Centrifugation Shearing Exposes Filamentous Networks in Cortical Regions of Crane-Fly Spermatocytes

ARThUR R. STRAUCH and JAMES R. LAFOUNTAIN, JR.
Division of Cell and Molecular Biology, State University of New York at Buffalo, Buffalo, New York 14260

ABSTRACT Spermatocytes of the crane-fly, Nephrotoma suturalis, were attached to electron microscope grids and then sheared by applying centrifugal force. Transmission electron microscopy of exposed regions of the cell cortex revealed networks containing arrays of filamentous structures. Networks were present in sheared spermatocytes at all stages of meiosis. The networks of dividing spermatocytes (meta- through telophase) were denser and appeared to contain more aggregated material than networks of prophase cells. The appearance of networks in spermatocytes resembled actin-containing networks of sheared and detergent-extracted human erythrocytes. Networks treated with myosin subfragment 1 under conditions in which muscle F-actin was clearly decorated were not distinguishable from those of untreated cells. Exposure to deoxyribonuclease-I caused the disruption of networks in sheared spermatocytes as well as in erythrocytes. The results of deoxyribonuclease experiments are interpreted as an indication that actin is a component of the cell cortex in crane-fly spermatocytes.

Our biochemical studies (34) have shown that actin comprises ~1% of the total protein in crane-fly spermatocytes and that ~80% of the actin in these cells is sedimentable. Those results indicated that spermatocyte actin is in a polymerized or aggregated state in vivo. Our two-dimensional peptide maps of several types of actin revealed close homologies between actin purified from spermatocyte homogenates and human erythrocyte actin. In contrast, fewer homologies were observed between spermatocyte actin and either skeletal muscle actin or actin isolated from a cytosolic extract of Dictyostelium discoideum. The latter results raised the possibility that the properties of spermatocyte actin might be more closely related to erythrocyte actin than either muscle or Dictyostelium actin.

Erythrocyte actin is one of the major components of the red cell cortex (3, 19, 20, 36). Although a consensus on the supramolecular state of erythrocyte actin has not yet been reached, recent evidence suggests that erythrocyte actin may be organized in situ in the form of short oligomers (3, 5). Those results provide one explanation for why actin filaments have been difficult to identify in thin sections of erythrocytes (36, 37).

The native supramolecular and distribution of actin in crane-fly spermatocytes also has been difficult to characterize in thin-sections. While Forer and Behnke (10) have shown that actin filaments and filament bundles can be visualized in the cortex of thin-sectioned cells after glycine and treatment with heavy meromyosin (glycerol/HMM), actin filaments were not identified in untreated cells (10). This discrepancy has raised the possibility that the glycerol/HMM treatments may have induced the polymerization of actin from a nonfilamentous state. Forer and Behnke (9, 10) themselves have acknowledged that the glycerol/HMM technique could be a source of artefacts. Cells extracted with glycerol are subject to severe shrinkage that could cause the relocation of actin-containing structures (11). In addition, either glycerol (16) or HMM (35, 39) can polymerize G-actin into free, recognizable filaments.

In view of the potentially complicating influences of glycerol and HMM treatments, we have approached questions regarding the organization and distribution of spermatocyte actin in situ using alternative methods. We have avoided use of glycerol and have used only physiological buffers, which have been used by ourselves and others (2) in studies on chromosome movement in living spermatocytes. Monolayers of spermatocytes attached to poly-L-lysine-coated grids were sheared using centrifugal force. Regions of the spermatocyte cortex that became exposed during shearing contained filamentous networks that resemble filamentous networks found in the cortex of human erythrocytes. Spermatocyte networks were sensitive to deoxyribonuclease-I (DNase-I) which has been used previously by Sheetz (30) to remove actin from erythrocyte membrane cytoskeletons. We interpret these results as an indication that the spermatocyte cortical networks revealed by centrifugation shearing contain actin.
Preparation of Sheared-Cell Monolayers

