Fibronectin as a carrier for the transglutaminase from human erythrocytes

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ABSTRACT Non denaturing electrophoresis was used to demonstrate that, immediately upon exposure to plasma, the transglutaminase (protein-glutamine:amine γ-glutamyltransferase, EC 2.3.2.13) from erythrocytes undergoes a significant shift in mobility. The plasma effect shows saturable characteristics and depends entirely on the presence of fibronectin in plasma, indicative of complex formation between this protein and transglutaminase. The results suggest a specific carrier function for fibronectin that might be of physiological importance in determining the fate of a tissue transglutaminase accidentally discharged into plasma.

Normally, there is negligible transglutaminase (protein-glutamine:amine γ-glutamyltransferase, EC 2.3.2.13) activity in the fluid phase of blood. The thrombin-catalyzed conversion of fibrin-stabilizing factor (factor XIII) generating a similar transamidase activity (1), is confined to the area where clotting is required, and this enzyme (factor XIIIa) remains tightly bound to the clot matrix. However, there might be pathological situations in which a transglutaminase of tissue origin would become discharged into the plasma (2); since the presence of such a foreign enzyme in the circulation could be dangerous, we considered it important to examine in molecular terms the fate of a tissue transglutaminase in contact with plasma. The lysate of membrane-depleted human erythrocytes mixed with human plasma was chosen as a model of the hemolytic condition.

This paper describes a nondenaturing biochemical approach for studying the associations of erythrocyte transglutaminase with plasma components. It focuses on the observation that, upon mixing with plasma, the erythrocyte protein undergoes an appreciable, immediate shift in electrophoretic mobility that is entirely dependent on the presence of fibronectin in the plasma.

MATERIALS AND METHODS

Erythrocytes were obtained from just-outdated blood that was collected in CPDA-1 anticoagulant (Lifesource, Glenview, IL) and were washed as described (3). They were lysed by addition of 4 volumes of 10 mM Tris-HCl (Sigma)/1 mM EDTA (Sigma), pH 7.5, containing 12.5% of aprotinin (Trasyol; FBA Pharmaceuticals, New York) per ml. Ghosts were removed by centrifugation at 130,000 × g for 20 min at 4°C in a Beckman Ti 70 rotor.

Human plasma was obtained from whole blood collected in 3.8% sodium citrate, with a ratio of blood to anticoagulant of 9:1, and was separated by centrifugation at 1500 × g for 10 min at 4°C. It was stored at −70°C until needed.

Fibronectin was removed from plasma (4) by passing a 1.5-ml aliquot with 30 mM Tris-HCl/0.5 mM EDTA, pH 7.5, through a gelatin affinity column [0.9 × 4.2 cm, Affi-Gel 10, Bio-Rad (5 mg of gelatin per ml)] at 22°C. The volume of the fibronectin-depleted plasma was reduced to that of the original plasma with the aid of a Centricron 10 (Amicon) filter.

Erythrocyte transglutaminase was kindly prepared by J. Colaluca by using a modification of a published procedure (5). The product was dissolved in 50 mM Tris-HCl/1 mM EDTA, pH 7.5, and was stored at −70°C.

Reaction mixtures were prepared on ice or at room temperature just prior to (≤10 min) electrophoretic analysis. Human plasma dilutions were made with 150 mM NaCl for the experiments in Fig. 1 and with 50 mM Tris-HCl/0.5 mM EDTA, pH 7.5, for those in Fig. 2.

Nondenaturing electrophoresis was performed in gels made from 1% (wt/vol) agarose (Seakem LE; FMC, Rockland, ME) in 75 mM imidazole (Sigma)/1 mM EDTA, pH 7.5. For activity staining (6), 0.3% N,N′-dimethylcasein (7) was also included. Fifteen milliliters of the mixture were poured on a glass plate (10 × 10 cm) with the well former (1 × 6 mm) positioned 3 cm from the edge of the plate. Samples (10 µl) were applied and electrophoresed at 4 V/cm and 4°C until the free bromophenol blue tracking dye (Sigma) migrated to within 1.5 cm of the edge of the gel.

Transglutaminase activity was detected by staining with dansylcysderavine (Sigma; converted to the fumarate salt by K. N. Parameswaran) essentially as described by Lorand et al. (6). The gels were immersed for 3 hr at 37°C in 50 mM Tris-HCl, pH 7.5/10 mM dithiothreitol (Sigma)/10 mM CaC2/1 mM dansylcysderavine. For detection of fluorescence, unbound dansylcysderavine was removed by washing with several changes of 20% (vol/vol) methanol and 10% (vol/vol) acetic acid. The destained gels were photographed under UV light (UVL-56, Blak-Ray, long wave UV-366 nm, Ultraviolet Products, San Gabriel, CA) by using a Vivitar VMC deep-yellow filter no. 15(G).

For immunoblotting, proteins were transferred from agarose gels to nitrocellulose (BA83, 0.2-µm pore size, Schleicher & Schuell) either with the use of an LKB Transphor electroblotting unit (Bromma, Sweden) as in Fig. 1 or simply by placing the gel above the nitrocellulose on a porous polyethylene sheet (Fisher Scientific, Chicago) and applying a uniform pressure of 30 g/cm² for 3–6 hr as in Fig. 2. Electrophoretic transfers were performed (8) with 25 mM Tris/192 mM glycine (Sigma)/20% methanol, pH 8.3, over a period of 2 hr at 0.6 A and 4°C.

Nonfat dry milk (Carnation, Los Angeles) at concentrations of 3% (wt/vol) in 10 mM Tris-HCl/150 mM NaCl, pH 8.0 (as used in Fig. 1) or of 1% in 25 mM Tris-HCl/200 mM NaCl, pH 7.5 (as used in Fig. 2) was used for 30 min to block the free sites left on the nitrocellulose. Incubation with the primary antibodies was carried out overnight; then, excess IgG was removed by three 10-min washes in the corresponding buffer without the milk product.

