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PLASMIN REGULATING SYSTEM FROM EMBRYONAL CARCINOMA F9 CELLS: PLASMINASES A,B AND EMBRINOGEN

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SUMMARY - Two plasmin inactivators, plasminase A and B, and their inhibitor embrinogen were isolated from embryonal carcinoma F9 cells by preparative two-dimensional electrophoresis. Plasminases A and B have molecular weights of 160,000 and 82,000, respectively. Both are serine proteinases which digest the light chain of plasmin in a time dependent inactivation process. The heavy chain of plasmin is not affected by this action. Plasminases A and B show similar specificity towards synthetic and natural polypeptide inhibitors. The interaction of the two enzymes leads to their inhibition. Embrinogen (m.w. 84,000) inhibits both plasminases A and B as well as urokinase and plasmin. Its activation by trypsin creates embrin, a proteinase directed against plasmin heavy chain.

Plasmin-catalyzed proteolysis affects the function of major plasma protein effector systems as coagulation, fibrinolysis, complement system. Plasmin degrades cell membrane proteins (1,2), immunoglobulins (3) somatotropin, glucagon, ACTH (4,5), fibronectin (6) and it activates collagenase (7). Plasmin modifies cell morphology and the interaction of cells with their substratum by action on both (8,9). Its presence in the medium disturbs monolayer ordered morphology of cells (10).The secretion of plasminogen activator(s) converting plasminogen to plasmin is connected to important events like differentiation (11), ovulation (12) early embryogenesis (13), malignant transformation (14) or hormonedirected changes (15). Plasminogen activator, absent from embryonal carci noma stem F9 cells, appears simultaneously with the differenciation induced by retinoic acid and cyclic AMP (11). Accordingly, its appearance in the culture medium is considered as a marker of differentiation.

Abbreviations: SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; DFP, diisopropylfluorophosphate; 2D-, two-dimensional.

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In this paper we describe a system of proteinases and their inhibitor from embryonal carcinoma F9 cells which regulate plasmin activity.

MATERIALS AND METHODS

Cell cultures and tumors. Embryonal carcinoma F9 cells were cultured as previously described (16). Tumors were produced after subcutaneous injection of $2x10^6$ F9 cells to three weeks old male 129 mice. The collected tumors were stored at -20°.

Preparative electrofocusing. 6g of solid tumor were washed with saline-phosphate buffer, then homogenized in 30 ml of lysis buffer containing 9,5 M urea, 2% (w/v) Nonidet P-40 and 0,2% (v/v) ampholine. After centrifugation for 10 minutes at 12,000 g the supernatant was completed to 95 ml with 4 M urea, then 2,5 ml of each ampholines pH 3,5-10 and 4-6 (LKB) were added. Electrofocusing was performed according to the method described for LKB 2117 Multiphor. 200µl of each fraction were incubated with 2 ml of distilled water and pH was measured with Metrohm Herisau pH-meter.

Preparative gel electroporesis was done in BRL 1100PG apparatus on the column of 10% running polyacrylamide gel (5 cm in height) and 5% stacking gel (1 cm in height) according to technical description in BRL Instruction Manual. The separation was done in Tris-glycine buffer described by Laemmli (17). For the elution this buffer was made 6,5% in glycerol. The current was 6 mA, elution rate 12 ml per hour, fraction volume was 2 ml.

Analytical electrophoresis was done according to Laemmli (17) with running gel containing 10% polyacrylamide. Apparent molecular weights were estimated from the relative migration of internal standards including β-galactosidase (120,000), phosphorylase (97,000), bovine serum albumin (66,000), aldolase subunit (40,000) and chymotrypsinogen (25,000). Gels were stained with Coomassie brilliant blue.

Assays. Proteolytic activity was determined with porcine plasminogen (Sigma) as substrate using the SDS-polyacrylamide gel electrophoresis as previously described (18). Plasmin activity was measured using the chromogenic tripeptide H-D-Val-Leu-Lys-p-nitroanilide (S2251 from Kabi, Sweden) as substrate (19). Protein concentration was determined according to Lowry et al. (20).

RESULTS AND DISCUSSION

Purification of plasminases A, B and of embrinogen: Plasminases A,B and embrinogen were fractionated from the crude extract in the first step by a preparative electrofocusing using a pH gradient 3-10. Both plasminases migrated in the pH region 5.6, embrinogen in the pH region 5,2-5,4. Further purification of the corresponding fractions was obtained by preparative electrophoreses in BRL columns. Details of the purification procedure will be published elsewhere. The molecular weights of purified plasminases A,B and of embrinogen were found to be 160,000, 82,000 and 84,000, respectively (Fig. 1).

