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CHARACTERIZATION OF A PLASMA MEMBRANE-ASSOCIATED PLASMINOGEN ACTIVATOR ON THYMOCYTES

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Summary

Plasma membranes isolated from normal thymocytes of hamster and rats were found to exhibit neutral protease activity toward ^{125}I-labeled casein. The plasma membrane-associated proteases were completely inhibited by the serine protease inhibitors, diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride and *p*-nitrophenyl-*p*-guanidinobenzoate, partially inhibited by soybean trypsin inhibitor and antipain, but were only weakly inhibited by L-1-tosylamino-2-phenylethyl chloromethyl ketone. The plasma membrane-associated proteases were also completely inhibited by $ZnCl_2$ (75—100 μM), but they were not affected by several other divalent cations. The plasma membrane fraction contained a plasminogen activator activity which was specifically localized in this fraction. The plasma membrane-associated plasminogen activator activity was inhibited by all of the inhibitors which inhibited plasma membrane-associated proteases except L-1-tosylamido-2-phenylethyl chloromethyl ketone. Labeling of plasma membrane-associated serine esterases with [^{3}H]diisopropyl fluorophosphate followed by separation of the proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed that this fraction contained a single major ^{3}H-labeled protein of M_r 105 000. Both the plasminogen activator and the M_r 105 000 esterase were shown to be glycoproteins by affinity chromatography on lentil lectin-Sepharose. These results indicate that the plasmino-

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Abbreviations: SDS, sodium dodecyl sulfate; DFP, diisopropyl fluorophosphate; PMSF, phenylmethyl sulfonyl fluoride; NPGB, *p*-nitrophenyl-*p*-guanidinobenzoate; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; TLCK, *p*-tosyl-L-lysine chloromethyl ketone; Hepes, *N*-2-hydroxyethylpiperazine-*N*-2′ ethanesulfonic acid.

gen activator of thymocytes is a glycosylated serine protease with an active site-containing subunit of M_r 105 000 which is specifically localized in the plasma membrane.

Introduction

Cell surface proteases have been detected on a large number of cell types, including lymphocytes, monocytes, basophils, and cultured cell lines from several tissue sources [1—5]. The surface proteases of basophils and several cell lines have been shown to function as plasminogen activators in vitro [4,5]. The fact that proteases with plasminogen activator activity are present on the surface of cells derived from several distinct tissues from several species suggests that these proteases may represent constitutive enzymes which participate in the basic metabolic and regulatory functions of the surface membrane. While the physiological functions of these surface proteases have not been established, proteases which act at the cell surface have been implicated in a variety of cellular processes, such as shedding of plasma membrane proteins [6], regulation of membrane-bound enzymes [7,8], and control of cell growth in lymphocytes [4,10,11] and in other cell types [12,13].

In a previous report [14], we examined the cell surface proteases exhibited by viable thymocytes and lymph node cells of hamsters and rats and demonstrated the presence of cell surface protease activity(s), one of which could function as a plasminogen activator. The present report describes the partial characterization of the proteases associated with the plasma membrane fraction isolated from normal thymocytes of hamsters and rats. On the basis of inhibitor sensitivity, the plasma membrane-associated proteases are similar to the proteases which were previously detected on viable cells [14]. One of these proteases has plasminogen activator activity and is enriched in the plasma membrane fraction of thymocytes. We have also demonstrated that the plasminogen activator is a glycoprotein, suggesting that it is an ecto-enzyme present at the thymocyte surface.

Materials and Methods

Materials. Normal female MHA hamsters and CD rats (4—8 week-old) were obtained from Charles River Breeding Colony (Vineland, NJ). [^{3}H]DFP (3.9 Ci/mmol) was obtained from Amersham-Searle Corp. (Arlington Heights, IL). Unlabeled DFP and PMSF were purchased from Calbiochem (La Jolla, CA). Soybean trypsin inhibitor was purchased from Worthington Biochemicals (Freehold, NJ). NPGB was purchased from U.S. Biochemical Corp. (Cleveland, OH). Antipain was obtained from Peninsula Laboratories (San Carlos, CA). Porcine plasminogen (4.3 CTA units/mg protein) was purchased from Sigma Chemical Company (St. Louis, MO). Reference standard urokinase (2240 Plough units/vial; lot No. 001282) was purchased from Calbiochem (La Jolla, CA) and was solubilized in Hepes-buffered saline (2240 Plough units/ml) and stored at —70°C in aliquots. Purified casein was purchased from Difco Laboratories (Detroit, MI). All reagents for polyacrylamide gel electrophoresis were obtained

from Eastman Kodak Co. (Rochester, NY). Sephadex G-25, G-50, G-100, Sepharose CL-4B and ampholines (pH 3—10) were obtained from Pharmacia (Uppsala, Sweden). All other reagents used were commercially available reagent grade.

Cell isolation. Thymi were obtained from normal female hamsters or rats and single cell suspensions were prepared as previously described [14]. Thymocyte preparations utilized in this study consisted of greater than 98% small lymphocytes, 1% red blood cells, and did not contain morphologically identifiable macrophages (less than one per 10^3 counted cells). Cell viability was greater than 95% by the trypan blue exclusion test.

Plasma membrane isolation. Thymocytes were disrupted by N_2 cavitation and the plasma membranes were isolated essentially as described by Schmidt-Ullrich et al. [15] except that sucrose gradients, rather than dextran gradients, were used for equilibrium density ultracentrifugation. Briefly, thymocytes were washed twice and resuspended in Hepes-buffered saline (10 mM Hepes, 0.075 M NaCl, 0.065 M KCl, pH 7.2) containing 0.5 mM $MgCl_2$. The cell suspension ($4.0—8.0 \cdot 10^7$ cells/ml) was homogenized in a Kontes Minibomb (Vineland, NJ) equilibrated with 425 lb/inch^2 N_2 for 15 min at 4°C. Differential centrifugation was performed exactly as described by Schmidt-Ullrich et al. [15]. The large granule pellet (largely lysosomes and mitochondria) was resuspended in 1.0 ml Hepes-buffered saline and layered onto a 30% (w/w) sucrose cushion and centrifuged at 200 000 × *g* for 3.5 h at 4°C in order to recover plasma membrane which initially pelleted with the large granules. The band at the interface was collected with a pasteur pipette and combined with the microsomal pellet. The pellet at the bottom of the tube was resuspended in Hepes-buffered saline and washed once at 10 000 × *g* for 20 min at 4°C and designated large granules. The combined microsomes were washed in hypotonic buffer, first in 10 mM Hepes, pH 7.5, then in 1 mM Hepes, pH 7.5, in order to remove cytoplasmic constituents trapped within the membrane vesicles. The washed microsomes were resuspended in 1.0 ml of 10 mM Hepes containing 0.1 mM $MgCl_2$, pH 7.5, diluted with an equal volume of 40% sucrose (w/w in 10 mM Hepes containing 0.1 mM $MgCl_2$, pH 7.5) and layered onto discontinuous sucrose gradients with 2.0-ml steps of 30 and 40% (w/w) sucrose. The gradients were developed at 200 000 × *g* for 4.5 h at 4°C; the resulting bands at the 20—30% and 30—40% interfaces were collected with a pasteur pipette and designated plasma membrane and endoplasmic reticulum, respectively. Thus, this fractionation yielded five subcellular fractions: nuclear pellet, cytosol, large granules, endoplasmic reticulum and plasma membrane.