The procedure we have used to prepare monolayers of sheared cells is illustrated in Fig. 1. Formvar coated (0.4%) 200-mesh copper finder grids (Maxtaform, H-2, Ernest Fullam, Inc., Schenectady, NY) were used to prepare monolayers of spermatocytes and erythrocytes. Grids were coated with poly-L-lysine (type VII-B, Sigma Chemical Co., St. Louis, MO, 1 mg/ml in 7 mM sodium acetate, pH 4.8) for 15 min and then rinsed in five drops (~200 μl) of distilled water followed by five drops of Tricine buffer (2). Tricine buffer consisted of 70 mM NaCl, 45 mM sucrose, 25 mM Tricine, 15 mM NaHCO₃, 8 mM KCl, 8 mM MgCl₂, 2 mM EGTA, 2 mM CaCl₂, pH 7.2. In Tricine-EGTA buffer, 10 mM EGTA was substituted for 6 mM CaCl₂. Grids were coated with poly-L-lysine immediately before use. To prepare spermatocyte monolayers, a coated grid first was placed in the chamber of a Plexiglas grid holder (23) that had been previously filled with Tricine buffer. Spermatocytes then were added to the chamber by rupturing one testis (~7 × 10⁸ cells) just beneath the surface of the buffer. Cells released into the buffer were dispersed by gentle agitation and then pelleted onto the grid surface by centrifuging the grid holder in a swinging bucket rotor at 650 g for 30 min at room temperature. In some experiments, centrifugation was interrupted after 15 min so that dividing cells attached to the finder grid could be located and their positions recorded. The position of selected dividing cells was recorded on Panatomic-X (Eastman Kodak, Rochester, NY) using a Zeiss Universal microscope equipped with a 16/0.40 Neofluar objective. After recording, the grid was returned to the grid holder and centrifugation was resumed.

After attachment of the cells to the grid, the grid holder was transferred to another centrifuge tube that contained a silicone rubber (3112 RTV, Dow Corning, Midland, MI) cushion cast at a 45° angle (see Fig. 1). Spermatocytes attached to the grid were sheared by centrifuging the grid holder at 650 g for 30 min at room temperature in a swinging bucket rotor. After shearing, monolayers either were fixed and processed for electron microscopy or were transferred to one of the extraction solutions described below.

Erythrocytes were attached to grids and sheared in one step by centrifuging dilute suspensions of cells in 5 mM sodium phosphate, pH 8.0 containing 2% Triton X-100, rinsed twice with ice-cold 5 mM sodium phosphate, pH 8.0, and then either processed for electron microscopy or treated with DNase-I solutions as described below.

Transmission Electron Microscopy

Sheared-cell monolayers were fixed in a 4% solution of glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2 for 1 h at room temperature. Fixed preparations were either rinsed directly in 0.1 M sodium cacodylate, pH 7.2, and then processed as described below, or stored in fixative overnight at 4°C with rinsing and dehydration done the next day. In contrast to the results of Lehrer (17), results obtained after overnight exposure to cacodylate-buffered glutaraldehyde were indistinguishable from those obtained after only 1 h of fixation. Preparations of fixed cells were dehydrated in a graded ethanol series, and dried from liquid carbon dioxide in a Samdri 780 critical point drying apparatus (Tousimis Research Corp., Rockville, MD). Dried preparations immediately were rotary shadowed with platinum-palladium at an angle of 75° in a Varian VE-10 vacuum evaporator and examined in a Hitachi H500L transmission electron microscope using a 50-nm objective aperture.

Extraction of Sheared-Cell Monolayers

Deoxyribonuclease I (type 2006D, Worthington Biochemical Corp., Freehold, NJ) and ovalbumin (type V, Sigma Chemical Co. St. Louis, MO) were used without further purification and were dissolved in Tricine buffer immediately before use at a concentration of 1 mg/ml. The protease inhibitor, phenylmethyl sulfonylfluoride (PMSF, Sigma Chemical Co.) at 0.01 mM or 1 mM, was included in DNase-I solutions used in experiments on spermatocytes. Pronase E (protease type XIV, Sigma Chemical Co.) was autodigested at 10 mg/ml in Tricine buffer for 1 h at 37°C and then diluted to 1 mg/ml with Tricine buffer before use. Grids containing monolayers of sheared cells were incubated with 200–500 μl of one of the above protein solutions for 15 min at room temperature. Controls were incubated in Tricine buffer alone. After treatment, grids were washed briefly in 1–2 ml of Tricine buffer, fixed, and prepared for transmission electron microscopy as described above.