Rabbit antiserum against the human erythrocyte transglutaminase was prepared by Ole B. Bjerrum, and IgG was purified on a ZetaChrom 60 DEAE disk (AMF Laboratory

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We developed different modalities for probing the presence of transglutaminase in human erythrocyte lysates after non-denaturing electrophoresis. In Fig. 1A, positions of transglutaminase-related antigens were identified by immunoblotting with a rabbit IgG raised against the purified human erythrocyte transglutaminase itself. By contrast, the experiments presented in Fig. 1B and C reflect on the enzymatic potential of transglutaminase in human erythrocyte lysates after non-denaturing electrophoresis. In Fig. 1A, positions of transglutaminase-related antigens were identified by immunoblotting with a rabbit IgG raised against the purified human erythrocyte transglutaminase itself. By contrast, the experiments presented in Fig. 1B and C reflect on the enzymatic potential of transglutaminase in human erythrocyte lysates after non-denaturing electrophoresis. In Fig. 1B, positions of transglutaminase-related antigens were identified by immunoblotting with a rabbit IgG raised against the purified human erythrocyte transglutaminase itself. By contrast, the experiments presented in Fig. 1B and C reflect on the enzymatic potential of transglutaminase in human erythrocyte lysates after non-denaturing electrophoresis. In Fig. 1B and C, positions of transglutaminase-related antigens were identified by immunoblotting with a rabbit IgG raised against the purified human erythrocyte transglutaminase itself. By contrast, the experiments presented in Fig. 1B and C reflect on the enzymatic potential of transglutaminase in human erythrocyte lysates after non-denaturing electrophoresis.

RESULTS AND DISCUSSION

mobility occurred instantaneously within the experimental time frame. Moreover, absence of transglutaminase in the area between the two discrete electrophoretic zones marked I and II could be taken as a sign of tight complex formation. The findings presented in relation to membrane-depleted lysate could be reproduced with purified erythrocyte transglutaminase. The isolated enzyme, which at pH 7.5 migrated rapidly as a doublet in the anodic direction (Fig. 2, lane 1), underwent an instantaneous reduction in electrophoretic mobility to about 50% with admixture of plasma (Fig. 2, lane 3). The important breakthrough in the search for the transglutaminase carrier in plasma came with the observation that selective removal of fibronectin by a gelatin affinity column (4) caused a complete loss of the ability of plasma to influence the electrophoretic behavior of transglutaminase (Fig. 2, lane 2). Subsequent experiments (data not shown) in binary mixtures of the purified enzyme and isolated plasma fibropectin confirmed the conclusion that, indeed, the transglutaminase-binding property of plasma resided with the fibronectin molecule. Even proteolytic fragments derived from fibronectin could interfere with the ability of plasma to influence the electrophoretic behavior of transglutaminase. This result was confirmed by the finding that addition of several protease inhibitors, including P-1600, aprotinin, and leupeptin, to the plasma samples prior to electrophoresis significantly reduced the ability of plasma to influence the electrophoretic behavior of transglutaminase (data not shown).}

Fig. 1. Change in the mobility of the transglutaminase of membrane-depleted erythrocyte lysate upon mixing with plasma. Nondenaturing electrophoresis was carried out at pH 7.5 in 1% sodium phosphate, pH 6.3/150 mM NaCl/30 mM NaOAc. Mouse monoclonal IgG against dansyl (9) was a gift of Fred Karush (University of Pennsylvania) and was purified from ascites fluid by using the monoclonal antibody purification system (MAPS; Bio-Rad) according to the manufacturer's instructions. Rabbit antiserum against guinea pig liver transglutaminase was kindly prepared by Pauline T. Velasco and stored after the addition of 15 mM NaN3, pH 4.0. Goat anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate (Sigma) and peroxidase (Vector Laboratories, Burlingame, CA) conjugates and sheep anti-mouse IgG (whole molecule) alkaline phosphatase conjugate (Sigma) were used as secondary antibodies. The nitrocellulose sheets were incubated with the secondary antibodies (1:2000 dilution) in the milk powder-containing buffer solutions for 2 hr. This was followed by three washes (each 10 min) in buffer. The color resulting from alkaline phosphatase action was developed for the experiment in Fig. 1 A by immersion in 0.37 mM 5-bromo-4-chloro-3-indolyl phosphate (Sigma)/0.39 mM nitro blue tetrazolium (Sigma)/100 mM Tris-HCl/100 mM NaCl/5 mM MgCl2, pH 9.5. Color development was stopped by placing the sheets in 0.002% 4-chloro-1-naphthol (Sigma; dissolved in methanol)/0.015% H2O2/25 mM Tris-HCl/200 mM NaCl, pH 7.5/17% methanol. Color development was stopped by placing the sheets in distilled water.
Furthermore, there is no evidence that factor XIII occurs in a fibronectin-complexed form in plasma; in fact, this protein is thought to circulate in combination with fibrinogen (12). The binding of erythrocyte transglutaminase to fibronectin, as presented in this paper, does not require the unmasking of its catalytic site by Ca\textsuperscript{2+} ions.

It is interesting that complexation could be readily demonstrated between human fibronectin and a transglutaminase from as diverse an origin as guinea pig liver. Thus, from the pathophysiological point of view, fibronectin may play a significant role in chaperoning tissue transglutaminases through the fluid phase of blood. It remains to be seen whether forming a complex with fibronectin might down-regulate some activities of transglutaminases or might hasten in any way their removal from plasma.

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