Distinct Cytoskeletal Domains Revealed in Sperm Cells

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ABSTRACT Antibodies against different cytoskeletal proteins were used to study the cytoskeletal organization of human spermatozoa. A positive staining with actin antibodies was seen in both the acrosomal cap region and the principal piece region of the tail. However, no staining was obtained with nitrobenzoxadiazol-phallacidin, suggesting that most of the actin was in the nonpolymerized form. Most of the myosin immunoreactivity was confined to a narrow band in the neck region of spermatozoa. Tubulin was located to the entire tail, whereas vimentin was only seen in a discrete band-like structure encircling the sperm head, apparently coinciding with the equatorial segment region. Surface staining of the spermatozoa with fluorochrome-coupled Helix pomatia agglutinin revealed a similar band-like structure that co-distributed with the vimentin-specific staining. Instead, other lectin conjugates used labeled either the acrosomal cap region (peanut and soybean agglutinins), both the acrosomal cap and the postacrosomal region of the head (concanavalin A), or the whole sperm cell surface membrane (wheat germ and lens culinaris agglutinins and ricinus communis agglutinin I). In lectin blotting experiments, the Helix pomatia agglutinin–binding was assigned to a 80,000-mol-wt polypeptide which, together with vimentin, also resisted treatment with Triton X-100.

Only the acrosomal cap and the principal piece of the tail were decorated with rabbit and hydridoma antibodies against an immunoanalogue of erythrocyte α-spectrin (p230). p230 appeared to be the major calmodulin-binding polypeptide in spermatozoa, as shown by a direct overlay assay of electrophoretic blots of spermatozoa with ^{125}I-calmodulin.

The results indicate that spermatozoa have a highly specialized cytoskeletal organization and that the distribution of actin, spectrin, and vimentin can be correlated with distinct surface specializations of the sperm cells. This suggests that cytoskeleton may regulate the maintenance of these surface assemblies and, hence, affect the spermatozoan function.

Mammalian spermatozoa are highly specialized cells which have many unique properties (for a review, see reference 18). The major compartments of a fully developed spermatozoon are the head, containing the nucleus and the sharply demarcated acrosomal and postacrosomal domains, the neck region, and the tail which contains mitochondria in its middle piece (18). The acrosomal region undergoes drastic changes during acrosomal reaction upon contact with the oocyte (18, 30, 64). These consist of a dissolution of the acrosomal plasma membrane and exposure of the acrosomal sac which subsequently becomes fused with the oocyte membrane (e.g., reference 30).

Although the structure and function of spermatozoa have been the subjects of an intensive study during recent years, many aspects of their motility, activation, and membrane

organization are still poorly understood (18, 23, 30). This is especially true for the structural basis of the extraordinary compartmentalization of the spermatozoon. This includes distinct cell surface domains that are revealed by the freeze-fracture method (23) and by fluorescence microscopy with monoclonal antibodies (25, 52, 58), lectin conjugates (37, 53, 60), and probes for anionic lipids (6, 7).

In this study we used antibodies to cytoskeletal proteins, combined with immunofluorescence microscopy and immunochemical analysis, to elucidate the role of cytoskeleton in the maintenance of the structural compartmentalization of spermatozoon. The results reveal a highly polarized cytoskeletal organization in human spermatozoon. Furthermore, the results suggest that distinct cytoskeleton–cell surface assem-

blies may be involved in the regulation of the cell surface events associated with acrosomal reaction and fertilization.

MATERIALS AND METHODS

Cells

We isolated human spermatozoa from ejaculates by first washing in NaCl-P buffer (140 mM NaCl, 10 mM sodium phosphate, pH 7.4), then overlaying on 2 vol of 15% (wt/vol) sucrose and centrifuging at 600 g for 20 min (33). In some experiments, the cells were extracted with 0.5% Triton X-100 (BDH Chemicals Ltd., Poole, England), buffered with 50 mM Tris-HCl, pH 7.4, and supplemented with 1 μM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO).

Immunofluorescence Microscopy

RABBIT ANTIACTIN ANTIBODIES: Actin was isolated from the acetone powder of chicken gizzard using gel filtration in 0.1% SDS, as described earlier (2). Antiserum was raised in rabbits and was affinity-purified on an actin-Sepharose 4B-column (3). In indirect immunofluorescence (IIF)[1] microscopy of cultured cells, typical staining of stress fibers could be seen with this antiserum (3, 43). Alternatively, F-actin was visualized by applying 7-nitrobenz-2-oxa-1-,3-diazole (NBD)phallacidin (Molecular Probes Inc., Plano, TX) on paraformaldehyde-fixed and detergent-treated specimens (4).

RABBIT ANTIMYOSIN ANTIBODIES: Myosin was also prepared from chicken gizzards and the heavy chains were isolated by gel filtration in 0.1% SDS. Antibodies were raised in rabbits as described previously (3). In IIF of cultured fibroblasts, myosin antibodies stained stress fibers in a typical way (43).

RABBIT ANTI-α-ACTININ ANTIBODIES: α-actinin was isolated from chicken breast muscle and antibodies were raised in rabbits as described (2).

RABBIT ANTITUBULIN ANTIBODIES: Tubulin was purified from calf brain using a depolymerization/polymerization method. Antibodies were raised in rabbits and were rendered monospecific by affinity-chromatography as described earlier (2, 3). In IIF of cultured fibroblasts, the antibodies gave a typically fibrillar, cytoplasmic staining (75).

RABBIT ANTIVINCULIN ANTIBODIES: Vinculin was purified from chicken gizzard according to Feramisco and Burridge (19), and antibodies were raised in rabbits. In immunoblotting of electrophoretically separated fibroblast polypeptides, the antiserum reacted with a single 130,000-mol-wt polypeptide (43).

RABBIT ANTIVIMENTIN ANTIBODIES: Vimentin was purified from the detergent-resistant cytoskeletons of cultured human fibroblasts and antibodies were raised in rabbits as described earlier (76). In immunoblotting of cultured fibroblasts, the antiserum bound only to the 58,000-mol-wt subunit protein of intermediate filaments (76).

