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A BIOLOGICALLY ACTIVE THROMBIN CLEAVAGE PRODUCT OF HUMAN SERUM SPREADING FACTOR

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SUMMARY: Purified human serum spreading factor preparations consisting of two immunologically-related, biologically-active proteins of molecular weights approximately 65,000 and 75,000 were incubated with purified hydrolytic enzymes: papain, neuraminidase and thrombin. Biologically active products of the enzymatic digestions were obtained in each case. Digestion of serum spreading factor preparations with thrombin produced a single active form of molecular weight approximately 57,000. Generation of a single molecular weight form of serum spreading factor by thrombin cleavage of the two higher molecular weight forms should simplify studies of the biochemistry and biology of this protein, and may represent a reaction of physiological significance.

Serum spreading factor (SF) is a glycoprotein isolated from human plasma or serum that markedly affects the attachment, spreading, and proliferative and differentiative potential of a variety of cell types in culture in serum-free medium (1-10). Tissue-associated SF also has been localized at cell surfaces and in tissue slices (11). Two forms of the protein of molecular weights approximately 65,000 (SF65) and 75,000 (SF75) are isolated from serum by a four-step chromatographic procedure (8). SF65 and SF75 are each independently active in cell spreading-promoting assays and in anti-SF monoclonal antibody-binding assays using a monoclonal antibody capable of inhibiting SF-promoted cell spreading in culture (6,7). In this communication we report the thrombin-catalysed conversion of serum SF preparations (SF65+SF75) to a single 57,000 dalton protein (SF57) retaining both cell spreading-promoting and anti-SF monoclonal antibody-binding activities.

ABBREVIATIONS

SF, spreading factor; SF75, 75,000 dalton form of serum spreading factor; SF65, 65,000 dalton form of serum spreading factor; SF57, 57,000 dalton form of serum spreading factor; PMSF, alpha-toluenesulfonyl fluoride; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

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MATERIALS AND METHODS

Serum SF and monoclonal anti-SF were prepared as described (7,8). Insolubilized neuraminidase-agarose and papain-carboxymethylcellulose and highly purified bovine and human thrombin were obtained from Sigma Chemical Company. Alpha-toluene sulfonyl fluoride (PMSF) was obtained from Kodak.

Cell spreading assays using HeLa human carcinoma and anti-SF monoclonal antibody immunoblots for identification of SF in protein samples after sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were carried out as described previously (2,7,8). Electrophoresis was carried out using 10% polyacrylamide gels. The following molecular weight standards appear in the figures (top to bottom): phosphorylase B, 92,500 daltons; bovine serum albumin, 66,500 daltons; ovalbumin, 45,000 daltons; carbonic anhydrase, 31,000 daltons.

RESULTS AND DISCUSSION

In a survey of effects of incubations of purified serum SF with a number of hydrolytic activities we identified three enzymes--papain, neuraminidase and thrombin--capable of acting on serum SF without destroying the biological (cell spreading-promoting) activity of the preparations. Unreduced, unboiled control serum SF migrated upon SDS-PAGE in a diffuse band running somewhat slower than unreduced bovine serum albumin (Fig. 1). Reduction of unboiled samples allowed resolution of the two components (SF65 and SF75). Neuraminidase treatment resulted in serum SF preparations that migrated slightly faster than untreated preparations, presumably representing a reduction in molecular weight by removal of sialic acid from the molecules. Treatment with papain resulted in material that migrated in a manner identical to that of control samples if unreduced, but migrated as fragments of molecular weights less than 30,000 after reduction. Short incubations of serum SF with bovine or human thrombin resulted in a decrease in the SF65 and SF75 bands and the appearance of a new protein migrating in a manner consistent with a molecular weight of approximately 57,000. All preparations illustrated in Fig. 1 containing proteins in the molecular weight range 57,000 to 75,000 were active in both cell spreading-promoting and monoclonal antibody-binding assays; reduction of papain-treated material with mercaptoethanol resulted in loss of both activities (not shown).

