

**Assays for Procoagulant Activities—Clotting was performed as follows.** Twenty μl of human citrated plasma was mixed with 40 μl of the plasma, cells, plasma fraction or the CHAPS-solubilized activator (1 mg protein/ml) and incubated at 37 °C for 30 s. Then 20 μl of 25 mM CaCl2 was added, and the time required for clot formation was measured. Tissue factor activity was evaluated by monitoring increases in amido-typtic activity of factor VIIa (22, 23) using a chromogenic substrate (S-2288; Kabi) described previously (15). Activation of factors VII and IX was measured by a chromogenic substrate (VPR-pNA) after incubation of purified bovine factor VIIa (Kogyo) with the membrane. Activation of factor X was evaluated as described for thrombin (see below) except that the chromogenic substrate used was Boc-Leu-Gly-Arg-p-nitroanilide (Seikagaku Kogyo).

**Assay for Thrombin—** In typical assay conditions, human prothrombin (10 μM in TBS containing 5 mM CaCl2) was incubated with plasma membranes of 8C cells at 37 °C. After an appropriate incubation period, the reaction was terminated by the addition of 1/10 volume of 100 mM EDTA. Thrombin generated was quantified by measuring its amidolytic activity of factor VIIa using a chromogenic substrate (S-2288; Kabi) as described previously (15). Activation of factors VII and IX was measured by a chromogenic substrate (VPR-pNA) after incubation of purified bovine factor VIIa with the membrane. Activation of factor X was evaluated as described for thrombin (see below) except that the chromogenic substrate used was Boc-Leu-Gly-Arg-p-nitroanilide (Seikagaku Kogyo).

**Treatment with Protease Inhibitors—** The plasma membrane was incubated with inhibitors for 30 min at ambient temperature. Then it was washed with TBS containing 5 mM CaCl2 and subjected to SDS-PAGE. Membrane-associated Prothrombin Activator in Mammalian Cells

**Treatment with Activated Protein C—** In order to exclude the effect of possible contamination by factor VVa, we conducted treatment with activated protein C (APC). This would inactivate this factor. The membranes (1 mg protein/ml in TBS) were incubated with activated protein C (10 μg/ml) in the presence of 1 mg/ml protein S and 5 mM CaCl2 at ambient temperature for 1 h. Then we added diisopropyl fluorophosphate to 10 mM so as to inactivate activated protein C and left to stand for 1 h. The membranes were then washed and subjected to the assay for prothrombin activator. The same treatment greatly reduced factor V activity expressed on activated platelets, which was employed as the positive control, within a few minutes, as reported by Suzuki et al. (24).

**Protein Sequencing—** Activated prothrombin was subjected to SDS-PAGE, electrophoretically onto a poly(vinylidene difluoride) membrane (Milipore Corp.) and stained with Amido Black (25). The band that comigrated with authentic α-thrombin was cut out and subjected to sequencing in an Applied Biosystems protein sequencer model 473A.

**Gei Filtration—** The prothrombin activator solubilized with CHAPS was applied to a column of Superdex 200pg equipped with a fast protein liquid chromatography system (Pharmacia) that had been equilibrated with TBS containing 0.3% CHAPS and eluted with the same buffer at a flow rate of 1 ml/min. One-ml fractions were collected and subjected to the assay of prothrombin activator.

**Results**

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It was found that a membrane fraction from 8C fibroblasts contained a lethal component when injected intravenously into mice. The 8C-injected mice experienced severe thrombosis. Ap-Parent fibrin deposition in vessels was seen in some tissues of injected animals; abundantly in the lung, to some degree in the liver, and less in the kidney (Fig. 1). The lethal component of 8C membranes is presumably a thrombogenic factor. Indeed, the membranes provoked clotting of normal human plasma in vitro. No direct action on fibrinogen was observed, ruling out the presence of thrombin-like substances. We next evaluated the mechanism to induce plasma clotting using various purified coagulation factors. Conversion of prothrombin to thrombin by membranes of 8C cells was clearly observed, as described below, but activation of other coagulation factors, namely factors VII, IX, X, and X3, was not detected (see "Experimental Proce-
Membrane-associated Prothrombin Activator in Mammalian Cells