Preparation of Myosin Subfragment 1

Myosin subfragment 1 (S₁) was prepared by proteolytic digestion of chicken muscle myosin using papain (Sigma Chemical Co.) solubilized and activated in 0.2 M ammonium acetate, 10 mM cysteine, 4 mM EDTA, pH 7.2 (22). S₁ was
purified by ammonium sulfate fractionation and stored at −20°C in 50% glycerol, 10 mM imidazole, 1 mM EDTA, 1 mM dithiothreitol, pH 7.0. For use in experiments on sheared cells, a 1-ml aliquot of S₁ was dialyzed against Tricine buffer to remove glycerol and then adjusted to a concentration of 0.8–1.0 mg/ml. Sheared-cell monolayers were incubated with ~1 ml of S₁ at room temperature for 15 min. After washing in 1–2 ml of Tricine buffer, specimens were fixed and processed for electron microscopy as described above. F-actin prepared according to the procedure of Spudich and Watt (33) was used to test the actin-binding activity of S₁.

RESULTS

Monolayers of cells were prepared by pelleting cell suspensions onto electron microscope grids that had been coated with poly-L-lysine (Fig. 1). Centrifugation of grids with attached cells at an angle of 45° (shearing) caused most of the cytoplasm of many cells to be displaced in the direction of centrifugal force (Fig. 2). Some weakly attached cells were completely removed from the grid during shearing (Fig. 2). Shearing exposed the cortex of many attached cells (Fig. 3).

High magnification micrographs of exposed regions of the cortex revealed filamentous networks (Fig. 4). Some of the filaments in a network had numerous branching points. The arrangement of filaments in some networks was polarized or oriented in the direction of shear force (Fig. 4A). In contrast, other networks contained filaments that were overlapped or clumped (Fig. 4B). Long, thick filaments having a width of ~25–50 nm occasionally were seen in the exposed cortex (Fig. 4A). The straight trajectory and thickness of those filaments suggested that they perhaps could be microtubules.

Visualization of filamentous networks was possible only in those cells from which the plasma membrane was removed during shearing. An intact plasma membrane overlying a network prevented the coating of filaments with platinum-palladium and thus precluded resolution of the network. Treatment of sheared cells with Triton X-100 did not improve the visualization of filamentous networks (Fig. 4D). That result also demonstrated that network components were not lipid. Networks were completely extracted with protease (see below).

Since the Tricine buffer used to obtain the above results contained a relatively high concentration of calcium (6 mM), we investigated the possible effects of 6 mM calcium on filamentous networks. Schliwa and van Blerkom (28) have shown that 10 mM EGTA aids the preservation of cytoskeletal structures in epithelial and fibroblast cells. Cortical networks prepared in Tricine-EGTA (Fig. 4C) were indistinguishable from networks prepared in Tricine with 6 mM CaCl₂.

The exact stages of division of the cells depicted in Fig. 3B–D and Fig. 4 were not determined. They were assumed to be in meiotic prophase, since the testes used in making those preparations were small and contained few dividing spermatocytes or spermatids (see reference 34 for criteria used in selection of testes). To determine whether networks could be found in other stages of meiosis (metaphase, anaphase, telophase, and cytokinesis), spermatocytes from slightly older larvae were prepared as monolayers on finder grids. Phase contrast microscopy was used to identify and locate cells in desired stages before shearing. Networks were present in sheared cells at all stages of division, but in stages other than prophase they appeared very dense and always contained aggregated material (Fig. 5A and B). Spermatocytes in metaphase, anaphase, telophase, and cytokinesis also appeared to be more resistant to shear force than prophase cells.