Increasing the thrombin concentration in the incubation mixtures from a molar ratio of about 1 thrombin molecule per six serum SF molecules to over 1

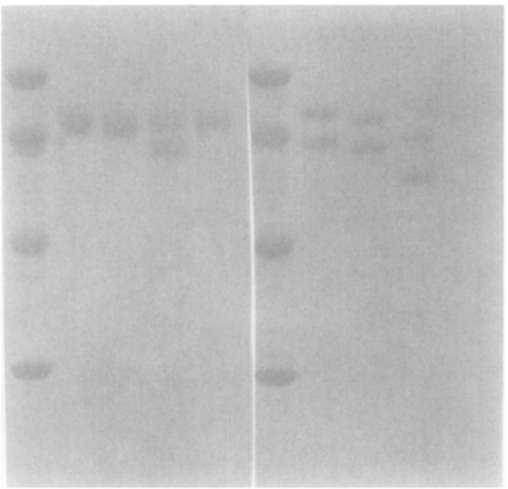
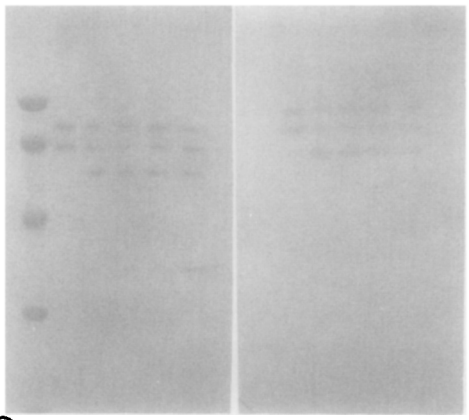


Fig. 1. Products of enzymatic hydrolysis of human serum SF. Serum SF (300 µg/ml) was incubated with bovine thrombin (20 µg/ml), insolubilized neuraminidase-agarose (1 unit/ml) or insolubilized papain-cellulose (6.4 units/ml) for two hours at 37°C. Total volume of the reaction mixtures was 250 µl. At the end of the incubation, PMSF (2 mM) was added to the reaction mixtures, samples were chilled and the enzyme-linked beads centrifuged from suspension and discarded. Samples containing 12 µg serum SF in each case were diluted in SDS-containing buffer and analysed by SDS-PAGE, either unreduced or after reduction with 1% mercaptoethanol. Left Panel: unreduced samples; (A) molecular weight standards (see MATERIALS AND METHODS), (B) serum SF incubated without added enzymic activities, (C) serum SF incubated with neuraminidase, (D) serum SF incubated with thrombin, (E) serum SF incubated with papain. Right Panel: samples as in (A)-(E), reduced with mercaptoethanol before electrophoresis.

thrombin molecule per SF molecule did not increase the extent of conversion to SF57 in a two-hour incubation, and longer incubations were required to complete conversion to the lower molecular weight form (Figs. 2 and 3). Thrombin-catalysed conversion of SF65 and SF75 to SF57 was inhibited by the protease inhibitor PMSF. SF preparations converted completely to SF57 by incubation with thrombin were effective at promoting cell spreading in culture (Fig. 4), and assay of the spreading-promoting activity of material eluted from gel slices after SDS-PAGE of these preparations established that the biological activity was associated with SF57.

The ability of thrombin to cleave serum SF preparations to a smaller biologically-active fragment may indicate a physiological activity of SF relevant to the clotting process. Thrombin cleavage may be involved, for instance, in the release from platelets of the platelet-associated form of SF we have identi-