RABBIT ANTIBODIES AGAINST P230, AN IMMUNOANALOGUE OF ERYTHROID α-SPECTRIN: p230 was isolated from fetal bovine lenses as described earlier (45). Antibodies were raised in rabbits and rendered monospecific by an immunoaffinity technique as described earlier (45). These antibodies recognized a 230,000-mol-wt polypeptide from cultured fibroblasts and lens epithelial cells, and also reacted with the α-subunit of the erythroid spectrin as shown by immunoblotting and immunoprecipitation techniques (45).

MONOCLONAL ANTIBODIES AGAINST VIMENTIN AND P230: 9-wk-old BALB/C mice were immunized intraperitoneally at 2-wk intervals with 100 μg of p230 emulsified in Freund's complete adjuvant. 3 d after the second immunization, the spleens were removed and the cells were suspended and fused with the myeloma cell line P3/NS-7/1-Ag4-1, using the procedure of Köhler and Milstein (39). Hybrid selection was initiated 24-h later using standard techniques. Antibody-producing hybrids were selected by solid-phase immunoassay and IIF microscopy of cultured cells. Positive hybrids were cloned by the limiting dilution method and the culture supernatants were collected and used in the present study. Hybridoma antibodies against vimentin were raised by the same technique by using detergent-resistant cytoskeletal polypeptides of Madin-Darby canine kidney cells as an antigen (reference 47 and I. Virtanen, R. Paasivuo, and V.-P. Lehto, manuscript in preparation). Hybrids, producing antivimentin antibodies, were selected on the basis of the typical staining of cultured fibroblasts in IIF microscopy. In immunoblotting of electrophoretically separated polypeptides of human erythrocyte ghosts (67), the monoclonal anti-p230 antibodies reacted with the α-subunit of erythrocyte

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[1] *Abbreviations used in this paper:* FITC, fluorescein isothiocyanate; HPA, *Helix pomatia* agglutinin; IIF, indirect immunofluorescence; NBD, 7-nitro-2-oxa-1-,3-diazole; TRITC, tetramethylrhodamine isothiocyanate; WGA, wheat germ agglutinin.

spectrins (46). Monoclonal antivimentin antibodies reacted only with the 58,000-mol-wt subunit protein of intermediate filaments of fibroblasts (47).

Characterization of the Antibodies

The different antibodies used for this study were characterized both by using immunoblotting with electrophoretically separated polypeptides of whole human fibroblasts and by absorption of the antibodies with purified antigens.

In immunoblotting, the affinity-purified rabbit antiactin antibodies (5 μg ml^{-1}) revealed a single 43,000-mol-wt polypeptide among fibroblast polypeptides (Fig. 1, lane 2). In contrast, affinity-purified rabbit antitubulin antibodies (5 μg ml^{-1}) revealed a single polypeptide with M_r = 52,000 in our gel system (Fig. 1, lane 3). Both monoclonal antivimentin antibodies (culture supernatant diluted 1:20; Fig. 1, lane 4) and rabbit antivimentin (5 μg ml^{-1}) antibodies reacted only with a 58,000-mol-wt polypeptide. Rabbit antimyosin antibodies (dilution 1:600) and rabbit anti-p230 antibodies (5 μg ml^{-1}) revealed among fibroblast polypeptides a 210,000- and a 230,000-mol-wt polypeptide, respectively (Fig. 1, lanes 5 and 6).

For absorption experiments vimentin was purified from detergent-resistant cytoskeletons of human fibroblasts using a preparative gel electrophoretic technique as described earlier (reference 76; Fig. 2, lane 2). Both actin from bovine muscle and myosin from rabbit muscle used for absorption experiments were obtained from Sigma Chemical Co. The purity of these polypeptides was confirmed by gel electrophoresis (Fig. 2, lanes 1 and 3). p230 polypeptide was isolated from bovine eye lenses as described earlier (45). Briefly, cytoskeletal elements were enriched by stepwise extractions with Triton X-100 and low and high ionic buffers. After this, p230 was isolated as a homogenous polypeptide using hydroxylapatite chromatography (Fig. 2, lane 4). Overnight incubations of the antibodies with the respective antigens (working dilution of the antibodies
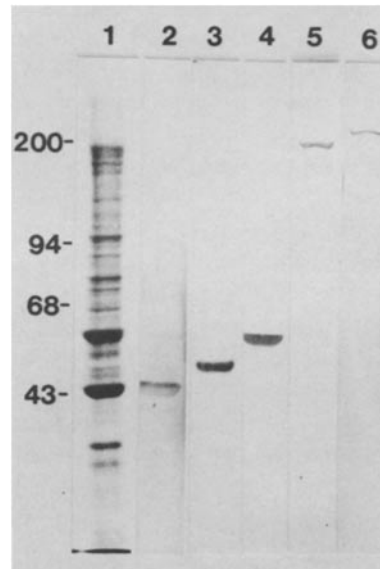

FIGURE 1 Immunoblotting of electrophoretically (8%) separated polypeptides from human embryonal fibroblasts transferred to nitrocellulose and stained with amido black for protein (lane 1), or immunostained for actin (lane 2), tubulin (lane 3), vimentin (lane 4), myosin (lane 5), and p230 (lane 6). Molecular weight markers are indicated on the left-hand side ($M_r \times 10^{-3}$).
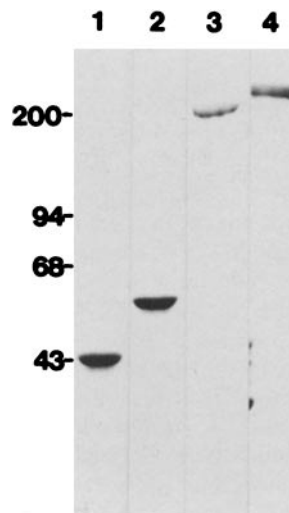

FIGURE 2 PAGE (8%) profiles of polypeptide preparations used to absorb the antibodies; lane 1, actin from bovine muscle; lane 2, vimentin from detergent-resistant cytoskeletons of cultured fibroblasts; lane 3, myosin from rabbit muscle; lane 4, p230 from bovine eye lens. ($M_r \times 10^{-3}$.)

together with 100 µg ml⁻¹ of the antigen) completely abolished the positive staining reaction both with cultured fibroblasts and sperm cells.