Characterization of membrane fractions. The distribution of subcellular membranes was followed by analysis of marker enzymes as suggested by Schmidt-Ullrich et al. [15]. Plasma membrane was monitored with alkaline *p*-nitrophenylphosphatase (EC 3.1.3.1) as assayed by Bosmann et al. [16] and endoplasmic reticulum was monitored by NADH: lipoamide oxidoreductase (EC 1.6.4.3) assayed as described in Ref. 17. β-D-Glucuronidase (EC 3.2.1.31) was used as the lysosomal marker and was assayed as described in Ref. 18 using *p*-nitrophenyl-β-D-glucuronide as substrate. Succinate dehydrogenase (EC 1.3.99.1) was used as a mitochondrial marker (inner membrane) and was assayed by the spectrophotometric method of Slater and Bonner [19]. Protein

was determined by using the method of Lowry et al. [20], using bovine serum albumin as the standard.

Plasminogen. Two sources of plasminogen were used in these experiments, affinity-purified rat plasminogen and a commercial preparation of porcine plasminogen. Porcine plasminogen (4.3 CTA units/mg protein) was solubilized in Hepes-buffered saline (4 mg protein/ml) and was treated with 5 mM DFP at 4°C, overnight, to inhibit contaminating plasmin activity, and was dialyzed to remove excess DFP before use. After this treatment, a low level of residual caseinolytic activity was still present as can be seen in the plasminogen controls in Fig. 1A. The dialyzed plasminogen was divided into aliquots and stored at −70°C and was stable for approx. 1 month.

Rat plasminogen was isolated from fresh, citrated, rat plasma by affinity chromatography on lysine-Sepharose essentially as described by Deutsch and Mertz [21] for the isolation of human plasminogen. In a typical isolation, 15 ml of fresh, citrated, rat plasma were diluted with an equal volume of 0.3 M sodium phosphate, pH 7.5, and DFP was added to a final concentration of 5 mM. The sample was passed through a column of lysine-Sepharose 4B (1.0 × 20 cm containing 15 ml packed lysine-Sepharose) prepared as described in Ref. 21. The column was washed with 0.3 M sodium phosphate, pH 7.5, until the absorbance at 280 nm was less than 0.01 (approx. 75 ml). Absorbed plasminogen was eluted in a sharp peak with 0.025 M ϵ-aminocaproic acid in 0.3 M sodium phosphate, pH 7.5 [22]. The final yield of plasminogen was 1.5—1.8 mg (0.1—0.12 mg/ml plasma). The specific activity of this plasminogen preparation was 21.4 CTA units/mg protein by comparison with the porcine plasminogen of known activity when assayed with ^{125}I-labeled casein in the presence of excess urokinase. Using SDS-polyacrylamide gel electrophoresis to monitor purity of the isolated plasminogen, it was observed that these plasminogen preparation contained less than 1% contamination by other proteins as determined by Coomassie blue staining or ^{125}I labeling (Fig. 3). This rat plasminogen preparations contained no detectable plasmin activity in a 3 h assay at 37°C with 75 μg ^{125}I-labeled casein as substrate, but was readily activated by both urokinase and rat thymocyte plasma membrane fractions (Fig. 1B). This plasminogen preparation was stable when stored as a solution (0.5—1.0 mg/ml) in 0.1 M sodium phosphate, pH 7.2, at −70°C for at least 3 weeks.

Preparation of radioactively labeled protease substrates. Casein was solubilized in Hepes-buffered saline at a concentration of 20 mg/ml and deaggregated by centrifugation at 100 000 × g for 2 h at 4°C. Deaggregated casein was iodinated with Na^{125}I by the chloramine T procedure [23] to a specific activity of approx. 100 000 cpm/μg protein. Iodinated casein was desalted and separated from a low molecular weight contaminant by chromatography on a column of Sephadex G-100 (1.5 × 35 cm) equilibrated in Hepes-buffered saline. The final product was sterilized by passage through a 0.45 μm Millipore filter and stored at 4°C.

Iodinated rat plasminogen was prepared by chloramine T iodination of affinity-purified rat plasminogen with Na^{125}I to a specific activity of 50 000 cpm/μg protein [23]. The ^{125}I-labeled plasminogen was desalted on a column of Sephadex G-25 (1.0 × 20 cm) equilibrated in 0.1 M sodium phosphate, pH 7.2. Iodinated plasminogen was used immediately or was stored at −70°C.

Assay of neutral protease and plasminogen activator activity. Neutral protease and plasminogen activator were assayed as previously described [14]. The assay employed was a caseinolytic assay using ^{125}I-labeled casein as substrate. Protease activity was measured as the release of 5% trichloroacetic acid-soluble ^{125}I-labeled digestion products from the ^{125}I-labeled casein. To measure neutral protease activity (pH 7.2) in the subcellular fractions of thymocytes, 25—75 μg sample protein were incubated with ^{125}I-labeled casein in a total volume of 0.25 ml Hepes-buffered saline containing 0.5% Nonidet P40, pH 7.2, for 0—3.5 h at 37°C. At 0, 1, 2, and 3 or 3.5 h after the addition of substrate, 0.5 mg unlabeled casein was added and the reaction mixture was precipitated with an equal volume (0.3 ml) of 10% cold trichloroacetic acid. The precipitates were incubated for at least 2 h at 4°C (identical results were obtained when precipitates were incubated at 4°C overnight). The resulting precipitate fractions were removed by centrifugation at 1500 × *g* for 10 min and 100-μl aliquots of the

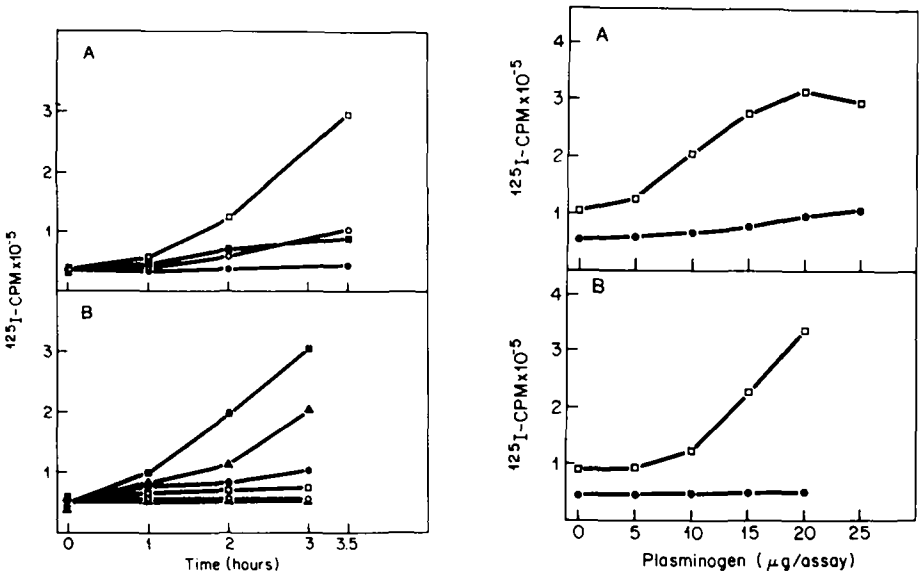


Fig. 1. Kinetics of protease and plasminogen activator assays. (A) ^{125}I-labeled casein hydrolysis in the presence of 75 μg ^{125}I-labeled casein for 0—3.5 h at 37°C: (●) buffer only; (■) 20 μg porcine plasminogen; (○) 50 μg rat thymocyte plasma membrane; (□) 50 μg plasma membrane + 20 μg plasminogen. Assays were performed in 0.25 ml Hepes-buffered saline/Nonidet P40, and were stopped at the indicated times by addition of 50 μl unlabeled casein (0.5 mg) and 0.3 ml cold 10% trichloroacetic acid. Aliquots (100 μl) of the soluble fraction were counted for ^{125}I and multiplied by 6 to calculate total cpm released/assay. Values represent average determinations of triplicate assays. Standard deviation was less than 10%. (B) ^{125}I-labeled casein hydrolysis in the presence of 75 μg ^{125}I-labeled casein for 0—3 h at 37°C: (○) buffer only; (△) 20 μg rat plasminogen; (□) 0.1 U urokinase; (●) 75 μg rat thymocyte plasma membrane; (□) 0.1 U urokinase + 20 μg plasminogen; (▲) 75 μg plasma membrane + 20 μg plasminogen. Assays were performed as described in A. Values represent average determinations of triplicate assays. Standard deviation was less than 10%.