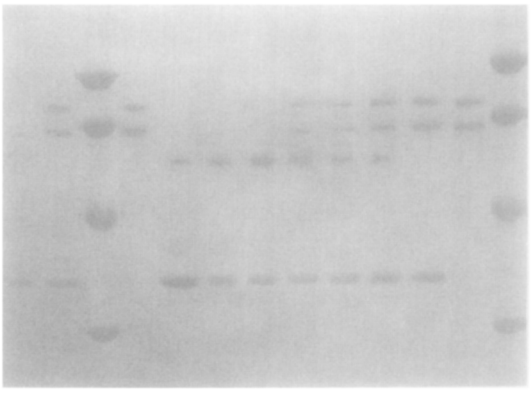


Fig. 2. Thrombin concentration-dependence of cleavage of serum SF. Serum SF (75 µg/ml) was incubated with the indicated thrombin concentrations for two hours at 37°C, the reaction stopped and reduced samples analysed by SDS-PAGE as described for the experiment of Fig. 1. A duplicate gel was processed for immunoblotting using monoclonal anti-serum SF and peroxidase-linked second antibody as described. Left Panel: gel stained for protein. Right Panel: Immunoblot of duplicate gel. (A) molecular weight standards (see MATERIALS AND METHODS), (B) serum SF incubated in the absence of thrombin, (C) serum SF incubated with 5 µg/ml thrombin, (D) serum SF incubated with 10 µg/ml thrombin, (E) serum SF incubated with 20 µg/ml thrombin, (F) serum SF incubated with 50 µg/ml thrombin.

Fig. 3. Timecourse of thrombin cleavage of serum SF. Thrombin (50 µg/ml) was incubated with serum SF (88 µg/ml) at 37°C under the conditions indicated below. Samples were reduced and analysed by SDS-PAGE. (A) thrombin incubated in the absence of serum SF for 4 hours, (B) serum SF + thrombin + 2 mM PMSF incubated 4 hours, (C) molecular weight standards (see MATERIALS AND METHODS), (D) serum SF incubated 25 hours, (E) serum SF + thrombin incubated 25 hours, (F) serum SF + thrombin incubated 8 hours, (G) serum SF + thrombin incubated 4 hours, (H) serum SF + thrombin incubated 1 hour, (I) serum SF + thrombin incubated 0.5 hours, (J) serum SF + thrombin incubated 0.1 hour, (K) serum SF + thrombin + 2 mM PMSF at time 0, (L) unincubated serum SF, (M) molecular weight standards.

fied (7) and SF may be related to previously identified platelet-associated thrombin substrates and cleavage products of molecular weights similar to SF75, SF65 and SF57 (12,13). The presence of endogenous SF57 in some SF preparations (6,7), presumably created by the action of thrombin in the starting material used for the purifications, suggests that the phenomenon we have observed in vitro with purified serum SF and purified thrombin probably also occurs in blood under more physiological conditions. The availability of a single 57,000 dalton molecule possessing the biological and monoclonal antibody-binding properties of SF65 + SF75 preparations also should facilitate biochemical analysis of serum SF toward the goal of defining structure-function relationships for this protein.

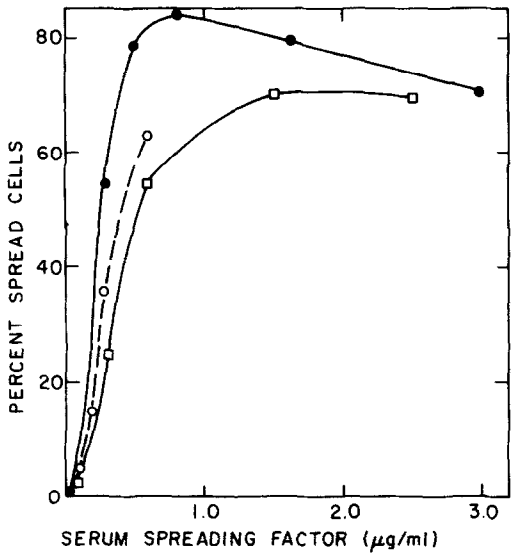


Fig. 4. Cell spreading-promoting activity of thrombin cleavage product of human serum SF. SF57 preparations were produced from SF65 + SF75 preparations by incubation with 20 μg/ml thrombin at 37°C. Complete conversion to SF57 was established by SDS-PAGE of the incubated preparations with subsequent protein staining and immunoblot using monoclonal anti-SF. HeLa cell spreading-promoting activity of SF preparations was assayed as described. (▫) SF preparation converted to SF57 by incubation with thrombin; (○) SF65+SF75 preparation incubated in the absence of thrombin; (●) unincubated serum SF preparation.

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