Comparison of the areas exposed during shearing using simple subjective criteria (small area vs. large area) indicated that ~95% of the prophase cells contained large exposed regions.
FIGURE 3 Electron micrographs of sheared spermatocytes. (A) The prophase cell labeled 3 A in Fig. 2 B. After displacement of cytoplasm during shearing, portions of the cell that were attached to the grid become exposed. The stages of the cells in (B, C, and D) were not determined. Under identical conditions of shearing, some cells had larger exposed regions than others. For example, compare A with B and C. (D) A sheared cell after only 1 h of fixation, in comparison to A through C, which were fixed and then stored overnight in fixative. Overnight exposure to cacodylated-buffered glutaraldehyde had no detectable effect on the morphology of sheared cells. × 5,000.
FIGURE 4  Electron micrographs of the exposed regions of four sheared spermatocytes illustrating networks of short, bifurcated filaments. (A) Networks occasionally contain 25- to 50-nm thick filaments (arrowheads) that follow straight trajectories through the network of thinner filaments. The polarized appearance of this network is interpreted to be the result of centrifugal force used to shear the cell. (B) A network that is less polarized than the network illustrated in A. Some regions of the network appear to contain dense clusters of filaments (arrowhead). (C) A network of a cell that was prepared in Tricine-EGTA. The network is indistinguishable from those in A and B above. (D) A region of a spermatocyte network that had been extracted with Triton X-100 after shearing. Treatment of monolayers with detergent after shearing did not improve the visualization of filamentous networks, indicating that the plasma membrane became removed during shearing and that lipid apparently is not a major component of networks. × 15,000.
FIGURE 5  Electron micrographs of filamentous networks observed in sheared spermatocytes at metaphase (A) and telophase (B). The insets in (A) and (B) are photomicrographs of the same cells before shearing and preparation for TEM. Networks observed in cells at metaphase, anaphase, telophase, and cytokinesis were dense and contained both filaments and aggregated material. \( \times 25,000 \); Insets: \( \times 650 \).

compared to only 30% of the cells at other stages of meiosis. In addition, cells in metaphase, anaphase, telophase, and cytokinesis frequently became detached from the grid during shearing.

The overall appearance of networks found in sheared spermatocytes resembled networks found in sheared and detergent-extracted human erythrocytes (Fig. 6). Triton X-100 was used to expose the red cell cortex (3, 12, 40) because the cortex of fresh erythrocytes could not be exposed by centrifugal force alone. Hainfeld and Steck (12) also reported difficulties in their attempt to flatten and rupture fresh erythrocytes by attaching them to poly-L-lysine-coated surfaces. While shearing did not disrupt the erythrocyte plasma membrane, we found that this treatment significantly improved the visualization of cortical networks.

The morphology of networks in sheared spermatocytes also is similar to the cortical networks that have been found in a variety of cells, including Dictyostelium amoebae (4). F-actin is a component of Dictyostelium cortical networks. To investigate whether spermatocyte networks contain F-actin, sheared cells were treated with myosin S. Using conditions under which muscle F-actin was clearly decorated with S (data not shown), the appearance of S-treated spermatocyte networks was not obviously different from that of untreated preparations (Fig. 7). The possible significance of this result will be considered in the Discussion.

DNase-I also was used as a probe for actin in this investigation. DNase-I has been used to dissociate not only F-actin (14, 21) but also actin Mg\(^{2+}\) paracrystals (13), actin-containing erythrocyte membrane cytoskeletons (30), and sedimentable actin in homogenates of crane-fly spermatocytes (34). Those results indicate that the specific interaction of DNase-I with actin is independent of both the source and supramolecular state of actin. DNase-I disrupted the sheared erythrocyte network (Fig. 8). That result is due to the specific release of actin from the cortex, as previously documented by Sheetz (30) and confirmed in this laboratory (data not shown). Sheared-spermatocyte networks also were disrupted after exposure to DNase-I (Fig. 9; Table I). While most of the network was removed following DNase-I treatment, some thick filaments appeared resistant to DNase-I (Fig. 9). As expected, treatment of sheared cells with ovalbumin, a protein that has no apparent effect on the structure of actin, did not dissociate the network (Table I). The effect of protease on sheared cells indicated that at least some components of the DNase-I labile network were proteins (Table I).