Fig. 2. Concentration dependence of plasminogen activation by rat thymocyte plasma membrane. (A) Activation of porcine plasminogen; (B) activation of rat plasminogen. Plasminogen at the concentrations indicated, was incubated with buffer only (●) or 75 μg plasma membrane protein (□) for 3 h at 37°C in a total volume of 0.25 ml Hepes-buffered saline/Nonidet P40. Soluble cpm/assay were determined as in Fig. 1. Values represent average determinations of triplicate assays; individual values did not vary more than 10%.

supernatant fractions were assayed for ^{125}I in a Nuclear Chicago gamma spectrometer. In preliminary experiments, ^{125}I-labeled casein was titrated in this assay with each of the subcellular fractions. In all cases, the rate of ^{125}I-labeled casein hydrolysis was dependent on the substrate concentration and 75 μg ^{125}I-labeled casein/assay (300 μg casein/ml) provided an excess of substrate such that less than 5% of the total cpm were released during the course of the assay. Total releasable cpm in the presence of excess trypsin (1 mg/ml) was approx. 85%. The characteristics of this assay in the presence of 50 μg rat thymocyte plasma membrane protein are illustrated in Fig. 1. Digestion of ^{125}I-labeled casein was linear from 0—3.5 h. Based on these experiments, a standard 3 h assay was performed with 75 μg ^{125}I-labeled casein in order to quantitate neutral protease activity in the various subcellular fractions. To calculate the rate of proteolysis, spontaneous background release (soluble cpm in the absence of added proteases) was subtracted from the experimental values and the rate was calculated according to Eqn. 1:

$$\frac{\text{experimental cpm} - \text{control cpm}}{\text{time of assay (min)} \times \text{mg protein}} = \text{cpm released/min per mg protein} \quad (1)$$

All assays were performed in triplicate and individual values did not vary by more than 10%. All experiments were performed at least three times with similar results.

In order to determine plasminogen activator activity in the subcellular fractions, the caseinolytic assay described above was performed in the presence of plasminogen. This represented a modification of the plasminogen activator assays described by Remert and Cohen [24] and Johnson et al. [25]. Any plasminogen-dependent increase in ^{125}I-labeled casein hydrolysis was taken as a measure of plasminogen activation. Plasminogen controls were included in all assays. The background activity in controls was subtracted from the experimental values in order to calculate the rate of ^{125}I-labeled casein hydrolysis in the presence of plasminogen (as in Eqn. 1). To calculate net plasminogen activator activity, the rate of ^{125}I-labeled casein hydrolysis observed in the absence of plasminogen (neutral protease activity) was subtracted from the rate observed in the presence of plasminogen. Thus, both protease and plasminogen controls were subtracted from the reported values for plasminogen activator. The activation of plasminogen by thymocyte plasma membrane fractions was linear after a short lag period (Fig. 1) and a standard 3 h assay in the presence of excess plasminogen (20 μg/assay, Fig. 2A) was employed for the quantitation of plasminogen activator in the subcellular fractions of thymocytes. All assays were performed in triplicate and individual values did not vary by more than 5%. All experiments were performed at least three times with similar results.

Cleavage of ^{125}I-labeled plasminogen by rat thymocyte plasma membrane or by urokinase was performed by incubation of 15 μg ^{125}I-labeled plasminogen with 75 μg plasma membrane protein, 0.1 Plough units urokinase or buffer (control) for 3 or 6 h at 37°C in 0.25 ml Hepes-buffered saline containing 0.1% Nonidet P40. At the end of the incubation period the reaction was stopped by boiling for 2 min in the presence of 2.0% SDS. Each sample was divided into two aliquots, 20 μl of 2-mercaptoethanol were added to one aliquot and all

samples were boiled for an additional 2 min. Samples were subsequently analyzed by SDS-polyacrylamide gel electrophoresis on 9% polyacrylamide gels. The gels were cut into fractions and each fraction was analyzed for ^{125}I in a Nuclear Chicago gamma spectrometer.

Labeling with [^3H]DFP. The isolated subcellular fractions were labeled with [^3H]DFP by incubating each fraction (50—200 μg protein) in 0.25 ml Hepes-buffered saline with 10 μCi (10 μM) [^3H]DFP for 60 min at 37°C. The labeled fractions were solubilized in 1.0% SDS (2 min, 100°C), dialyzed and concentrated by vacuum dialysis prior to electrophoretic analysis.

Lentil lectin affinity chromatography. Lectin from *Lens culinaris* was affinity purified from lentil beans and coupled to CNBr-activated Sepharose 4B at 2 mg protein/ml Sepharose as described by Hayman and Crumpton [26]. Cell lysates or Nonidet P40-solubilized membranes were subjected to affinity chromatography on lentil lectin-Sepharose columns equilibrated in Hepes-buffered saline containing 0.1% Nonidet P40. The non-adherent fraction was washed through the column with the same buffer and saved. The adherent fraction was eluted with 0.25 M α-methylmannoside in the starting buffer. The adherent and non-adherent fractions were dialyzed and concentrated by vacuum dialysis as described above.

Polyacrylamide gel electrophoresis. Polyacrylamide disc gel electrophoresis in the presence of SDS was performed as described by Shapiro et al. [27]. Dansylated, cross-linked immunoglobulin light chains were used as internal molecular weight standards. These were prepared as follows: a human lambda-type Bence-Jones protein was reduced and alkylated with 0.22 M dithiothreotol and 0.5 M iodoacetamide. The reduced and alkylated protein was dansylated using the method of Kinoshita et al. [28] and then cross-linked with dimethylsuberimidate dihydrochloride as described by Carpenter and Harrington [29]. When the dansylated, cross-linked light chain was subjected to SDS-polyacrylamide gel electrophoresis in the presence or absence of 2-mercaptoethanol, fluorescent bands of M_r 23 000, 46 000, 69 000, 92 000, 115 000 and 138 000, corresponding to monomers to hexamers, respectively, of the light chain, were detected. The standards were visualized with an ultraviolet lamp and their positions were marked by injecting a small amount of india ink at the position of each fluorescent band prior to slicing the gels. Alternatively, a mouse IgM myeloma protein (HP 76), internally labeled with ^3H-labeled amino acids (μ chain = 80 000, λ chain = 23 000), was employed as an external molecular weight standard. The gels were sliced into 1.5 mm fractions using a Savant Autogel Divider and the fractions were analyzed for ^3H by liquid scintillation in 5.0 ml Beckman Redisolv-EP in a Beckman LS350 spectrometer or ^{125}I in a Nuclear Chicago gamma spectrometer.

Isoelectric focusing in polyacrylamide gels. Isoelectric focusing in polyacrylamide gels was performed essentially as described by O'Farrell [30] except that urea was omitted from all solutions and 15% sucrose (w/v) was added as a mild stabilizing agent. The gels were prefocused as described in Ref. 30. Equal quantities of [^3H]DFP-labeled and unlabeled plasma membrane were mixed, solubilized in the Nonidet P40-containing sample buffer and 250 μg total membrane protein were focused to equilibrium at 400 V for 13 h followed by 1 h at 600 V in 6 mm × 11 cm gels. The gels were sliced into 50 fractions using a

Savant Autogel Divider and each fraction was assayed for pH using a Radiometer pH meter. The fractions (0.5 ml each) were neutralized by addition of 50 μl of 1.0 M sodium phosphate, pH 7.2. One-half of each fraction was counted for ^{3}H and the other half was assayed for either protease or plasminogen activator activity as described above except that assays were incubated for 0—16 h at 37°C, due to the low levels of enzyme present in each fraction. Hydrolysis of ^{125}I-labeled casein in the presence or absence of plasminogen was linear for up to 16 h at 37°C, and results are reported as cpm released/h.

