DNase I-DEPENDENT DISSOCIATION OF ERYTHROCYTE CYTOSKELETONS

MICHAEL P. SHEETZ. From the Physiology Department, University of Connecticut Health Center, Farmington, Connecticut 06032

ABSTRACT

The human erythrocyte contains a complex of peripheral membrane proteins which forms an extensive network or cytoskeleton on the cytoplasmic membrane surface. When I treat erythrocyte cytoskeletons with deoxyribonuclease I (DNase I), the cytoskeletons dissociate and erythrocyte actin is solubilized. The dissociation of the cytoskeletons by DNase I parallels the disruption of actin filaments in vitro by DNase I and is blocked by the addition of actin to the DNase I. Large protein complexes remain after DNase I disrupts the cytoskeletons, but these complexes are no longer visible in the light microscope nor sedimentable and are selectively depleted with respect to actin. From these studies, I suggest that DNase I binds to and solubilizes actin, which serves as a structural link between protein complexes in the erythrocyte cytoskeleton.

KEY WORDS DNase I • actin • erythrocyte membrane • cytoskeleton

Extraction of erythrocytes (12, 13) and erythrocyte membranes (19) by nonionic detergents yields structures which contain most of the erythrocyte membrane spectrin and actin. Most lipids and integral membrane proteins are solubilized by this treatment. We designated the detergent-insoluble structure as the "erythrocyte cytoskeleton" (12), since it is morphologically similar to the intact cell in certain cases (6). In addition, the major components of the cytoskeletal complex, spectrin and actin, are involved in membrane shape changes (14, 1, 2) and hereditary shape abnormalities (7, 15). It has been suggested that actin plays a central role in the ATP-dependent, erythrocyte shape changes either in complex with an unidentified myosin (13, 4, 11) or as a polymer whose polymerization-depolymerization is coupled to membrane shape (1, 2). It is not known, however, whether erythrocyte actin exists in vivo in a filamentous (8, 9), profilamentous (18), or other form and, further, what proteins are bound to actin. Because many aspects of the organization of actin and associated proteins are likely to be affected by changes in salt concentration but not by the nonionic detergent Triton (17), we have studied the organization of actin in Triton cytoskeletons prepared directly from intact cells in a medium which approximates the intracellular ionic environment (12, 13). The removal of the lipid barrier by Triton allows the addition of proteins which will bind to actin and alter its organizational state (e.g., DNase I). There is a strong association between globular actin and DNase I which prevents actin polymerization, and DNase I will also bind to and depolymerize filamentous actin (5). Pinder et al. (9) used DNase I to block polymerization of erythrocyte actin. I find, however, that DNase I is also capable of solubilizing erythrocyte actin.

MATERIALS AND METHODS

Fresh human blood was centrifuged (1,500 g for 10 min) and the supernate and buffy coat were aspirated. Where noted, cells were suspended in an N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES)-Ringer solution, incubated with 2 mM diisopropylfluorophosphate (DFP) for 30 min at 37°C and, after DFP treatment, cells were pelleted and resuspended with Iso Tris (140 mM NaCl, 24 mM Tris, pH 7.4) twice. A 15% suspension of red cells in Iso Tris was mixed with an equal volume of 4% Triton X-100 in 140 mM KCl, 24 mM HEPES, 1.0 mM MgCl₂, 0.5 mM EGTA, 0.05 mM CaCl₂, 0.5 mM dithiothreitol (DTT) (pH 7.0) at 4°C. The mixture was applied to a linear sucrose gradient.)
(10–66% sucrose) in the extraction buffer without Triton and centrifuged at 100,000 g for 1 h. The light-scattering band at a density of ~1.27 g/ml was isolated, diluted (1:10) in phosphate-buffered saline (PBS) (145 mM NaCl, 5 mM PO₄, pH 7.4) with 0.5 mM Mg, 0.5 mM EGTA, 0.05 mM CaCl₂, and 0.2 mM DTT, and centrifuged at 35,000 g for 10 min. The pellet was resuspended in the PBS dilution buffer at a concentration of ~2 mg/ml. DNase I (Sigma Chemical Co., St. Louis, Mo., grade DN-C1) was dissolved in Iso Tris with 0.5 mM MgCl₂, 0.5 mM EGTA, 0.05 mM CaCl₂, 0.2 mM DTT, and 2 mM a-phenylmethyl sulfonyl fluoride (PMSF) was added from a stock solution in ethanol. The DNase I sample was then dialysed against two changes of 200 vol of the Iso Tris buffer (final activity, 2,640 Kunitz U/mg protein). Actin was prepared by the method of Spudich and Watt (16). SDS-polyacrylamide gel electrophoresis was performed according to the procedure of Fairbanks et al. (3).