Immunofluorescence Microscopy

For IIF microscopy with cytoskeletal antibodies, the spermatozoa were air-dried on an objective glass and then fixed with methanol, cooled to −20°C, or with 3.5% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 10 min. In control experiments, paraformaldehyde-fixed sperm cells were also reacted with cytoskeleton antibodies. No staining was seen in these experiments. The methanol-fixed specimens were first exposed to rabbit or hybridoma antibodies for 30 min, washed, and then reacted with fluorescein isothiocyanate (FITC)-coupled goat anti-rabbit IgG (Cappel Laboratories, Inc., Cochranville, PA) or FITC-anti-mouse IgG (Cappel), respectively, for 30 min. Affinity-purified rabbit antibodies were used at a concentration of 50 µg ml⁻¹ and antisera at a dilution of 1:40 to 1:80. Culture supernatants of monoclonal antibodies were used for immunostaining. The conjugates alone did not give any detectable staining on either paraformaldehyde- or methanol-fixed sperm cells. To visualize Helix pomatia agglutinin (HPA)-binding glycoconjugates of spermatozoa, paraformaldehyde-fixed specimens were exposed to tetramethylrhodamine isothiocyanate (TRITC)-HPA (EY-Laboratories, San Mateo, CA), for 30 min. To further double-stain for vimentin, the specimens were then treated with 0.1% Nonidet P-40, to permeabilize the cells, and stained for vimentin using monoclonal antibodies and FITC conjugate as above. FITC-peanut agglutinin, FITC-lens culinaris agglutinin, FITC-ricinus communis agglutinin I, FITC-wheat germ agglutinin (WGA), and FITC-soybean agglutinin were from Vector Laboratories, Inc. (Burlingame, CA). Surface staining with lectin conjugates (100 µg ml⁻¹) was performed either on unfixed sperm cells at 0°C or on paraformaldehyde-fixed specimens. Both methods gave similar staining results.

The specimens were embedded in glycerol-veronal buffer (1:1, pH 8.4) and examined in a Zeiss Universal Microscope equipped with an epi-illuminator IIIRS and filters for FITC- and TRITC-fluorescence.

Gel Electrophoresis and Immuno- and Lectin-blotting

PAGE in the presence of SDS was performed according to Laemmli (40) using 6.5 or 8% slab gels. Electrophoretic transfer of polypeptides onto nitrocellulose paper was performed according to Towbin et al. (74), using a Trans-Blotᴿ apparatus (Bio-Rad Laboratories, Richmond, CA). For protein staining, 0.1% amido black was used. For immunostaining, the nitrocellulose sheets were first incubated overnight in NaCl-P buffer containing 3% BSA (Sigma). Then, the sheets were exposed to rabbit or monoclonal antibodies (5 µg ml⁻¹ for affinity-purified antibodies, 1:600 dilution for antisera, and 1:10 dilution for hybridoma supernatants) in NaCl-P buffer, supplemented with 2% BSA and 0.2% Triton X-100, for 60 min. After washing, the sheets were further exposed to peroxidase-coupled rabbit anti-mouse or swine anti-rabbit immunoglobulins (diluted in the above buffer 1:100; Dakopatts, Copenhagen, Denmark) for 60 min, and the peroxidase reaction was developed according to Towbin et al. (74).

For lectin staining experiments of blotted proteins, the nitrocellulose sheets were first exposed to biotinylated WGA or HPA, (5 µg ml⁻¹; E-Y Laboratories), and after washing, to an avidin-peroxidase conjugate (5 µg ml⁻¹; E-Y Laboratories). The peroxidase reaction was developed as above. Preincubation of the lectin conjugates with the corresponding inhibiting monosaccharide (0.2 M N-acetyl glucosamine or 0.2 M N-acetyl galactosamine in NaCl-P buffer, respectively) led to a complete inhibition of the staining reaction.

¹²⁵I-Calmodulin Overlay Assay

For overlay experiments with ¹²⁵I-calmodulin the nitrocellulose sheets were exposed to ¹²⁵I-calmodulin (25 µCi ml⁻¹; 25–50 µCi µg⁻¹; Radiochemical Centre, Amersham, Buckinghamshire, U.K.) in the presence of Ca²⁺ or EDTA according to Glenney and co-workers (26, 27) for 3 h. After a thorough washing, the sheets were dried and exposed to Kodak X-Omat film.

RESULTS

Actomyosin System of Spermatozoon

In IIF microscopy of spermatozoa, a bright fluorescence was seen in the acrosomal cap and in the principal piece of the tail with antiactin antibodies (Fig. 3, a and b). NBD-phallacidin, a fluorescent probe for F-actin (4) which decorates, for instance, the stress fibers of cultured fibroblasts (4, 45), failed, however, to stain the spermatozoa (data not

shown). A distinctly different staining pattern was obtained with antimyosin antibodies: most of the immunoreactivity was confined to a narrow, band-like staining at the neck region of spermatozoa in addition to a fainter staining of the sperm head and tail regions (Fig. 3, c and d). The presence of both myosin and actin in sperm cells was also confirmed in immunoblotting: immunostaining of the electrophoretically separated polypeptides of spermatozoa with antiactin and antimyosin antisera revealed polypeptides of $M_r = 43,000$ and 210,000, respectively (Fig. 5, lanes 1–3). On the other hand, preabsorption of antiactin and antimyosin antibodies with the corresponding antigens completely abolished the staining reaction (for actin, see Fig. 4, a and b). No staining of spermatozoa was seen with antivinculin or anti-α-actinin antibodies (data not shown).

Tubulin and Intermediate Filaments in Human Spermatozoon

Antitubulin antibodies gave a bright staining of the entire tail region of spermatozoa, extending to the neck region of the sperm head (Fig. 3, e and f). No staining was seen in the sperm head.