Results

Isolation and characterization of plasma membrane

Thymocytes were disrupted by N_2 cavitation (425 lb/inch^2, 15 min, 4°C) and plasma membrane was isolated as described in Materials and Methods; the subcellular fractions (plasma membrane, endoplasmic reticulum, large granules, nuclei and cytosol) were characterized by marker enzyme analysis. The subcellular distribution of marker enzymes obtained from hamster thymocytes is shown in Table I. Approx. 45% of the plasma membrane marker enzyme alkaline phosphatase was recovered from the sucrose gradient at the 20—30% interface (bouyant density = 1.12 g/ml). This fraction contained approx. 2% of the homogenate protein, yielding a 19-fold purification of the plasma membrane marker enzyme. The membrane band at the 30—40% interface (bouyant density = 1.18 g/ml) contained significant activities of plasma membrane, endoplasmic reticulum, lysosomal and mitochondrial marker enzymes. Similar fractionation of rat thymocytes (Table II) yielded separation of the marker enzymes similar to that obtained with hamster thymocytes. The plasma membrane fraction from rat thymocytes contained less contamination by endoplasmic reticulum and lysosomal marker enzymes (1—2% of total). The major difference between the results from the two species was at the level of the large granule fraction. Latent β-glucuronidase activity, indicating intact lysosomes, could be demonstrated with the large granule fraction of rat thymocytes but not with the large granule fraction of hamster thymocytes. This result may indicate that the hamster lysosomes were damaged during cell disruption or fractionation.

Plasma membrane localization of thymocyte plasminogen activator

When neutral protease and plasminogen activator activities of the subcellular fractions from both hamster and rat thymocytes were determined (Tables III and IV), plasminogen activator activity was found to be distributed in a manner analogous to the plasma membrane marker enzyme alkaline phosphatase. The plasma membrane fraction contained approx. 50% of the total homogenate plasminogen activator activity (purification factor = 20—25); however, this fraction contained only 5% of the total neutral protease activity. Thus, the thymocyte plasminogen activator fractionated as a plasma membrane-localized enzyme. Thymocyte plasma membrane fractions contained plasminogen activator activity equivalent to approx. 1.3 Plough units urokinase/mg protein (rat) and 1.7 Plough units urokinase/mg protein (hamsters). In contrast, neutral protease activity was enriched in the large granule fraction; however, this fraction contained only 15% of the total plasminogen activator activity.

TABLE I

MARKER ENZYME ANALYSIS OF SUBCELLULAR FRACTIONS OF HAMSTER THYMOCYTES

Total activity represents values for $1 \cdot 10^9$ cells: nmol *p*-nitrophenol released/min for alkaline phosphatase and β-glucuronidase; nmol NADH oxidized/min for NADH: lipoamide oxidoreductase; and nmol $K_3Fe(CN)_6$ reduced/min for succinate dehydrogenase. Specific activity is expressed as nmol/min per mg protein. Reported values represent average determinations of three experiments. Values did not vary by more than 15%.

Fraction	Protein (mg)	Alkaline phosphatase			NADH: lipoamide oxidoreductase			Succinate dehydrogenase			β-Glucuronidase		
		Specific activity	Total activity	Percent	Specific activity	Total activity	Percent	Specific activity	Total activity	Percent	Specific activity	Total activity	Percent
Homogenate	17.2	17.5	300.5	100	69.8	1201	100	30.2	519.3	100	16.7	287.3	100
Nuclei	7.2	4.6	32.8	10.9	12.8	92.1	7.7	18.8	135.4	26.1	15.6	112.4	39.1
Cytoplasm	5.5	3.6	20.0	6.6	0	0	0	0	0	0	9.4	52.0	18.1
Large granules	1.65	23.0	37.9	12.6	273.3	391.5	32.6	170.0	281.0	54.1	32.2	53.1	18.5
Endoplasmic reticulum	0.46	110.2	50.7	16.9	855.0	393.3	32.7	175.8	80.8	15.6	98.5	45.3	15.8
Plasma membrane	0.40	336.2	134.5	44.8	163.2	65.3	5.4	0	0	0	36.2	14.5	5.0
Recovery	15.21		275.9	91.8		942.2	78.4		497.2	95.7		277.3	96.5

TABLE II

MARKER ENZYME ANALYSIS OF SUBCELLULAR FRACTIONS OF RAT THYMOCYTES

Total activity represents values for $1 \cdot 10^9$ cells: nmol *p*-nitrophenol released/min for alkaline phosphate and β-glucuronidase; nmol NADH oxidized/min for NADH: lipoamide oxidoreductase; and nmol $K_3Fe(CN)_6$ reduced/min for succinate dehydrogenase. Specific activity is expressed as nmol/min per mg protein. Reported values represent average determinations of two experiments. Values did not vary by more than 10%.

Fraction	Protein (mg)	Alkaline phosphatase			NADH: lipoamide oxidoreductase			Succinate dehydrogenase			β-Glucuronidase		
		Specific activity	Total activity	Percent	Specific activity	Total activity	Percent	Specific activity	Total activity	Percent	Specific activity	Total activity	Percent
Homogenate	16.6	12.8	212.8	100	149.8	2487	100	51.6	856.5	100	13.9	230.1	100
Nuclei	7.8	2.9	22.4	10.5	35.4	276.3	11.1	20.9	163.0	19.0	10.4	80.9	35.2
Cytoplasm	5.5	0	0	0	4.9	27.1	1.1	0	0	0	4.1	22.7	9.9
Large granules	1.27	24.1	30.6	14.4	870.9	1106	44.5	417.6	530.4	61.9	69.5	88.3	38.4
Endoplasmic reticulum	0.37	82.7	30.6	14.4	2086	771.8	31.0	283.2	104.8	12.2	34.6	12.8	5.6
Plasma membrane	0.30	332.0	99.6	46.8	133.0	39.9	1.6	0	0	0	9.0	2.7	1.2
Recovery	15.24		183.2	86.1		2221	89.3		798.2	93.2		207.4	90.1

TABLE III

SUBCELLULAR DISTRIBUTION OF HAMSTER THYMOCYTE PROTEASES AND PLASMINOGEN ACTIVATOR

Neutral protease activity was determined with 10—50 μg of sample protein and 75 μg ^{125}I-labeled casein in 0.25 ml Hepes-buffered saline containing 0.1% Nonidet P40 for 3 h at 37°C. Total activity represents cpm/min per 10^9 thymocytes. Values represent average determinants of two experiments. Plasminogen activator activity was determined under the same conditions as neutral protease activity, except that 20 μg porcine plasmnogen were present in the assay.

Fraction	Protein (mg)	Protease activity			Plasminogen activator		
		Specific activity	Total activity	Percent	Specific activity	Total activity	Percent
Homogenate *	10.1	101.2	1022	100	43.5	439.0	100
Cytosol	5.4	33.9	183.2	17.9	3.0	16.1	3.7
Large Granules	1.8	393.9	709.0	69.4	35.6	64.1	14.6
Endoplasmic reticulum	0.46	301.9	138.9	13.6	190.2	87.5	19.9
Plasma membrane	0.40	134.6	53.8	5.3	542.2	216.9	49.4
Total	8.06 (79.8%)		1085	106.2		384.6	87.6

* Homogenate after removal of nuclear pellet at 1800 X *g* for 10 min at 4°C.