RESULTS AND DISCUSSION

Washed human erythrocytes were extracted with Triton in isotonic media by a modification of the procedure of Yu et al. (19). Cytoskeletons were separated from detergent, cytoplasm, and solubilized lipid by sedimentation into a linear sucrose density gradient in an isotonic salt solution where they banded at a density of 1.27 g/cm³. SDS polyacrylamide gel analyses of the cytoskeletons indicated that they contained spectrin (60%), actin (component 5 of Fairbanks et al. [3]) (10%), component 3 (14%), component 4 (8%), and several minor components (13). By dark-field illumination (Fig. 1), the cytoskeletons appeared as discrete polymorphic structures of 2–3 μm in diameter. When samples were incubated with DNase I at 37°C for 40 min, these structures were not observed. Moreover, after dissociation by DNase I, the cytoskeletal structures were no longer pelletable by centrifugation at 35,000 g for 20 min which indicated that the size of the complex was significantly decreased. If the DNase I was first inactivated by the addition of an equimolar amount of g-actin (globular) or by dialysis against EDTA, then no dissociation occurred. Because proteases are a common contaminant of DNase I preparations, DNase I samples were pretreated with 2 mM PMSF. Subsequent analysis of the DNase I samples for proteolytic activity showed that PMSF treatment had inactivated the proteases (10). Dissociation of cytoskeleton structures required that DNase I was capable of binding to actin and was independent of proteases in the DNase I preparations.

FIGURE 1 These dark-field light micrographs are of cytoskeletons prepared from intact erythrocytes (a) before, (b) after incubation for 40 min at 37°C with 0.125 mg of DNase I/mg cytoskeleton protein, and (c) after the same incubation with a DNase I-actin complex (0.19 mg of actin and 0.125 mg of DNase I per mg of cytoskeletal protein). x 750.

DNase I treatment released actin from the cytoskeleton. After DNase I treatment, the mixture of cytoskeletons and DNase I was chromatographed on a 4% agarose column (Bio Rad A-15M, Bio-Rad Laboratories, Richmond, Calif.). The majority of cytoskeletal components were in the void volume fraction (i.e., >15 million daltons), whereas actin and DNase I were in the included volume fraction (Fig. 2). From densitometer scans of the Coomassie Blue-stained SDS gels, we estimated that the content of actin in the void volume fraction was only 40–60% of that in the original cytoskeletal material, with the remainder in the included volume. Other minor components were lost after DNase I treatment and work is continuing to determine whether they are normally linked to actin. But actin alone of the major proteins was solubilized from the protein complex by DNase I (Fig. 2a and b). Although the solubilization of actin destroyed shell structure, spectrin, components 3 and 4, and the remaining actin were in a multimolecular complex and only small amounts of components 3 and 4 and spectrin were...
Figure 2 The SDS polyacrylamide gels show the Coomassie Blue-staining pattern of (a) the cytoskeletons incubated without DNase I, the material in the (b) excluded, and (c) included volume fractions from a 4% agarose column run in Isotris, 0.2 mM DTT and 1.0 mM EGTA, and 0.1 mM CaCl₂ of cytoskeletons and DNase I after 50 min at 37°C (0.2 mg DNase I/mg of shell protein). Of 580 µg of shell protein applied, 400 µg was recovered in the excluded volume fraction. Column fractions were concentrated for gel electrophoresis by precipitation with 7.5% TCA. The DNase I enzyme alone was applied to gel d.

found in included volume fractions. Analysis of dissociated cytoskeletons by negative-stain electron microscopy (1% uranyl acetate, pH 4.5) and by velocity sedimentation in a model E ultracentrifuge revealed that large complexes containing fibrous elements remained after DNase I treatment. When cytoskeletons are dissociated by phosphatase and protease action, spectrin is selectively solubilized from the cytoskeleton and actin is found in an oligomeric fraction which pellets at 35,000 g for 20 min and contains 50- to 70-Å diameter filaments (13). Thus, under conditions where actin normally would form filaments or associate with the other shell components, DNase I will solubilize the actin.