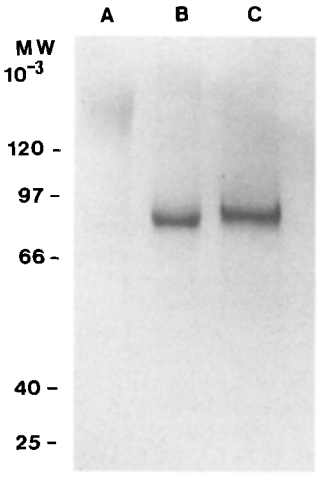


Fig. 1. Analytical gel electrophoresis of plasminases A, B and embrinogen.
After preparative electrophoresis the corresponding fractions were concentrated by ultrafiltration (Amicon UM-10). The proteins were precipitated in 10% trichloroacetic acid at 4°C and analyzed in 10% PAGE.
A - plasminase A; B - plasminase B; C - embrinogen.

Plasminase A yielded a diffuse band that was poorly stained by Coomassie blue. When this preparation was dialyzed to eliminate SDS-containing buffer, incubated with ^{14}C-DFP and analyzed by SDS-PAGE, the radioactivity was found in two bands (mw. 160,000 and 82,000, not shown). This together with the fact that plasminases A and B were isolated from the same fraction of preparative electrofocusing indicate that plasminase B may originate from plasminase A.

Action of plasminases A and B on plasminogen and plasmin. Incubation of plasminogen with urokinase results in conversion of its single chain into heavy (65,000) and light (25,000) plasmin chains (Fig. 2E). Incubation of plasminogen with plasminase A or B results in the formation of the heavy chain and the degradation of the light chain (Fig.2A,C). When plasminogen was incubated with the mixture of plasminase A and B, this activity was inhibited (Fig. 2D). On the contrary, urokinase did not inhibit the activity of plasminases (Fig. 2F,G).These results show that plasminases A and B split selectively the light chain in the activated plasmin or in plasminogen.

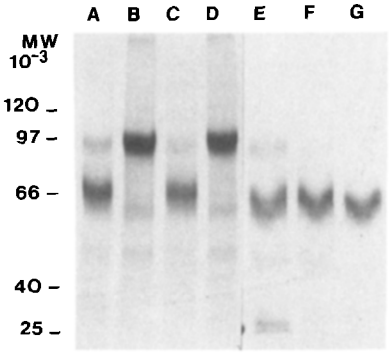


Fig. 2. Action of plasminases A and B on plasminogen and plasmin. Plasminogen (40µg) was incubated at pH 7,5 and 37°C for 2 hrs with 0,8µg of plasminase A (A), alone (B), with 0,4µg of plasminase B (C) or with the mixture of both (D). Plasminogen was activated with urokinase (0,01U) for 15 min. at 37°C; the activation mixture was incubated for 2 hrs alone (E), with plasminase A (F) or B (G).

Since the active site of plasmin is situated in the light chain (21), the effect of plasminases A and B on plasmin activity using synthetic substrate H-D-Val-Leu-Lys-p-nitroanilide was studied. Plasminases A and B were found to inactivate plasmin in a time-dependent process which corresponds to the proteolytic degradation of plasmin rather than to the formation of an inactive complex (Fig. 3). This is in agreement with the observation that the light chain of plasmin disappears after the incubation with plasminases (Fig. 2).

The results presented in Fig. 2 and 3 prove that the effect of plasminases A and B opposes that of plasminogen activators: urokinase generates plasmin from plasminogen by a limited proteolysis, whereas plasminases destroy this generated plasmin activity by a more extensive proteolysis of the light chain leaving the heavy chain apparently intact (Fig. 2).

Affinity of plasminases A and B towards proteinase inhibitors: The formation of the heavy chain of plasmin as the only macromolecular product was used for the assay of the inhibition of plasminase activity under the same conditions as described in Fig. 2. The results in Table I show that both plasminases are sensitive to the same inhibitors. Their inhibition by DFP and NPGB on one hand and the absence of inhibi-