DISCUSSION

Crane-fly spermatocytes contain filamentous networks in regions of the cell cortex that were exposed by centrifugation shearing. Networks were disrupted after exposure to DNase-I. Others have shown that DNase-I can dissociate F-actin (14, 21) and actin Mg\(^{2+}\) paracrystals (13). We have confirmed the
FIGURE 6  Electron micrographs illustrating filamentous network structure in crane-fly spermatocytes and human erythrocytes. (A) A network observed in a sheared and detergent-extracted erythrocyte. These structures contained both filamentous and globular components. (B) A portion of a sheared-spermatocyte network. The appearance and substructure of this network resembled the sheared and detergent-extracted erythrocyte network illustrated in (A). × 24,000.

FIGURE 7  Electron micrographs depicting an untreated control (A) and S$_r$-treated (B) spermatocyte network. No change in the structure of the network was detected after treatment with S$_r$. × 36,000.
FIGURE 8  Electron micrographs illustrating the effect of DNase-I on sheared-erythrocyte networks.  (A) Control networks incubated in buffer alone for 15 min.  (B) Networks treated with DNase-I for 15 min. Long, wavy filaments (inset) having a width of ~10-20 nm appeared to be resistant to DNase-I.  x9,000; Inset x 24,000.

reports of Sheetz (30) that DNase-I can dissociate actin from other components in the erythrocyte membrane cytoskeleton.  DNase-I also has been used in studies on a variety of nonmuscle cells as a diagnostic tool for investigating both structural (7, 24, 27) and contractile (15) properties of actin.  In our previous biochemical studies, DNase-I was used to depolymerize sedimentable actin in homogenates of spermatocytes (34).  In view of these properties of DNase-I, we interpret our results on sheared-cell monolayers as an indication that networks in the exposed cortex of spermatocytes contain actin.  DNase-I labile components in spermatocyte networks probably are not either DNA or chromatin (25) because nuclei are sheared away from cortical regions at 650 g and they are expected to remain intact under these conditions of centrifugation (26, 38).

We believe that it is unlikely that the effect of DNase-I on spermatocyte networks is due to protease contamination.  To investigate the possible influence of contaminating proteases on results obtained with DNase-I solutions, three control experiments were done.  First, F-actin and DNase-I at a molar ratio of 0.9 were incubated at 22°C for 60 min.  A 10-fold decrease in the specific viscosity of F-actin induced by DNase-I was not accompanied by proteolysis of depolymerized actin.  Proteolysis was assessed by the appearance of proteolytic fragments on SDS PAGE gels.  Second, after treatment of Triton-extracted erythrocyte networks with DNase-I for 15 min at 22°C, SDS gels of polypeptides released by DNase-I contained a single 43 K-dalton band.  Third, DNase-I disrupted the spermatocyte network in the presence of high (1 mM) concentrations of protease inhibitor.

Thick filaments in networks appear to be more resistant to DNase-I than thin network filaments.  While this result suggests that thick filaments may not be composed of actin, it is also possible that these structures contain actin that is resistant to DNase-I.  For example, actin paracrystals are disrupted more slowly by DNase-I than erythrocyte or spermatocyte actin-containing networks (A. R. Strauch, unpublished data).  Structures resembling actin paracrystals have not been observed in thin sections of spermatocytes (9, 10).

