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Fibronectin as a carrier for the transglutaminase from human erythrocytes

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ABSTRACT **Nondenaturing 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Erythrocytes were obtained from just-outdated blood that was collected in CPDA-1 anticoagulant (Lifesource, Glenview, IL) and were washed as described (3).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 units of aprotinin (Trasylol; 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 50 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 Centricon 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 dansylcadaverine (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 $CaCl_2$/1 mM dansylcadaverine. For detection of fluorescence, unbound dansylcadaverine 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 J. Bjerrum, and IgG was purified on a ZetaChrom 60 DEAE disk (AMF Laboratory

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Products, Meriden, CT) by following the manufacturer's instructions. It was taken up in the same volume of 15 mM sodium phosphate, pH 6.3/150 mM NaCl/30 mM NaN_3 as the antiserum from which it was derived and was stored at 4°C. 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 NaN_3 at 4°C.

Goat anti-rabbit IgG (whole molecule) alkaline phosphatase (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 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 $MgCl_2$, pH 9.5. Color due to peroxidase action was developed for the experiment in Fig. 2 in 0.002% 4-chloro-1-naphthol (Sigma; dissolved in methanol)/0.015% H_2O_2/25 mM Tris·HCl/200 mM NaCl, pH 7.5/17% methanol. Color development was stopped by placing the sheets in distilled water.

RESULTS AND DISCUSSION

We developed different modalities for probing the presence of transglutaminase in human erythrocyte lysates after nondenaturing electrophoresis. In Fig. 1*A*, 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. 1 *B* and *C* reflect on the enzymatic potential of transglutaminase by utilizing the Ca^{2+}-specific incorporation of dansylcadaverine into a casein substrate. Fig. 1*B* shows the fluorescent activity bands as seen directly on the gels in UV light after destaining in acidic methanol (6). Fig. 1*C* represents a method for detecting the dansylcadaverine-modified casein product by blotting with an antibody to the dansyl residue after electrophoretic transfer (8) to nitrocellulose. Inasmuch as free dansylcadaverine is not transferred by this procedure, prior destaining of the gel could be omitted. The only qualitative difference between the approaches shown in Fig. 1*B* and Fig. 1*C* is the presence of fluorescence near the origins in lanes 1–6 in Fig. 1*B* for which there are no immunodetectable counterparts in Fig. 1*C*. As of now, we have no explanation for the difference; the fluorescence near the origins in Fig. 1*B* might be due to noncovalently bound dansylcadaverine, or, if covalently bound, the material from these regions of the gel did not become transferred to the nitrocellulose sheet.

However, the experiments in Fig. 1 provide unambiguous evidence that erythrocyte transglutaminase, upon mixing with plasma, undergoes a major shift in its electrophoretic migration properties. In fact, the anodic mobility (pH 7.5) of the erythrocyte protein was reduced approximately to half of its value in the presence of plasma. As best seen in Fig. 1*C*, when the relative amount of plasma to erythrocyte lysate was increased, the fast-moving free transglutaminase species (marked at position I) was gradually replaced by the slower migrating one (marked at position II), suggesting that saturable complexation between transglutaminase and some plasma constituent might have taken place. This idea was reinforced by the observation that the shift in electrophoretic