When antibodies against different types of intermediate filament proteins (for antibodies, see Reference 76) were applied on methanol-fixed spermatozoa, only rabbit and hybridoma antivimentin antibodies were found to react: a narrow, band-like staining could be seen encircling the sperm head, as judged by differential focusing (Fig. 3, g and h). This staining pattern apparently corresponds to the equatorial segment region (18). However, when either paraformaldehyde-fixed or unfixed sperm cells were reacted with antivimentin antibodies, a positive staining reaction could not be seen (Fig. 4, c and d). In line with the IIF findings, immunostaining of electrophoretic blots of spermatozoa with antivimentin antibodies showed a distinct reaction with a polypeptide of $M_r = 58,000$ (Fig. 5, lane 4).

The apparent localization of vimentin in the sperm head close to the plasma membrane prompted us to examine whether fluorochrome-coupled lectins could reveal a comparable regionalization of surface glycoconjugates of the sperm head. Interestingly enough, only TRITC-HPA gave a surface staining on sperm head that closely resembled that seen with antivimentin antibodies in permeabilized cells (Fig. 6, a and b). Furthermore, in double staining with TRITC-HPA and monoclonal antivimentin antibodies, a close co-distribution of the two staining patterns was seen (Fig. 6, b and c). Both the vimentin-containing elements and the HPA-binding sites also resisted extraction with Triton X-100 (Fig. 6, b, c, and insets). In contrast to this finding, FITC-soybean agglutinin, sharing a similar nominal saccharide specificity with HPA (28), distinctly bound in surface staining to the whole acrosomal region of the sperm head (Fig. 6, d and e). A similar staining pattern was also obtained with FITC-peanut agglutinin (Fig. 6, f and g), which bound to some terminal galactosyl residues (28). On the other hand, FITC-concanavalin A gave a bright staining reaction on the acrosomal region as well as on the posterior surface of the sperm head (Fig. 6, h and i) whereas FITC-lens culinaris agglutinin, FITC-WGA, and FITC-ricinus communis agglutinin I bound evenly to the whole surface membrane of spermatozoa, including the tail region (Fig. 6, j and k).

When the electrophoretic blots of spermatozoan polypeptides were stained with a lectin-peroxidase conjugate, a single
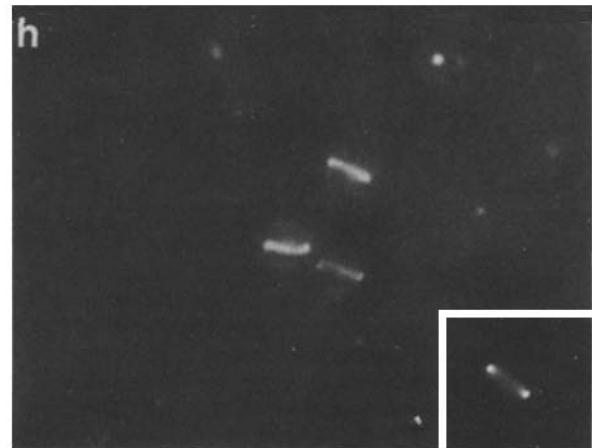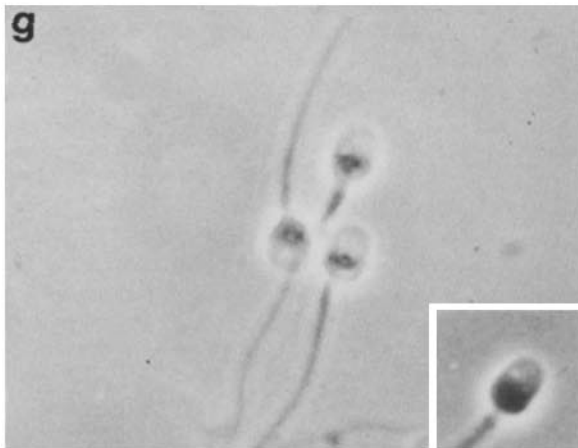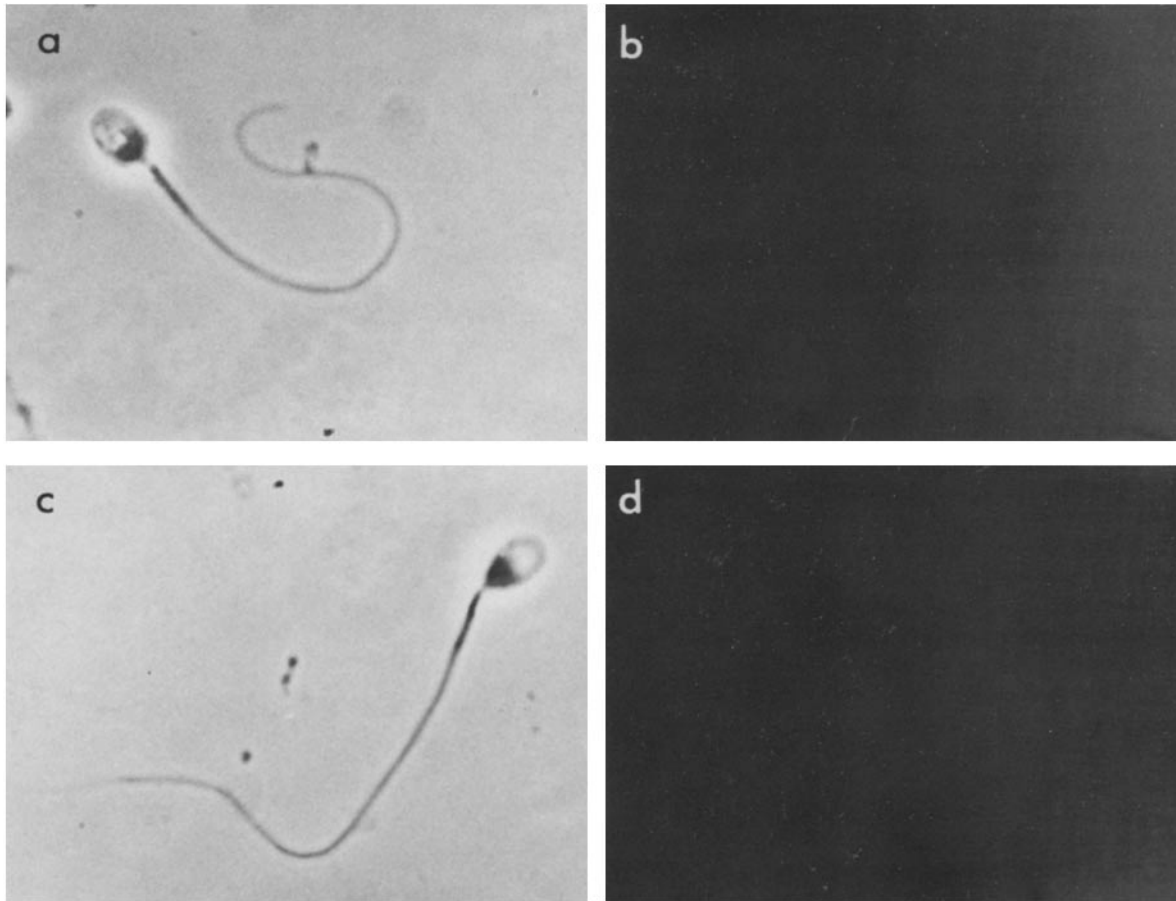
FIGURE 4 Staining of methanol-fixed spermatozoa with actin antibodies, preabsorbed with actin, does not give any staining reaction (a and b). No staining is seen in paraformaldehyde-fixed spermatozoa after staining with rabbit antivimentin antibodies (c and d). Note also the lack of unspecific binding by the conjugates. × 1,100.