Fig. 2. Activation of prothrombin by isolated membranes of 8C cells. A, time course of activation. Human prothrombin (10 μM) was incubated with plasma membranes (1 mg protein/ml) for the indicated time, and amidolytic activity of thrombin was then determined. For details, see “Experimental Procedures.” The amounts of thrombin generated are expressed as percentages of that of the same prothrombin preparation fully activated by factor Xa. B, SDS-PAGE of products of prothrombin incubated with 8C membranes. Prothrombin was activated as in A for the indicated time (lanes + 8C). Samples were then centrifuged at 18,000 x g for 20 min at 4 °C to remove the membranes. Supernatants were subjected to SDS-PAGE under either nonreducing (NR) or reducing (R) conditions, and proteins were stained with Coomassie Blue. In the control experiment, prothrombin was activated by factor Xa (100:1, w/w) in the presence of 5 μM CaCl2 and 10 μg/ml phosphatidylcholine/phosphatidylserine (3:1, w/w) for 3 h at 37 °C (lane + Xa). Lanes PT and α-T were authentic prothrombin and α-thrombin, respectively. Positions of molecular mass markers are shown on the right, and derivatives of prothrombin are identified on the left: PT, prothrombin; PreT, prothrombin-1, α-T, α-thrombin; F1, fragment 1; F2, fragment 2; B, the B-chain of α-thrombin.

The time course of activation of human prothrombin by isolated 8C membranes is shown in Fig. 2A; more than 80% of prothrombin was converted to thrombin within 30 min under our experimental conditions. As is shown in Fig. 2B, prothrombin was processed by an enzyme(s) present in the membrane. The derivatives of prothrombin cleaved by the putative 8C protease are indistinguishable from those generated by incubation with factor Xa. The band corresponding to α-thrombin is clearly seen in the nonreducing gel, and in the reducing gel the band corresponding to the B-chain is evident (Fig. 2B). The protein that comigrated with the authentic standard was shown to be α-thrombin by amino acid sequence analysis; two phenylthiohydantoin-derivatives, corresponding to the sequence of human prothrombin that starts at Thr286 and Ile221, were found in each sequencing cycle (see “Experimental Procedures”). This result indicates that correct and selective cleavage of prothrombin has taken place. The thrombin generated had clotting activity similar to that of authentic α-thrombin. Bovine prothrombin was also activated by the membranes, and the apparent K_m values of the human and bovine proteins were about 0.2 μM, well below the concentration in plasma (~2 μM). When the CHAPS-solubilized activator was subjected to a gel filtration column, the activity was eluted at the position of apparent molecular mass of 65–70 kDa (data not shown).

The activation of prothrombin had an absolute requirement for Ca^{2+} ions, and physiological concentrations of Ca^{2+} ions (in the millimolar range) were necessary (Fig. 3). Calcium ions are probably required by the substrate prothrombin and not by the activator, since prethrombin-1 (a derivative of prothrombin that lacks the major Ca^{2+}-binding site, the N-terminal γ-carboxyglutamatic acid domain) was found to be a poor substrate (data not shown). We next investigated the effect of various protease inhibitors in order to characterize the nature of this activator X was significant but much weaker than that on prothrombin. Furthermore, it is presently uncertain whether activation of prothrombin and (bovine) factor X is mediated by the same enzyme. We did not pursue activation of factor X any more in the present study.

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enzyme and to rule out the possibility of the involvement of factor Xa (prothrombinase complex), which was possibly adsorbed by the cells from the culture medium, in the activation. Potent inhibitors of factor Xa, namely p-amidinophenylmethanesulfonyl fluoride (1 mM) or Dns-EGRck (1 μM) (27), failed to inhibit the activity of the membranes under conditions where both amidolytic activity of factor Xa and prothrombin activation by reconstituted prothrombinase complex were completely blocked. Moreover, a high concentration of antithrombin III (0.1 mg/ml) in the presence of heparin (5 units/ml) also gave no inhibition. The preparation of membranes was thus apparently devoid of factor Xa. We can also negate the possibility of the existence of factor V/Va, which, if present, greatly amplifies the action of trace amounts of factor Xa. The activator was rather stable (it retained activity at least 1 week when stored at 4 °C), whereas factor V is very labile; exogenous addition of factor Xa resulted in only a slight increment of the ability to generate thrombin; and finally, treatment of the membranes with activated protein C did not reduce the activity. In addition, well established low molecular weight inhibitors for trypsin-like serine proteases, i.e. diisopropyl fluorophosphate (10 mM), phenylmethanesulfonyl fluoride (1 mM), benzamidine (10 mM), and leupeptin (1 mM), could not attenuate the activity, and pepstatin (1 mM) and iodoacetamide (10 mM) were also without effect. The activator thus appeared not to be a serine, carboxyl, or thiol protease. On the other hand, exposure of the solubilized enzyme to EDTA resulted in irreversible loss of the activity (Fig. 4), presumably by removal of the metal ion required for catalysis. Although unequivocal classification should await purification of the enzyme protein and determination of its structure, the enzyme is, therefore, most likely to be a metalloprotease. We are in the process of isolating this protein.