Inhibition of plasma membrane proteases and plasminogen activator

In a previous report, it was demonstrated that the cell surface protease activities of hamster and rat thymocytes were largely serine proteases and the sensitivity of these proteases to a variety of inhibitors was determined [14]. Therefore, the inhibitor sensitivity of the plasma membrane-associated proteases was examined in order to determine whether similar proteases were present at the

TABLE IV

SUBCELLULAR DISTRIBUTION OF RAT THYMOCYTE PROTEASES AND PLASMINOGEN ACTIVATOR

Neutral protease activity was determined with 10—50 μg of sample protein and 75 μg ^{125}I-labeled casein in 0.25 ml Hepes-buffered saline containing 0.1% Nonidet P40 for 3 h at 37°C. Total activity represents cpm/min per 10^9 thymocytes. Values represent average determinations of two experiments. Plasminogen activator activity was determined under the same conditions as neutral protease activity, except that 20 μg porcine plasminogen were present in the assay.

Fraction	Protein (mg)	Protease activity			Plasminogen activator		
		Specific activity	Total activity	Percent	Specific activity	Total activity	Percent
Homogenate *	9.0	79.2	715.3	100	33.6	302.4	100
Cytosol	5.6	29.2	162.3	22.6	2.7	14.9	4.9
Large granules	1.3	353.4	459.4	64.2	27.0	35.1	11.6
Endoplasmic reticulum	0.39	262.0	102.2	14.3	152.8	59.6	19.7
Plasma membrane	0.30	140.7	42.2	5.9	463.7	139.1	46.0
Total	7.59 (84.5%)		766.1	107.1		248.7	82.2

* Homogenate after removal of nuclear pellet at 1800 X *g* for 10 min at 4°C.

TABLE V

INHIBITION OF PLASMA MEMBRANE PROTEASES

Protease activity was determined with 40 μg plasma membrane protein and 75 μg ^{125}I-labeled casein for 3 h at 37°C. Inhibitors were added 30 min prior to the addition of substrate. All inhibitors were titrated over the effective dose range to determine the concentration resulting in 50% inhibition (ID_{50}) and the concentration resulting in maximum inhibition. n.d., not done. SBTI, soybean trypsin inhibitor.

Inhibotor	ID_{50} (μM)	Maximum inhibition of protease		
		Concentration * (μM)	Percent inhibition activity	
			Hamster	Rat
DFP	40	100	96	98
PMSF	65	100	93	92
NPGB	25	60	n.d.	98
TPCK	—	100	18	9
SBTI	2.7	3.6	76	79
Antipain	210	250	62	67
$ZnCl_2$	30	100	93	92

* Concentration which resulted in maximum inhibition.

cell surface and in the isolated plasma membrane. The plasma membrane-associated protease activity was completely inhibited by DFP, PMSF and NPGB (100 μM), indicating that these enzymes were serine proteases (Table V). Protease activity was also completely inhibited by $ZnCl_2$ (75—100 μM), and it was inhibited to a lesser extent by antipain and soybean trypsin inhibitor (100 μg/ml). These results are analogous to the inhibitor sensitivity which was observed for both hamster and rat thymocyte surface proteases [14]. In contrast, while the cell surface proteases detected on viable thymocytes were inhibited approx. 30% by TPCK, the plasma membrane-associated protease activity was relatively insensitive to inhibition by TPCK (Table V). Since a TPCK-sensitive protease(s) was enriched in the large granule fraction (data not shown), it is likely that this protease was detected in viable cell cultures due to release of intracellular contents from damaged cells. It is also likely that the presence of the TPCK-sensitive protease(s) in the plasma membrane fractions is indicative of lysosomal contamination, since the level of TPCK-sensitive protease activity roughly correlates with the level of β-glucuronidase activity detected in the plasma membrane fractions of hamster and rat thymocytes.

Inhibition of plasminogen activator activity in the plasma membrane fractions using the protocol described in Table V was relatively uninformative, since all of the compounds tested, except TPCK, also inhibit plasmin. Thus, it could only be stated with certainty that the plasminogen activator was not TPCK sensitive. While this information is valuable, it was of interest to perform experiments to determine whether the plasma membrane-associated plasminogen activator was a serine protease similar to other plasminogen activators which have been characterized [32—34]. Therefore, experiments were performed in which the plasma membrane fraction was preincubated with the covalent inhibitors DFP, PMSF, TPCK and NPGB and then washed twice by centrifugation at 100 000 × *g* to remove the excess inhibitor before protease and plasminogen activator activities were assayed. These experiments (Table

TABLE VI

EFFECT OF INHIBITOR PRETREATMENT AND REMOVAL ON PLASMA MEMBRANE-ASSOCIATED PROTEASE AND PLASMINOGEN ACTIVATOR ACTIVITIES

Rat thymocyte plasma membrane (200 μg protein per sample) was pretreated for 30 min at 37°C with the protease inhibitors indicated (500 μM) or with isopropanol for controls. The vesicles were washed twice by centrifugation at 100 000 × *g* for 10 min at room temperature and solubilized in 1.0% Nonidet P40 (10 min, 37°C). Protease and plasminogen activator activities were assayed with 30 μg plasma membrane protein and 75 μg ^{125}I-labeled casein in the presence or absence of 20 μg plasminogen for 3 h at 37°C in a total volume of 0.25 ml Hepes-buffered saline containing 0.5% Nonidet P40. Reported values represent the mean of triplicate assays. Values did not vary more than 10%.

Inhibitor	Percent of control	
	Protease	Plasminogen activator
DFP	12	2
PMSF	15	5
NPGB	4	0
TPCK	92	88

VI) demonstrated that the plasminogen activator was inhibited by DFP, PMSF and NPGB, and thus, represents a serine protease. These results are consistent with the inhibitor sensitivity of other plasminogen activators [32—34]. Inhibition by NPGB probably indicates that the plasminogen activator is a trypsin-like protease with specificity for arginine [37].

Separation of plasma membrane proteases and plasminogen activator by lentil lectin affinity chromatography

As shown by Hayman and Crumpton [26], approx. 85% of plasma membrane-associated carbohydrate is retained on lentil lectin-Sepharose columns and the adherent molecules can be specifically eluted with α-methylmannoside. Thus, lentil lectin affinity chromatography represents a useful method for

TABLE VII

LENTIL LECTIN SEPARATION OF RAT THYMOCYTE PLASMA MEMBRANE PROTEASES AND PLASMINOGEN ACTIVATOR

Rat thymocyte plasma membrane (1.4 mg protein) were solubilized in 1.0 ml of 1.0% Nonidet P40 in Hepes-buffered saline and fractionated on a 5.0 ml column of lentil lectin-Sepharose 4B. The adherent fraction was eluted with 0.25 M α-methylmannoside in Hepes-buffered saline containing 0.1% Nonidet P40. Neutral protease activity was determined with 10—50 μg of sample protein and 75 μg ^{125}I-labeled casein in 0.25 ml Hepes-buffered saline containing 0.1% Nonidet P40 for 3 h at 37°C. Total activity represents cpm/min. Specific activity is expressed as cpm/min per mg protein. Values represent average determinations of three experiments. Plasminogen activator activity was determined under the same conditions as neutral protease activity, except that 20 μg porcine plasminogen were present in the assay.