major HPA-binding polypeptide with M_r = 80,000 was revealed (Fig. 7, lanes 1 and 2). Other lectins, such as WGA, bound to distinctly different polypeptides (Fig. 7, lane 1 and 3).

Presence of α-Spectrin-like Polypeptides in Human Spermatozoon

Rabbit and hybridoma antibodies to p230 showed in IIF a distinct staining of the acrosomal cap and the principal piece of the tail (Fig. 8, a and b), a distribution that closely resembles that of actin (compare with Fig. 3, a and b). Surface stainings of sperm cells with anti-p230 antibodies did not give any positive staining reaction. Immunoblotting with anti-p230 antibodies showed a distinct reaction with a polypeptide of M_r = 230,000 of the electrophoretically separated polypeptides of whole spermatozoa (Fig. 9, lanes 1 and 2). Since the nonerythroid spectrins have turned out to be major calmodulin-binding proteins (15, 26, 27, 36, 55, 65), we also studied
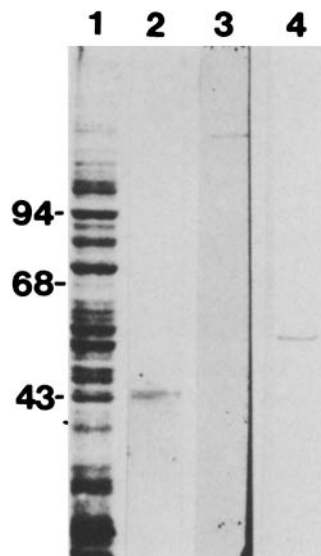

FIGURE 5 Polypeptides of human spermatozoa run in PAGE (8%), transferred to nitrocellulose paper, and stained for protein (lane 1). Immunostaining for actin (lane 2), myosin (lane 3), or vimentin (lane 4) reveals a single polypeptide with M_r = 43,000, 210,000, or 58,000, respectively.