In order to assess whether expression of the activator is specific to 8C cells or is ubiquitous, we have screened lysates of various cells for prothrombin activator; 8C cells showed prominent activity, and activation of prothrombin was also seen in some cell types of different origins, such as MDCK cells (canine, kidney epithelial) and T98G cells (human, glioblastoma) (Table I). This result strongly suggests that the same enzyme is present in various cell types. Some other cells also had activities, whereas some were negative (Table I), suggesting a specificity of expressing cell.

The enzyme expressed in T98G cells was characterized in detail. Enzymological features of the prothrombin activator of T98G were indistinguishable from those found in 8C. The T98G enzyme was solely recovered in membrane fractions and is present on the surface of intact cells; showed the same cleavage pattern of prothrombin; had similar Ca++-dependence and Km value for prothrombin; eluted at a similar position on gel filtration; and was inhibited by EDTA but not by other protease inhibitors above described. The T98G prothrombin activator must be the human counterpart of the 8C enzyme. With this human cell line, we were enabled to use antisera directed to factor X and could get further evidence that eliminated the possibility of involvement of factor Xa in the activation of prothrombin. The T98G membrane was subjected to SDS-PAGE followed by immunoblot analysis with antisera against human and bovine factor X. In this experiment, the lowest detection limit was 20 ng, and the estimated factor Xa contamination in the tested sample was 400 ng, assuming the activation was solely catalyzed by factor Xa (plus Ca++ ions and phospholipids; participation of factor V/Va was unlikely as described above). Neither human nor bovine factor X/Xa was detected. Taking this result and the pharmacological evidences together, we get firm confidence that the activator we identified is not factor Xa that may be synthesized by these cells or mere contamination from the culture medium but is a novel enzyme completely different from factor Xa.

**DISCUSSION**

We have shown that 8C fibroblasts express an activator of prothrombin on their surface. The action of this enzyme on prothrombin much resembles that of factor Xa, but all of the
pharmacological evidence argues against its participation. Furthermore, we could also rule out the possibility of involvement of another constituent of the prothrombinase complex, factor V Va. This enzyme is thus clearly disparate from the "classical" prothrombinase. Recently, Altieri (28, 29) has discovered a novel membrane-integral protein, EPR-1, which serves as a cellular receptor for factor Xa and potentiates the activation of prothrombin. However, the identity of EPR-1 and the prothrombin activator herein described is not understood. Expression of EPR-1 is seen in peripheral monocytes, monocytic-myoeloid cell lines (e.g. U-937 and HL-60), and some populations of T lymphocytes (28), but we scarcely observed prothrombin activation in cells of hematopoietic lineage including U-937 and HL-60 (Table 1). Moreover, EPR-1 is less potent in enhancing the generation of thrombin by factor Xa (29) and cannot explain the efficient activation of prothrombin seen in the present study, in that contamination by factor Xa is, if there is any, very low. We thus conclude that the 8C enzyme is a unique and hitherto novel prothrombin activator.

The presence of similar activities to generate thrombin in some other cell lines (Table 1) indicates that the activator is not unique to 8C cells and also suggests its involvement in certain physiological and/or pathological events in vivo. It is true, however, that 8C cells express the activator at high levels. We currently do not know why 8C cells are so active in this regard, but it should be pointed out that this continuous cell line has been transformed by Moloney murine sarcoma virus (21). It is possible that disruption of normal gene expression by the viral oncogene results in uncontrolled overproduction of the activator. It is of interest to note that certain malignant tumors express procoagulant activities and that patients in the late stages of cancer frequently manifest a coagulation disorder, disseminated intravascular coagulation (30). Deposition of fibrin on cancer cells is thought be an important strategy for their survival against attacks by the immune system, and participation of coagulation enzymes in metastasis has been suggested (30, 31). Most of these procoagulants are attributable to tissue factor (32), and an enzyme that directly activates factor VIIa (may be abbreviated as MAPA). A number of "exogenous" activators have been isolated from snake venoms and microorganisms (37), but, to the best of our knowledge, this is the first report of a candidate for another physiological activator specific for prothrombin in addition to factor Xa. This activator should generate thrombin independently of the coagulation cascade, and the thrombin generated would provoke various physiological events that are not restricted to hematopoietic processes. Such an alternative pathway would be highly significant for activation of cells, in particular those spatially separated from the bloodstream.

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