The characteristics of the DNase I interaction with the cytoskeletons are similar to those of the DNase I-filamentous actin interaction. The molar ratio of DNase I to actin is 1:1 in their complex (5). At DNase I to actin ratios of <1, a longer incubation time was required to cause dissociation of cytoskeletal structures as observed in the light microscope, which is consistent with the possibility that DNase I binds directly to actin. DNase I binds only weakly to filamentous actin with an association constant of 10⁸-10⁹ M (5). Likewise, at DNase I concentrations below 10⁻⁶ M, the incubation time for cytoskeleton dissociation increased dramatically (Fig. 3). The time required for DNase I to disrupt actin filaments was also on the order of 15 min, which can be explained as a direct effect of DNase I on actin filament stability (5). Although the dissociation of erythrocyte cytoskeletons by DNase I parallels the disruption of actin filaments by DNase I, another form of actin may be complexed with other components in the cytoskeletons which modify its interaction with DNase I.

If erythrocyte membranes are incubated with DNase I under similar conditions, no release of actin is detected. Accessibility of DNase I to the cytoplasmic face of the membranes was assured by lysis of the cells in DNase I solutions in 10 mM Tris (pH 7.4) and by the addition of DNase I in 10 mM Tris (pH 7.4) to unsealed ghosts. Hitchcock et al. (5) have observed that myosin completely and tropomyosin partially inhibit DNase I.
binding to actin filaments. Similarly, in the erythrocyte membrane the actin may be complexed with other components which prevent DNase I from binding to and solubilizing it. Detergent extraction may remove the components which are bound to actin or otherwise activate enzymes which cause the release of actin-binding proteins.

We have evidence that both phosphatases and proteases are present in the cytoskeleton preparations (12, 13). If red cells are preincubated with DFP before detergent extraction to inhibit cytoskeleton proteases, then longer incubation times with DNase I are required to cause dissociation. After the longer incubations, however, even DFP-treated samples show evidence of proteolysis in the SDS gels (Fig. 4). But it is important to note that shell morphology did not change during the incubation. About 40% of the actin was removed by DNase I from another portion of the same sample during an identical incubation and there was no evidence of additional proteolysis caused by the DNase I preparation. The addition of 0.1 mM $N\alpha$-$p$-tosyl-$\epsilon$-lysine chloromethyl ketone HCl (TLCK) and 0.1 mM $\epsilon$-tosylamide-2-phenyl-ethylchloromethyl ketone (TPCK) to cytoplasm prevented dissociation by DNase I except when the cytoskeletons were preincubated at 37°C without protease inhibitors. Preincubation in the absence of DNase I and protease inhibitors for 30 min in this case enabled DNase I to dissociate the cytoskeletons even in the presence of TLCK and TPCK. With shorter preincubations which produced less proteolysis, DNase I caused less cytoskeleton disruption. Other serine esteras in addition to proteases are inactivated by DFP, and such enzymes may also aid in exposing actin to DNase I. But the characteristic changes in the polypeptide pattern noted above are always observed when DNase I is effective. If cytoskeletons are prepared without proteolytic inhibitors and incubated for 3–5 h at 37°C, then cytoskeleton structure is lost and significant amounts of spectrin are solubilized whereas actin remains in a pelletable fraction. Thus, although proteolysis will not release actin from the cytoskeleton, proteolytic cleavage of a certain cytoskeletal component(s) appears to be a prerequisite for actin extraction and cytoskeleton disruption by DNase I.

On the basis of these findings, I suggest that erythrocyte actin provides a structural bridge between macromolecular complexes of other cytoskeletal components. Further, the removal of the actin-binding proteins from the actin by proteolysis enables DNase I to bind to the actin and, possibly, proteolysis also changes the organization of actin. DNase I can then solubilize the structural actin, thereby removing the links between the macromolecular complexes and dissociating the cytoskeleton.

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