FIGURE 3 IIF of methanol-fixed human spermatozoa with antibodies to actin (a and b), myosin (c and d), tubulin (e and f), and vimentin (g and h). Note that actin is confined to both the acrosomal and principal piece regions (b), whereas myosin is concentrated in the neck region (arrows in d) but is also present in the tail and head regions. Tubulin is found in the entire tail (f) whereas vimentin is found only in the head as a band-like formation, apparently coinciding with the equatorial segment (h). Upon differential focusing the vimentin-positive band is seen to encircle the whole head: when focused to the middle region of the head, only two bright fluorescent spots can be seen on both sides of the head in permeabilized sperm cells (insets in g and h). × 1,100.
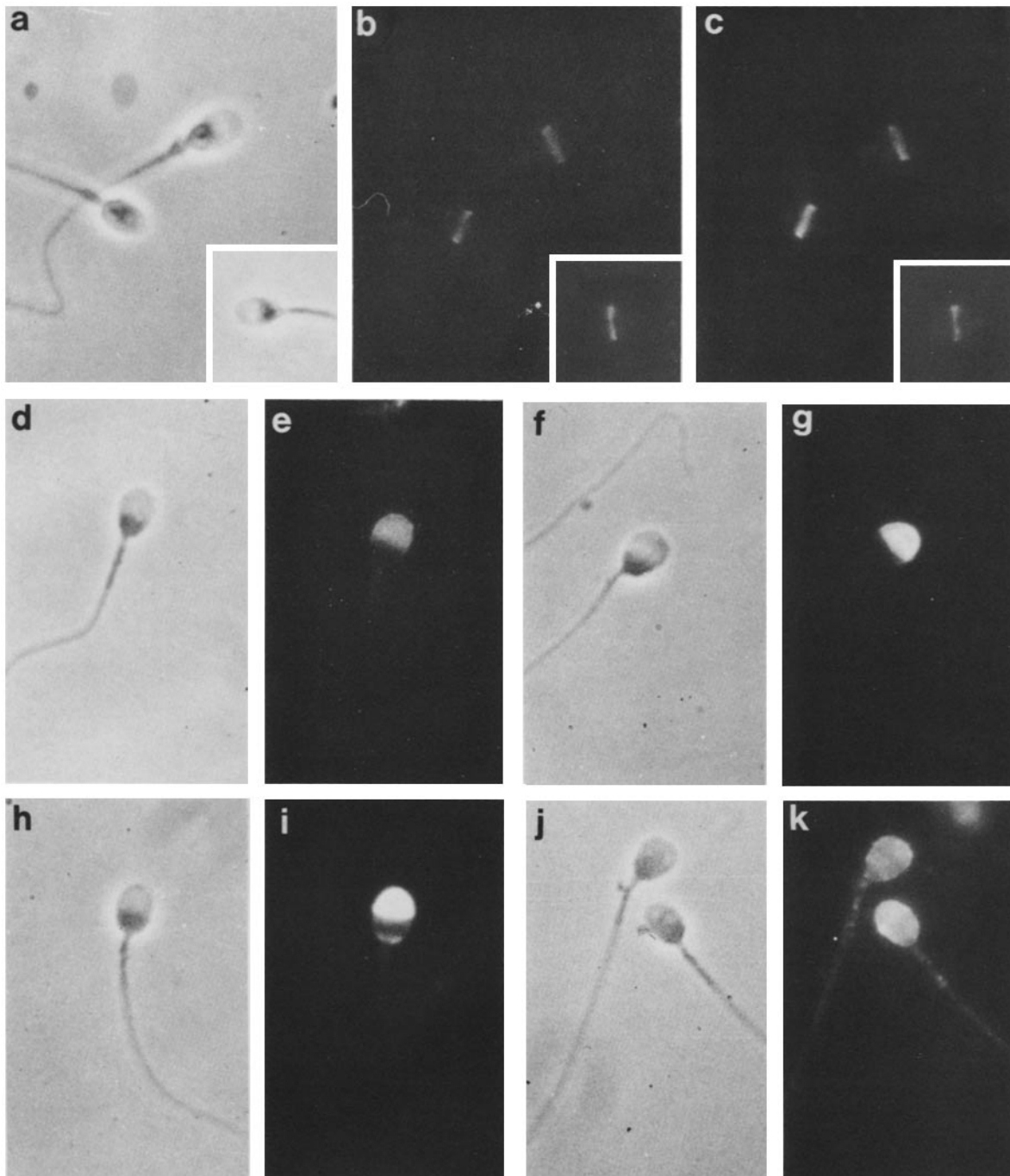
FIGURE 6   Double staining with antivimentin antibodies (a and b) and FITC-HPA (a and c) shows similar band-like formations in the sperm head which can also be visualized after extraction with Triton X-100 (insets, a–c). Both FITC-soybean agglutinin (d and e) and FITC-peanut agglutinin (f and g) bind exclusively to the acrosomal cap region of the sperm head, FITC-Concanavalin A binds in addition to the posterior surface of the sperm head (h and i), and ricinus communis agglutinin, WGA, and lens culinaris agglutinin (j and k) conjugates bind to the entire sperm cell surface. × 1,100.

the binding of iodinated calmodulin to electrophoretic blots of human spermatozoa. The main ¹²⁵I-calmodulin-binding polypeptide, revealed by the overlay assay, had an apparent molecular weight of 230,000 (Fig. 9, lane 3) co-migrating with the polypeptide revealed by immunostaining with anti-p230 of the adjacent lane (Fig. 9, lane 2). There also appeared to be some minor polypeptides binding ¹²⁵I-calmodulin (Fig. 9, lane 3). Binding of calmodulin to p230 appeared to be Ca^{2+} dependent since the binding to p230 was strongly reduced in the presence of a Ca^{2+} chelator (Fig. 9, lane 4).

DISCUSSION

Mammalian sperm cells undergo a complex maturation process beginning in the germinal epithelium of the testis, continuing in the epididymis, and resulting in fertilization-capable, terminally differentiated spermatozoa (18, 30). The freeze-etching technique (23), lectin conjugates (37, 53), fluorescent probes for anionic lipids domains (6, 7), and monoclonal antibodies in IIF (25, 38, 51, 58) have been used in attempts to elucidate the surface characteristics of mammalian sper-
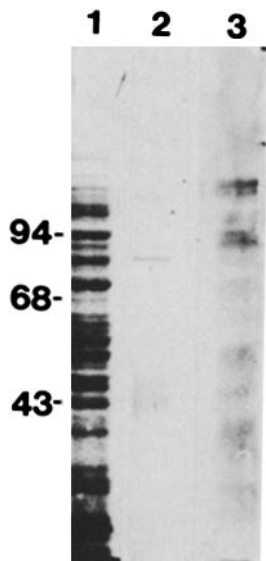
FIGURE 7 Polypeptides of human spermatozoa run in PAGE (8%) and transferred to nitrocellulose paper. Lane 1, amido black staining for protein; lane 2, HPA-binding polypeptides; lane 3, WGA-binding polypeptides.
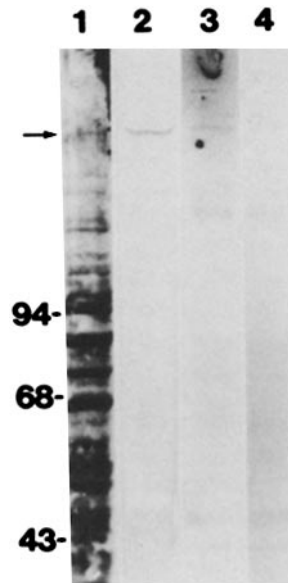