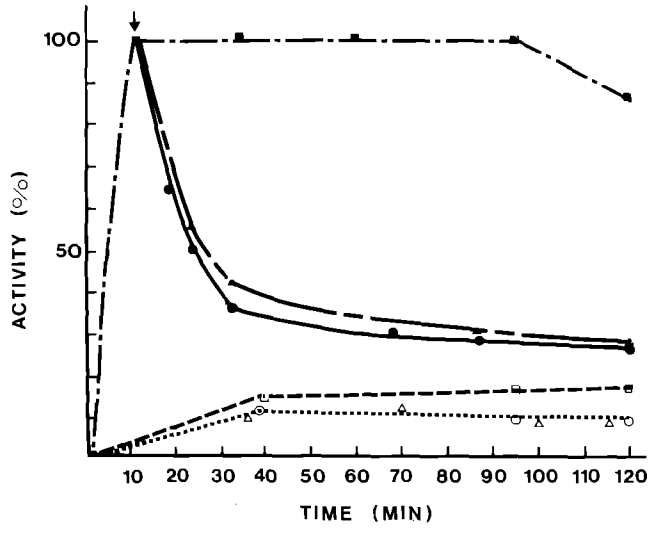


Fig. 3. Effect of plasminases A and B on plasmin activity towards its synthetic substrate H-D-Val-Leu-Lys-p-nitroanilide.
Plasminogen (2 mg/ml) was incubated with urokinase (0,2 U/ml) at pH 7,5 and 37°C. 30µl samples were withdrawn for the activity measurement (■——■). After 10 min of incubation was added either 1,6µg of plasminase A (▲——▲) or 0,8µg of plasminase B (●——●) and incubated under the same conditions. Plasminogen without activation (□-----□), plasminogen and plasminase A (○-----○) or B (△-----△).

tion by the inhibitors of metalloproteinases or thiol-proteinases (as EDTA and p-chloromercuribenzoate, respectively) indicate that they belong to the group of serine proteinases. Their inactivation by reducing compounds such as mercaptoethanol and dithiothreitol demonstrates the

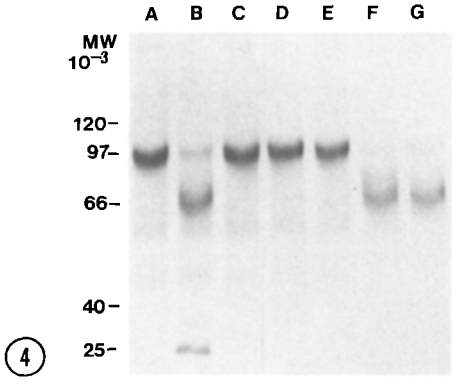
TABLE I
EFFECT OF INHIBITORS ON PLASMINASES A AND B

Inhibitor	molar conc.	plasminase A	plasminase B	urokinase (26)	plasmin (26)
DFP	2.10^{-3}	+	+	+	+
Benzamidine	2.10^{-3}	+	+	+	+
NPGB	1.10^{-3}	+	+	+	+
EDTA	7.10^{-2}	-	-	nd	nd
pCMB	2.10^{-3}	-	-	nd	nd
Dithiothreitol	2.10^{-2}	+	+	nd	nd
Mercaptoethanol	1.0	+	+	nd	nd
TLCK	8.10^{-3}	-	-	-	+
Heparin	1.10^{-5}	-	-	nd	nd
BPTI	3.10^{-5}	+	+	-	+
STI	1.10^{-5}	+	+	-	+
Ovomucoid	5.10^{-6}	-	-	nd	nd

NPGB, p-nitrophenyl guanidinobenzoate; EDTA, ethylenediamine tetraacetate; pCMB,p-chloromercuribenzoate; TLCK, tosyllysine chloromethylketone; BPTI, basic pancreatic trypsin inhibitor; STI, soybean trypsin inhibitor; nd - data not available.

importance of S-S bridges and intact tertiary structure for their activity.

Characterization of embrinogen from F9 cells: Embrinogen is a protein of m.w. 84,000 (Fig. 1C). Its incubation with plasminases A, B or with urokinase results in inhibition of their activities (Fig.4).Inhibition of plasmin by embrinogen was studied using synthetic substrate of plasmin. When plasminogen was first activated by urokinase and then embrinogen was added (Fig. 5), 45% inhibition of plasmin was observed within 2 minutes. The extent of inhibition was dependent on embrinogen concentration. In an equimolar ratio, 97% inhibition was achieved in one minute. This indicates that the inhibition of plasmin by embrinogen is due rather to the formation of an inactive complex than to its degradation.