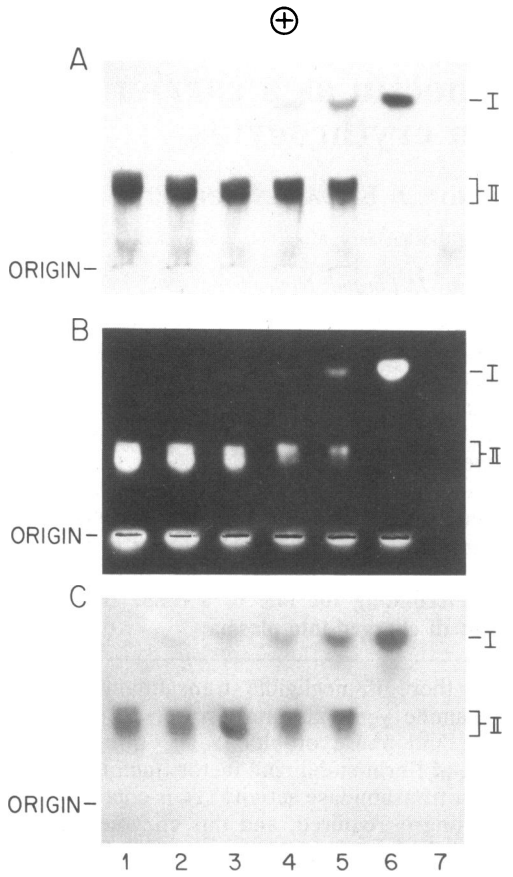

FIG. 1. Change in the mobility of the transglutaminase of membrane-depleted erythrocyte lysate upon mixing with plasma. Nondenaturing electrophoresis was carried out at pH 7.5 in 1% agarose alone (*A*) or with inclusion of 0.3% *N,N'*-dimethylcasein (*B* and *C*). Positions for transglutaminase were probed by immunoblotting with anti-human erythrocyte transglutaminase antibody (1:500 dilution) as in *A*; by Ca^{2+}-dependent, enzyme-specific fluorescence activity staining with dansylcadaverine (6) as in *B*; and by immunoblotting the dansylcadaverine-stained gels with a monoclonal antibody (1:1000 dilution) to the dansyl moiety as in *C*. Electrophoretic transfers (8) were to nitrocellulose. Reaction mixtures (40 μl) contained fixed amounts of the erythrocyte lysate (20 μl of 20% packed-cell equivalent) and plasma of various percentages (approximately 11% for lane 1, 9% for lane 2, 7% for lane 3, 5% for lane 4, and 4% for lane 5). Each lane corresponds to a 10-μl aliquot of the mixture. Lane 6 represents a control of the lysate alone, and lane 7 is the control for the highest percentage of plasma (11%).

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 fibronectin confirmed the conclusion that, indeed, the transglutaminase-binding property of plasma resided with the fibronectin molecule. Even proteolytic fragments derived

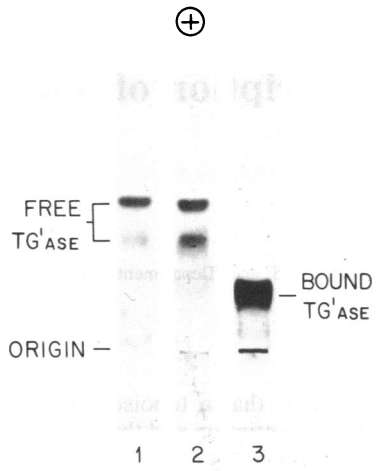

FIG. 2. Fibronectin-depleted plasma, in contrast to normal plasma, does not affect the mobility of transglutaminase (TG'ase). By using agarose as support for nondenaturing electrophoresis, human erythrocyte transglutaminase (5 pmol) was tested alone (lane 1), after mixing with fibronectin-depleted plasma (lane 2), or with normal plasma (lane 3), each corresponding to 80% of the plasma equivalent. Nitrocellulose transblots were incubated with rabbit antiserum (1:2000) to guinea pig liver transglutaminase and were labeled with peroxidase-conjugated anti-rabbit IgG as the second antibody.

from fibronectin could be shown to bind transglutaminase. Thus, the possibility is now open to define more precisely the complementary binding domains of the two partners in forming the fibronectin–transglutaminase complex.

It is important to draw a distinction between our findings and literature reports, often illustrated on linear models, that point to the factor XIIIa-reactive γ-glutaminyl sites in fibronectin as transglutaminase-binding domains (e.g., ref. 10). Confusion arises also from referring to factor XIIIa by the generic name: transglutaminase. Though tissue transglutaminase and factor XIIIa may function by similar catalytic mechanisms (see ref. 11), they are quite different proteins. 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^{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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