FIGURE 9 Polypeptides of human spermatozoa run in PAGE (6.5%) and transferred to nitrocellulose paper. Lane 1, amido black staining for protein; lane 2, immunostaining for p230; lane 3, overlay with ^{125}I-calmodulin in the presence of Ca^{2+}; lane 4, overlay with ^{125}I-calmodulin in the presence of EGTA. Note that immunostaining for p230 and ^{125}I-calmodulin overlay reveals a similar polypeptide (lanes 2 and 3; arrow in lane 1).
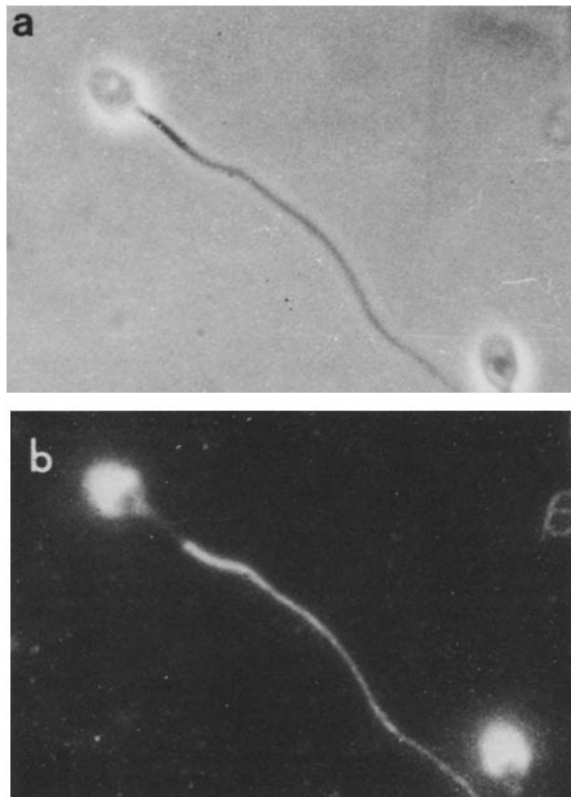

FIGURE 8 Distribution of α-spectrin-like immunoreactivity in methanol-fixed human spermatozoa visualized with anti-p230 antibodies. Note the distinct staining of both the acrosomal cap and principal piece regions (a and b). × 1,100.

matozoa. These studies have indicated that the acrosomal, the equatorial segment, the postacrosomal, and the tail regions each form a distinct surface domain with characteristic properties that are lacking in other surface regions (for a review, see reference 23). The results with lectin conjugates in the present study confirm these findings, with the acrosomal, equatorial segment, and postacrosomal regions showing distinct lectin-binding properties. But the structural basis of this remarkable regionalization has remained unelucidated. Some recent results, however, have clearly indicated that some of

the components of the sperm surface are truly mobile whereas others, possibly those showing a restricted localization, are not (e.g., reference 25).

Much interest has been devoted recently to the role of cytoskeletal elements in maintaining distinct surface membrane domains (10, 12, 27, 32, 44, 45; for a review, see reference 50). An important role in cytoskeleton–cell surface associations has been assigned to the so-called surface lamina structure, which consists of cytoskeletal proteins at the cytoplasmic aspect of the surface membrane and associated cell surface glycoproteins (10, 44). Among many cytoskeletal elements associated with the cell surface lamina, especially actin, spectrin-like proteins and intermediate filaments appear to be important in the cell surface–cytoskeleton interaction (10, 42, 45). Only a few attempts have been made thus far to systematically characterize the cytoskeletal organization of spermatozoa. Using human autoantibodies, researchers have suggested that actin is confined in human spermatozoa to the acrosomal region (e.g., reference 14), mainly to the postacrosomal areas (16, 17), and, together with myosin, to the centriolar region at the sperm neck (1, 13, 14). On the other hand, while microtubules could be demonstrated only in the sperm tail (78) both intermediate filament proteins (22) and spectrin-like polypeptides (59) have been reported to be lacking in mammalian sperm cells.

Actin, the major subunit protein of microfilaments (57), was found in this study to be localized in both the acrosomal and principal piece regions of spermatozoa. Surprisingly, however, most of the actin in human sperm cells (cf. reference 20) did not appear to be in the F-actin form, as indicated by the lack of binding of NBD-phallacidin, a specific probe for F-actin (4). In this respect, it is interesting that neither the sperm cells of echinoderms (71–73) nor those of boars (56, 69) contain polymerized actin until the acrosomal reaction has taken place, which induces a dramatic polymerization of actin (73). Furthermore, spermatozoa of a crane-fly appear to contain amounts of polymerized actin that diminish as the maturation proceeds (68). These results seemingly contradict earlier reports in which the presence of actin filaments in sperm cells was suggested (1, 8, 17, 62). These studies were based upon the visualization of binding of heavy meromyosin to actin, an experimental technique that may in some in-

stances cause an artifactual formation of actin fibers (for a review, see reference 21). The present results also suggest that myosin and actin have a different localization in spermatozoa, myosin being concentrated mostly in the neck region. In this respect our results are closely similar to those of Sandoz et al. (61) on the axonemes of ciliated cells from quail oviduct, but differ from those of Campanella and co-workers (13, 14) which also showed a strong staining of the entire head region. Our immunoblotting and absorption experiments demonstrated the highly specific reaction of both antimyosin and antiactin antibodies with spermatozoa, revealing only the corresponding antigens, and ruling out the possibility of an artifactual reaction in immunolocalization experiments.

Spermatozoa, like many other nucleated cells (77), also appeared to contain intermediate filaments of vimentin type, in contrast to the results of Franke et al. (22) obtained on tissue sections of guinea pig testis. The localization of vimentin in human spermatozoa was especially intriguing since only a very distinct, narrow, band-like structure encircling the sperm head was revealed in IIF, which apparently coincided with the equatorial segment region of the sperm head (18). This specialized surface region has previously been shown to differ in many respects from other surface domains of spermatozoa (23), and it has been suggested to play a specific role in fertilization (5): After the completion of acrosomal reaction, resulting in disappearance of most of the acrosomal surface membrane, the equatorial segment persists and appears to initiate the fusion of the spermatozoon with surface membrane of the egg (5). Interestingly, a corresponding, strictly delineated surface domain could be visualized in our study with the HPA-lectin conjugate, indicating a highly compartmentalized distribution of some N-acetyl galactosamine-containing glycoconjugates (cf. reference 28 for lectin specificity) at the surface of the equatorial segment region. According to the lectin-blotting experiments, the HPA-binding capacity could solely be assigned to a single polypeptide of $M_r = 80,000$. As both the band-like vimentin-specific staining and the HPA-binding sites resisted extraction with Triton X-100, the results suggest that the equatorial segment of the sperm head may be stabilized by a specific cell surface–cytoskeleton interaction. The localization of vimentin solely at the equatorial segment region also corresponds closely to the distribution of cAMP-dependent protein kinase in spermatozoa (70). This enzyme is responsible for vimentin phosphorylation, a posttranslational modification modulating the functional state of vimentin in many cells (11, 24, 54, 66), including Sertoli cells (66).