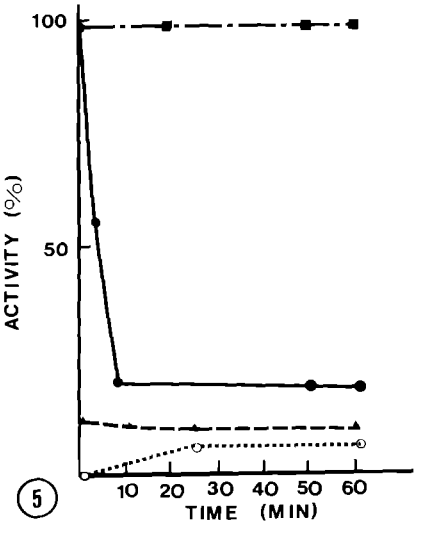


Fig. 4. Effect of embrinogen on urokinase and plasminases A and B. The conditions were as in Fig. 2. Embrinogen (7,5µg) was added simultaneously with plasminase A or B. A - plasminogen alone; B - plasminogen and urokinase; C - plasminogen and urokinase preincubated with embrinogen; D - plasminogen, plasminase A and embrinogen; E - plasminogen, plasminase B and embrinogen; F and G - the same as D and E respectively without embrinogen.

Fig. 5. Effect of embrinogen and embrin on plasmin activity towards H-D-Val-Leu-Lys-p-nitroanilide.
The same conditions as in Fig. 5 were used. Plasminogen(plasmin)-embrinogen ratio was 1,3:1 (w/w). (■———■), plasmin and embrin; (●———●), plasmin and embrinogen; (▲- - -▲), plasminogen and urokinase inhibited by preincubation with embrinogen; (○·····○), plasminogen and embrinogen.

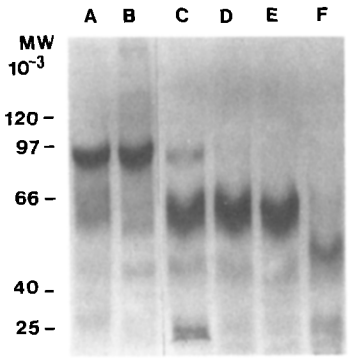


Fig. 6. Effect of embrin on plasmin.
The same conditions as in Fif. 4 and 6 were used. A - plasminogen; B - plasminogen and embrinogen (40:8); C - plasminogen and trypsin (40:1); D - plasmingen, plasminase A and trypsin (40:2:1); E - plasminogen, plasminase B and trypsin (40:2:1); F - plasminogen, embrinogen and trypsin (40:8:1).

Embrinogen is a precursor of a proteinase: When embrinogen is incubated with plasminogen, the latter shows no change in its molecular weight (Fig.6 A,B). On the contrary, when embrinogen was preincubated at 37°C at pH 7,5 for 1 hour with trypsin, then it degrades the heavy chain of plasmin (Fig.6 F). The addition of trypsin to plasminogen in the same proportion but in absence of embrinogen results only in its activation to plasmin (Fig.6 C). This is evidence for the activation of embrinogen into a proteinase (embrin), which in turn degrades (alone or in cooperation with trypsin) the heavy chain of plasmin. As shown in Fig. 5, incubation of plasmin with embrin does not decrease the activity of plasmin towards its synthetic substrate. Further study of the specificity of this proteinase is under investigation.

Differences between the plasminases-embrinogen-embrin system and the urokinase-nexin system: It was recently reported (22,23), that human foreskin cells release the proteinase nexin of m.w. 40,000 which binds urokinase (m.w. 54,000) into an inactive complex. On the other hand, the same cells also release into the culture medium an inactive urokinase precursor that generates urokinase activity under condition of plasminogen activator assay. Therefore nexin was suggested as an im-

portant component of the regulation of plasminogen activator activity in fibroblasts.

Our present results provide evidence for another system of plasmin regulation in undifferentiated embryonal carcinoma F9 cells. This system includes serine proteinases, their precursors and inhibitors.

Plasminase A of mol. weight 160,000 is a high molecular weight precursor of plasminase B (82,000) and inhibits the latter. Embrinogen (84,000) inhibits both plasminases as well as urokinase and plasmin. It also can be activated to form the proteinase embrin.

Plasminases A and B show the same specificity towards synthetic and polypeptide inhibitors. Both are serine proteinases which digest the light chain of plasmin containing the active site, and do not appear to affect the heavy chain. On the contrary, active embrin digests the plasmin heavy chain and does not change the activity of its light chain. As the first kringle of the heavy chain of plasmin contains a high affinity binding site for fibrinogen and fibrin (24,25), the plasmin regulating system may distinguish the fibrinolytic from other functions of plasmin. It controls the generation of plasmin by the action of embrinogen on urokinase, the inactivation of plasmin by plasminases A and B and eventuallythe discrimination between fibrinolytic and other plasmin specificities by action of embrin. Thus the plasmin regulating system may be one of the factors affecting functional changes of the cell.

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