	Protein (mg)	Protease activity			Plasminogen activator		
		Specific activity	Total activity	%	Specific activity	Total activity	%
Plasma membrane	1.4	123.2	172.5	100	406.5	569.1	100
Lectin adherent	0.2	390.0	78.0	45.2	2201.0	440.2	77.4
Lectin non-adherent	1.1	58.3	64.1	37.2	92.5	101.8	17.9
Total recovery	1.3 (92.8%)		142.1	82.4		542.0	95.2

separating plasma membrane glycoproteins from non-glycosylated components. When the plasma membrane fraction was solubilized in 1.0% Nonidet P40 and subjected to affinity chromatography on columns of lentil lectin-Sepharose, an adherent (glycoprotein) and a non-adherent fraction were obtained. As shown in Table VII, approximately equal quantities of protease activity were present in both fractions; however, essentially all of the plasminogen activator (81% of recovered activity) was present in the glycoprotein fraction resulting in an approx. 5-fold purification of the plasminogen activator over the unfractionated plasma membrane activity (6.2 Plough units/mg protein). These data indicate that the plasminogen activator represents a plasma membrane-associated glycoprotein. Since the majority of plasma membrane-associated carbohydrate resides on the external surface of the plasma membrane [31], these data are consistent with the interpretation that the plasminogen activator represents a cell surface protease on thymocytes.

The experiments described above were performed with an impure preparation or porcine plasminogen which could have contained contaminating proteases or zymogens other than plasminogen. While these experiments demonstrated that the plasma membrane fraction of thymocytes contained a unique proteolytic activity capable of generating increased caseinolytic activity from the crude plasminogen preparation, it was not directly demonstrated that this increased caseinolytic activity was due to the proteolytic conversion of plasminogen to plasmin. Therefore, homologous rat plasminogen was isolated from fresh, citrated, rat plasma by affinity chromatography on lysine-Sepharose as described in Materials and Methods. When this pure preparation of rat plasminogen was employed in the caseinolytic assay with rat thymocyte plasma membrane fractions (Fig. 1B), plasminogen activation was observed which was similar to that observed with porcine plasminogen. A lag phase of approx. 1 h was followed by a linear increase in casein hydrolysis. Also shown in Fig. 1B is the activation of rat plasminogen by urokinase. It can be seen that following the lag phase observed with the plasma membrane fractions, 75 μg plasma membrane protein displayed approximately the same rate of plasminogen activation as 0.1 Plough units of urokinase. This increased casein hydrolysis was directly dependent on the concentration of rat plasminogen added to the caseinolytic assay (Fig. 2B). These experiments demonstrate unequivocally that the plasma membrane fraction of rat thymocytes is capable of plasminogen activation.

In order to determine the mechanism of plasminogen activation by thymocyte plasma membrane, ^{125}I-labeled rat plasminogen was incubated with either rat plasma membrane or urokinase for 0—6 h at 37°C, and the ^{125}I-labeled plasminogen was subsequently analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 4, incubation of ^{125}I-labeled plasminogen with thymocyte plasma membrane resulted in the proteolytic cleavage of the plasminogen peptide chain into fragments which were virtually identical to those obtained when ^{125}I-labeled plasminogen was incubated with urokinase. The major cleavage products of ^{125}I-labeled plasminogen (M_r 92 000) were polypeptide chains of M_r 70 000 (plasmin heavy chain) and M_r 22 000 (plasmin light chain). These results are consistent with the reported values for the two polypeptide chains of plasmin [34]. The cleavage products of ^{125}I-labeled plasminogen obtained

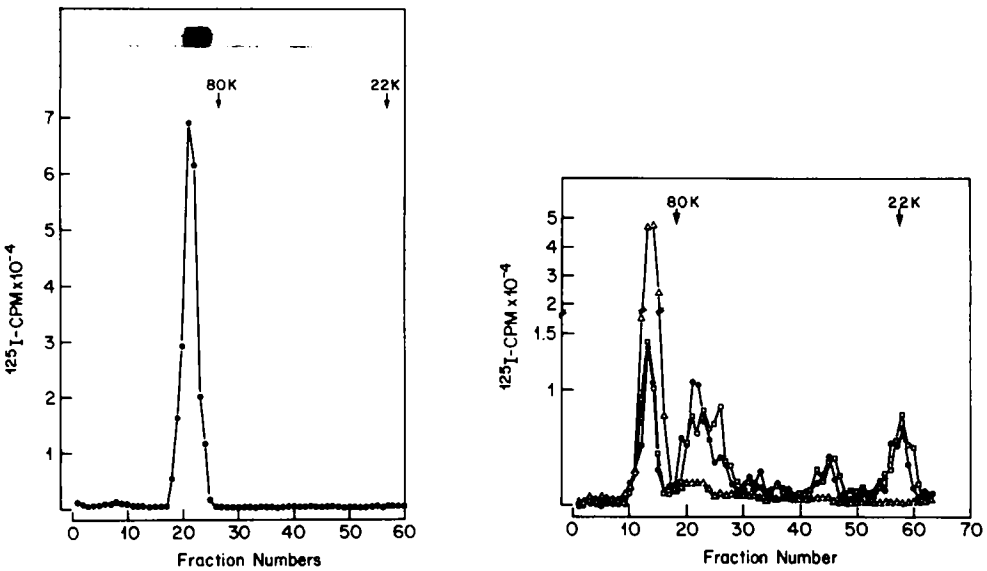


Fig. 3. SDS-polyacrylamide gel electrophoresis of rat plasminogen. Affinity purified rat plasminogen was iodinated with Na^{125}I as described in Materials and Methods. Unlabeled plasminogen (50 μg protein) and ^{125}I-labeled plasminogen (7.5 μg protein) were electrophoresed on 9% polyacrylamide gels containing 0.1% SDS in the absence of 2-mercaptoethanol as described in Materials and Methods. The gel containing unlabeled plasminogen was stained with Coomassie brilliant blue (0.025%) in 45% ethanol and 7% acetic acid, destained in the same solution without dye, and stored in 7% acetic acid with 2% glycerol. The gel containing ^{125}I-labeled plasminogen was sliced into 60 (2 mm) fractions and each fraction was analyzed for ^{125}I as described in Materials and Methods. External marker proteins were the heavy and light chain of reduced HP 76 (IgM myeloma protein). K, kdaltons.

Fig. 4. Proteolytic cleavage of rat plasminogen by thymocyte plasma membrane and urokinase. Rat plasminogen was affinity purified and iodinated with Na^{125}I as described in Materials and Methods. ^{125}I-labeled plasminogen (15 μg) was incubated with buffer only (△), 75 μg rat thymocyte plasma membrane (□), or 0.1 Plough units urokinase (●) for 6 h at 37°C in a total volume of 0.25 ml Hepes-buffered saline/Nonidet P40. Samples were prepared for electrophoresis as described in Materials and Methods, and separated on 9% polyacrylamide gels containing 0.1% SDS. Samples shown were reduced with 20 μl 2-mercaptoethanol (2 min, 100°C). Gels were sliced into 2-mm fractions and counted for ^{125}I as described in Materials and Methods. External marker proteins were reduced HP76 (IgM myeloma protein). K, kdaltons.

with both plasma membrane-associated plasminogen activator and urokinase were linked by disulfide bonds, since only one peak (M_r 92 000) was observed in the absence of 2-mercaptoethanol (data not shown, but identical to those of Fig. 3). These results are consistent with the reported properties of plasmin heavy and light chains [34]. A minor cleavage product (M_r 32 500) was detected in both the plasma membrane and urokinase samples which was also present only after reduction with 2-mercaptoethanol. This product may be the result of plasmin action, since these experiments were performed in the absence of Trasylol, a plasmin inhibitor [34]. The experiment depicted in Fig. 1 further illustrates the equivalent plasminogen activator activity of 75 μg rat thymocyte plasma membrane and 0.1 Plough units of urokinase. Based on the reduction of ^{125}I cpm remaining as intact plasminogen, approx. 5.0 μg of ^{125}I-labeled plasminogen were cleaved to plasmin during the 6 h incubation with either plasma

membrane-associated plasminogen activator or urokinase. At 3 h and 37°C, approx. 2.0 μg ^{125}I-labeled plasminogen were cleaved, indicating the linear nature of this cleavage. These experiments demonstrated that the plasminogen activator of thymocytes activated plasminogen by proteolytic cleavage of the plasminogen molecule in a manner analogous to the activation of plasminogen by urokinase [33,34].