The appearance of networks in the cortex of sheared spermatocytes is clearly unlike that of single actin filaments and filament bundles reported by Forer and Behnke (9, 10) in thin-sections of spermatocytes that were treated with glycerol/HMM.  The difference between our results and those of Forer and Behnke may be explained by the methods used to expose and identify cortex components.  We have employed an approach using physiological buffers that have less potential for perturbing the native state of actin than the glycerol/HMM treatments used by Forer and Behnke (9, 10).  Both glycerol (16) and HMM (35, 39) have been shown to be capable of inducing the polymerization of G-actin in vitro.  Actin-containing networks in human erythrocytes appear to be transformed after HMM treatment into free, recognizable filaments (36).

Actin filaments or bundles were not observed by Forer and Behnke (9, 10) in untreated cells, and this suggests that the
TABLE I

| Treatment  | Number of spermatocytes observed | Number of spermatocytes that contained a cortical network | Percentage with an exposed cortical network |
|------------|---------------------------------|--------------------------------------------------------|------------------------------------------|
| Controls   | 2087 (3)*                       | 1832‡                                                  | 88                                       |
| DNase-I    | 3699 (2)                        | 35                                                     | 1                                        |
| Ovalbumin  | 1733 (2)                        | 1582                                                   | 91                                       |
| Pronase E  | 1385 (2)                        | 199                                                    | 14                                       |

Spermatocyte monolayers were prepared from testes containing predominantly prophase cells. After the shearing step, cells were incubated in either DNase-I, ovalbumin, or Prase E at 1 mg/ml in Tricine buffer for 15 min at room temperature. Controls were incubated in Tricine buffer alone.

PMSF (0.01 mM) was included in DNase-I solutions to inhibit proteases. After treatment, grids were rinsed in Tricine buffer and prepared for TEM as described in Materials and Methods.

* The number in parentheses indicates the number of grids examined for each treatment.
‡ Not all sheared cells contained an exposed network. The criterion used for scoring network-containing cells was that they must appear similar to the cells illustrated in Figs. 3 and 4.

Native state of actin in spermatocytes may be something other than decoratable filaments. Using conditions under which muscle F-actin and nonmuscle actin filaments (1) can be decorated, we have been unable to visualize any detectable change in the structure of spermatocyte network filaments upon addition of myosin S1. It is possible that decorated filaments may be difficult to identify because of the dense, compact arrangement of the network (1). Alternatively, spermatocyte actin in its native state may be refractory to S1 treatment because it is in a nonfilamentous state and/or associated with other components that inhibit S1 binding (18, 32).

Under similar experimental conditions, erythrocyte actin also is difficult to decorate in situ (36, 37). However, erythrocyte actin can be decorated with S1 after purification and polymerization from an acetone powder of ghosts (36). Recent evidence obtained from reconstitution studies using purified proteins indicates that erythrocyte actin may exist in situ in a nonfilamentous state, perhaps in the form of short oligomers (5).

The resemblance in the structure of networks found in the cortical regions of spermatocytes and erythrocytes, coupled with the striking homologies between spermatocyte and erythrocyte peptide maps (34), lead us to hypothesize that the actin of spermatocytes and erythrocytes may be organized and used in similar ways. We do not know whether the cell cortex is the only site of actin localization in spermatocytes, as is known to be the case in erythrocytes (6, 19, 20, 36). There are limits in the extent of a transcellular cytoskeleton in spermatocytes since...
only ~1% of the total protein is actin (34). The localization of actin in the cortical regions of spermocytes seems logical in view of the dynamic changes in cell shape that occur during spermatogenesis. Spermocytes undergo two rounds of cytokinesis, and this is followed by a period when daughter cells begin to elongate into spermatids. While the filamentous network in spermocytes may function in cortical contraction, it is not clear how this could be accomplished with networks that appear to be structurally isotropic. Perhaps there are subtle localized changes in the organization of the cortex associated with contraction. Networks in cells at metaphase, anaphase, telophase, and cytokinesis are denser than networks in prophase cells, indicating a change in organization between these stages. It is possible that network components become anisotropic or are distributed differently within the cortex as the cell approaches cytokinesis (29). In this regard, it would be informative to investigate the deployment of myosin during meiosis and its contribution to the organization of the spermocyte cortex.

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