[^{3}H]DFP labeling and SDS-polyacrylamide gel electrophoresis of plasma membrane-associated proteases

Since the plasma membrane-associated protease activity was inhibited by DFP (Table V), covalent modification of the enzymes with [^{3}H]DFP could provide a useful radioactive marker for further biochemical characterization of the proteases. When the isolated plasma membrane fractions from rat thymocytes were labeled with [^{3}H]DFP and separated by SDS-polyacrylamide gel electrophoresis, the plasma membrane was found to contain one major ^{3}H-labeled component with an apparent molecular weight of 105 000 which represented approx. 65% of recovered cpm (Fig. 5). The mobility of this component was unaltered in the presence of 2-mercaptoethanol. Similar results were obtained with hamster thymocyte plasma membrane (data not shown). In contrast, when the unfractionated cellular homogenate was analyzed in this manner, at least ten electrophoretically distinct ^{3}H-labeled proteins (M_r 18 500—105 000) were detected [35]. The M_r 105 000 plasma membrane-associated esterase represented a minor component of the cellular homogenate (3% of incorporated cpm) and this component was enriched in the plasma membrane fraction approx. 20-fold on a cpm/μg protein basis (0.75 cpm/μg homogenate protein compared to 16.5 cpm/μg plasma membrane protein). The other ^{3}H-labeled components which were present in the cell homogenate were found in the other subcellular fractions and were not inactivated during plasma membrane isolation [35]. Since both the M_r 105 000 ^{3}H-labeled protein and the plasminogen activator activity were enriched in the plasma membrane fraction approx. 20-fold, it seemed likely that this esterase represented the plasma membrane associated-plasminogen activator.

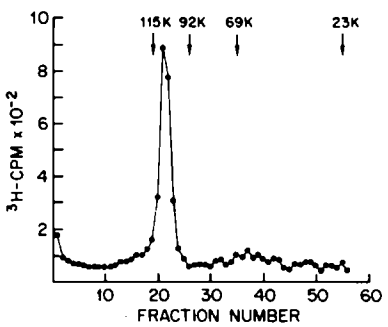


FIg. 5. SDS-polyacrylamide gel electrophoresis of [^{3}H]DFP-labeled thymocyte plasma membrane. Rat thymocyte plasma membrane (150 μg protein) in 0.25 ml Hepes-buffered saline was incubated with 10 μCi [^{3}H]DFP for 60 min at 37°C. Labeled plasma membrane was solubilized in 1.0% SDS, dialyzed and concentrated, and electrophoresed on 5% polyacrylamide gels for 15 h at 5 mA per gel as described in Materials and Methods. K, kdaltons.

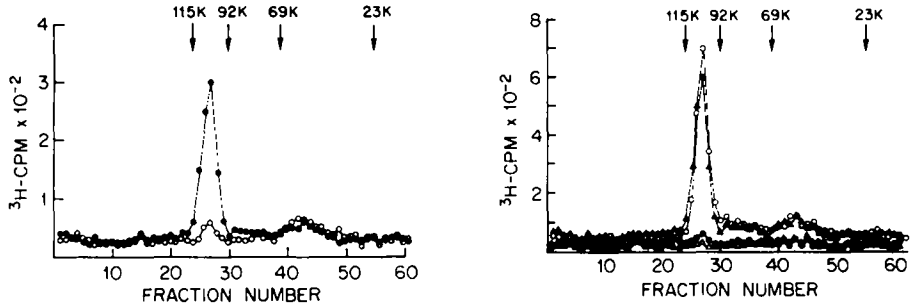


Fig. 6. SDS-polyacrylamide gel electrophoresis of lentil lectin fractions of [^{3}H]DFP-labeled thymocyte plasma membrane. Rat thymocyte plasma membrane was labeled with 10 μCi [^{3}H]DFP for 60 min at 37°C, solubilized in 1.0% Nonidet P40 and subjected to affinity chromatography on lentil lectin-Sepharose. The adherent (●) and non-adherent (○) fractions were dialyzed and concentrated, made to 1.0% in SDS and electrophoresed on 5% polyacrylamide gels as described in Fig. 1. K, daltons.

Fig. 7. Inhibition of [^{3}H]DFP incorporation into plasma membrane-associated esterase. Thymocyte plasma membrane (150 μg/sample) was preincubated for 30 min at 37°C in isopropanol (○), 100 μM NPGB (●), 100 μM PMSF (△), or 100 μM TPCK (▲); 10 μCi [^{3}H]DFP were added and the samples were incubated for 60 min at 37°C. Samples were solubilized in 1.0% SDS, dialyzed and concentrated, and electrophoresed on 5% polyacrylamide gels as described in Fig. 5. K, kdaltons.

Lentil lectin chromatography of [^{3}H]DFP-labeled plasma membrane

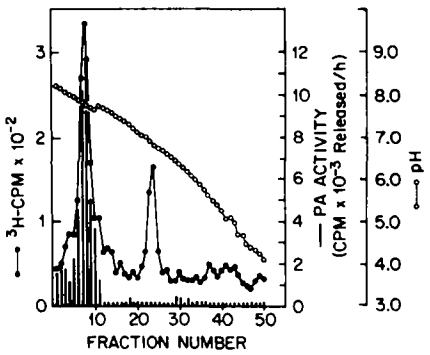
Since the plasma membrane-associated plasminogen activator was found to be adherent to lentil lectin, [^{3}H]DFP-labeled plasma membrane was subjected to lectin chromatography in order to determine whether the M_r 105 000 plasma membrane esterase was also glycosylated. As shown in Fig. 6, the ^{3}H-labeled protein of M_r 105 000 segregated into the lectin-adherent fraction, indicating that the plasma membrane-associated esterase, as well as the plasma membrane-associated plasminogen activator (Table VII), was a glycoprotein.

Inhibition of [^{3}H]DFP incorporation into the plasma membrane-associated serine esterase

In order to test further the correlation of the plasma membrane-associated plasminogen activator with the plasma membrane-associated serine esterase, plasma membranes were incubated in the presence of protease inhibitors prior to labeling with [^{3}H]DFP and analysis by SDS-polyacrylamide gel electrophoresis. These experiments demonstrated that incorporation of [^{3}H]DFP into the M_r 105 000 esterase was blocked by the same compounds which inhibited the plasminogen activator activity. As seen in Fig. 7, [^{3}H]DFP incorporation was inhibited by unlabeled PMSF and NPGB, compounds which inhibited the plasminogen activator activity (Table VI), and was not inhibited by TPCK, which did not inhibit the plasminogen activator activity. Incorporation of [^{3}H]-DFP was also inhibited by unlabeled DFP (data not shown). These results are consistent with the conclusion that the M_r 105 000 plasma membrane-associated esterase represents the plasma membrane-associated plasminogen activator.

Isoelectric focusing of plasma membrane-associated proteases

Preliminary experiments indicated that plasminogen activator activity could



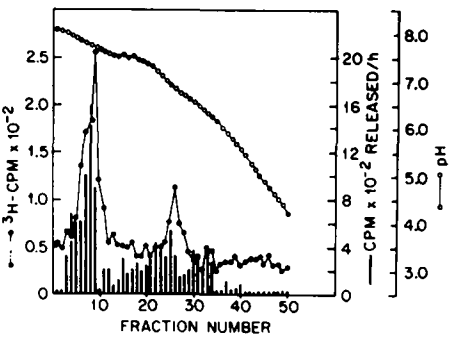


Fig. 8. Isoelectric focusing of plasma membrane-associated plasminogen activator. Rat thymocyte plasma membrane was labeled with 10 μCi [^{3}H]DFP as described. Unlabeled plasma membrane (125 μg protein) and [^{3}H]DFP-labeled plasma membrane (125 μg protein) were mixed, solubilized in 1.0% Nonidet P40 and subjected to isoelectric focusing as described in Materials and Methods. The gel was cut into fractions and one-half of each fraction was counted for ^{3}H and the other half was assayed for plasminogen activator (PA) activity as described in Materials and Methods.

Fig. 9. Isoelectric focusing of plasma membrane-associated proteases. Rat thymocyte plasma membrane was labeled with 10 μCi [^{3}H]DFP as described in Materials and Methods. Unlabeled plasma membrane (125 μg protein) and [^{3}H]DFP-labeled plasma membrane (125 μg protein) were mixed, solubilized in 1.0% Nonidet P40 and subjected to isoelectric focusing as described in Materials and Methods. The gel was cut into fractions and one-half of each fraction counted for ^{3}H ([^{3}H]DFP) and the other half was assayed for neutral protease activity as described in Materials and Methods.

not be recovered after exposure of plasma membrane fractions to SDS, therefore, it was not possible to elute plasminogen activator activity after SDS-polyacrylamide gel electrophoresis and to determine whether the protease(s) of M_r 105 000 contributed the plasminogen activator activity. Therefore, [^{3}H]DFP-labeled and unlabeled plasma membrane fractions were subjected to isoelectric focusing under conditions in which the plasminogen activator retains enzymatic activity. As seen in Figs. 8 and 9, the [^{3}H]DFP-labeled plasma membrane yielded two major ^{3}H-labeled components with isoelectric points of 7.75 and 6.85.

The major component, of which the p*I* ranged from 7.7 to 7.9, contained 60—65% of recovered cpm and thus, must represent the M_r 105 000 component detected by SDS-polyacrylamide gel electrophoresis (Fig. 5). Furthermore, SDS-polyacrylamide gel electrophoresis analysis of ^{3}H-labeled protein eluted from the isoelectric focusing gel at p*I* 7.7—7.9 demonstrated that the only detectable ^{3}H-labeled protein had an apparent M_r of 105 000 (data not shown). The minor component (p*I* = 6.8—7.0) could represent a more acidic component of the M_r 105 000 material or it could represent lower M_r ^{3}H-labeled component(s) (M_r 45 000—65 000) which are present in the plasma membrane fraction (see Figs. 6 and 7). The latter explanation seems most plausible since the components of p*I* 6.85 and M_r 45 000—65 000 both represent 15—20% of recovered cpm. Insufficient cpm were recovered from this area of the gel to permit an estimation of the apparent M_r of these molecules by SDS-polyacrylamide gel electrophoresis. The plasminogen activator activity of each fraction is also shown in Fig. 8. It can be seen that plasminogen activator activity co-eluted with the major ^{3}H-labeled component (p*I* 7.75) and was not

detected in other regions of the gel. Thus, the M_r 105 000 serine esterase detected by SDS-polyacrylamide gel electrophoresis must represent the plasma membrane-associated plasminogen activator. Detectable plasminogen activator activity was not associated with the minor component (p*I* 6.85); however, neutral protease activity was detected in this region of the gel (Fig. 9). Fig. 9 also illustrates that the region of the gel containing the plasminogen activator (p*I* 7.7—7.9) contains approx. 50% of the recovered neutral protease activity. These results were to be expected from the lentil-lectin separation experiments (Table VII) which indicated that a portion of the plasma membrane-associated protease activity (the non-adherent fraction) did not correlate with plasminogen activator activity, indicating the presence of at least two distinct proteases in the plasma membrane fraction. Thus, the relationship of these isoelectric forms to each other is not absolutely clear. The acidic component may represent an altered form of the plasminogen activator with little or no plasminogen activator activity or it could represent a distinct serine protease.

Discussion

The purpose of this study was to characterize the protease activities associated with the thymocyte plasma membrane in order to compare these activities to the cell surface proteases which were previously detected on viable cells [14]. The plasma membrane-associated proteases were shown to be serine proteases by inhibition with DFP and PMSF. Furthermore, these proteases appear to be trypsin-like since inhibition was also achieved with soybean trypsin inhibitor, antipain and NPGB; however, a trypsin-specific chloromethyl ketone, TLCK, was not inhibitory. This study has also demonstrated that the thymocyte cell surface plasminogen activator which was previously detected on viable cells is localized in the plasma membrane fraction of thymocytes. The plasma membrane-associated plasminogen activator activity segregated into the glycoprotein fraction of thymocyte plasma membrane, demonstrating that the plasminogen activator is a glycoprotein. These observations suggest that the plasma membrane-associated plasminogen activator resides on the external surface of the membrane.

Labeling of the isolated plasma membrane fraction with [^{3}H]DFP and analysis by SDS-polyacrylamide gel electrophoresis demonstrated that a single major serine esterase of M_r 105 000 was present in the plasma membrane fraction of both hamster and rat thymocytes. Several lines of evidence indicate that this component represents the plasma membrane-associated plasminogen activator: (1) both the plasminogen activator and the M_r 105 000 esterase were enriched in the plasma membrane fraction approx. 20-fold, (2) both the plasminogen activator and the M_r 105 000 esterase were shown to be glycoproteins, (3) inhibition of [^{3}H]DFP incorporation into the M_r 105 000 esterase was achieved with protease inhibitors which inhibited the plasminogen activator activity, but not with an inhibitor which did not inhibit the plasminogen activator activity, and (4) [^{3}H]DFP-labeled plasma membrane esterases and plasminogen activator activity co-elute in isoelectric focusing gels with an isoelectric point of 7.7—7.9. From these data, we conclude that the thymocyte plasminogen activator, which was previously detected on viable cells is a glycosylated serine protease

which is selectively localized in the plasma membrane of thymocytes. Labeling with [^{3}H]DFP indicates that the plasminogen activator has an apparent M_r of 105 000. The plasma membrane-associated esterases can be separated into two isoelectric forms of p*I* (7.75 and 6.85); however, only the former bears detectable plasminogen activator activity.

The plasminogen activator which is present in the thymocyte surface membrane appears to be biochemically distinct from the plasminogen activator which is secreted by macrophages [36] and various tissue culture cell lines [37], since the major species of plasminogen activator secreted by these cells have an apparent M_r of 48 000—50 000. Jaken and Black [38] have recently reported that SV40 transformed 3T3 mouse fibroblasts contain a membrane-associated plasminogen activator of M_r 105 000. The high molecular weight plasminogen activator of 3T3 cells was present in all of the subcellular membrane fractions; however, since the plasminogen activator assay employed by these authors was not characterized for linearity with time and enzyme concentration, enrichment of the plasminogen activator into a specific membrane fraction could not be determined. Nevertheless, the latter study may indicate that the M_r 105 000 plasma membrane-associated plasminogen activator described here may not be restricted to thymocytes.

The finding that the plasma membrane-associated plasminogen activator of thymocytes is both functionally and biochemically distinct from intracellular proteases suggests that this protease may perform functions which are unique to the surface membrane. Proposed functions of plasma membrane-localized proteases include regulation of membrane-bound enzyme systems [7,8], negative feed-back on extracellular peptide signals [39], control of cell surface 'shedding' [6] and regulation of cell proliferation [9—13]. These functions could be mediated by direct cleavage of membrane proteins or extracellular ligands by membrane-associated proteases or through interaction of the membrane proteases with soluble zymogens, such as plasminogen, resulting in an increase in soluble protease activity in the local environment of the membrane. Either mechanism could result in the proteolytic cleavage of cell surface and extracellular proteins which could positively or negatively affect the activity of plasma membrane enzyme systems, transport processes and ligand interactions. It is clear that plasma membrane-localized proteases are present on many cell types and it is also clear that these enzymes have the potential to perform important regulatory functions. Further characterization of these proteases will hopefully lead to a better understanding of their role in the physiology of the cell surface.

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