Many recent studies have indicated the presence of a family of polypeptides, immunologically and structurally related to erythroid α-spectrin, in diverse cells and tissues (9, 12, 26, 27, 29, 31, 32, 41, 45, 49, 59). In neural cells, α-spectrin-like polypeptides (fodrin) are involved in axonal transport (49) and it has been suggested that they provide mechanical support to cell cortex and plasma membrane in a manner analogous to that of erythroid spectrins (12, 41, 45, 49). They are also the major calmodulin-binding polypeptides in many cells (15, 26, 27, 36, 55, 65). In contrast to most other cells, some smooth muscle cells (45, 59) and sperm cells (59) have been reported to lack spectrin-like proteins. However, the present results with antibodies to p230, an immunoanalogue of erythroid α-spectrin (45), revealed α-spectrin-like immunoreactivity both in the sperm head and in the principal piece of the tail. This polypeptide also appeared to be the major calmodulin-binding polypeptide in spermatozoa as judged by the

direct overlay technique with ^{125}I-calmodulin. Our results, hence, also support the view of Tilney (71, 72) that the high molecular weight doublet of polypeptides associated with nonpolymerized actin in Thyone spermatozoa indeed represents spectrins.

In view of the calmodulin-binding capacity of the spectrin-like p230, it is interesting that Jones and co-workers (33, 34) have reported that there is a high content of calmodulin in spermatozoa of different species, and that calmodulin is localized mainly at the acrosomal region of the sperm head. Spectrin/calmodulin-complex–mediated phenomena that involve Ca^{2+} as a mediator are common in many cells (35). Since both α-spectrin and calmodulin (34) appear to share a similar localization in the sperm head, it is conceivable that they may also pay a critical role in the numerous Ca^{2+}-activated activities of spermatozoa, which include, for instance, capacitation and acrosomal reaction (48, 51). One such event could be the recently described activation of a Ca^{2+}-dependent protease that seems to be associated with the onset of acrosomal reaction (63). On the other hand, p230 may also serve as a structural component of the fibrous sheath of the tail, as suggested by the very intense p230-specific staining of the principal piece region. Until now, the chemical composition of this structure has remained unknown (18).

In summary, our results show the presence and the distinctly polarized organization of actin, myosin, vimentin, and nonerythroid α-spectrin in human spermatozoa. Furthermore, they suggest that plasma membrane-associated spectrin and vimentin may play an important role in the assembly and stabilization of the specialized cell surface domains of the spermatozoa and, hence, in the surface-associated events of fertilization.

REFERENCES

1. Baccetti, B., E. Bigliardi, A. G. Burrini, and V. Pallini. 1980. Actin filaments and mitochondrial movement in vertebrate spermiogenesis. Gamete Res. 3:203–209.
2. Badley, R. A., C. W. Lloyd, A. Woods, L. Carruthers, C. Allcock, and D. A. Rees. 1978. Mechanisms of cellular adhesion. III. Preparation and preliminary characterization of adhesions. Exp. Cell Res. 117:231–244.
3. Badley, R. A., A. Woods, L. Carruthers, and D. A. Rees. 1980. Cytoskeleton changes in fibroblast adhesion and detachment. J. Cell Sci. 43:379–390.
4. Barak, L. S., R. R. Yocum, E. A. Nothnagel, and W. W. Webb. 1980. Fluorescence staining of the actin cytoskeleton in living cells with 7-nitrobenz-2-oxa-1,3-diazole phallacidin. Proc. Natl. Acad. Sci. USA. 77:980–984.
5. Barros, C., and I. E. Franklin. 1968. Behavior of the gamete membranes during sperm entry into the mammalian egg. J. Cell Biol. 37:C13–C18.
6. Bearer, E. L., and D. S. Friend. 1980. Anionic lipid domains: correlation with functional topography in a mammalian cell membrane. Proc. Natl. Acad. Sci. USA. 77:6601–6605.
7. Bearer, E. L., and D. S. Friend. 1982. Modifications of anionic-lipid domains preceding membrane fusion in guinea pig sperm. J. Cell Biol. 92:604–615.
8. Behnke, O., A. Forer, and J. Emmersen. 1971. Actin in sperm tails and meiotic spindles. Nature (Lond.). 234:408–410.
9. Bennett, V., J. Davis, and W. E. Fowler. 1982. Immunoreactive form of erythrocyte spectrin and ankyrin in brain. Philos. Trans. R. Soc. Lond. B Biol. Sci. 299:301–312.
10. Ben-Ze'ev, A., A. Duerr, F. Solomon, and S. Penman. 1979. The outer boundary of the cytoskeleton; a lamina derived from the plasma membrane proteins. Cell. 17:859–865.
11. Browning, E. T., and M. M. Sanders. 1981. Vimentin: a phosphoprotein under hormonal regulation. J. Cell Biol. 90:803–808.
12. Burridge, K., T. Kelly, and P. Mangeat. 1982. Nonerythrocyte spectrins: actin-membrane attachment proteins occurring in many cell types. J. Cell Biol. 95:478–486.
13. Campanella, C., and G. Gabbiani. 1979. Motile properties and localization of contractile proteins in the spermatozoon of discoglossus pictus. Gamete Res. 2:163–175.
14. Campanella, C., G. Gabbiani, B. Bacetti, A. G. Burrini, and V. Pallini. 1979. Actin and myosin in the vertebrate acrosomal region. J. Submicrosc. Cytol. 11:53–71.
15. Carlin, R. K., D. C. Bartelt, and P. Siekevitz. 1983. Identification of fodrin as a major calmodulin-binding protein in postsynaptic density preparations. J. Cell Biol. 96:443–448.
16. Clarke, G. N., F. M. Clarke, and S. Wilson. 1982. Actin in human spermatozoa. Biol.

Reprod. 26:319–327.

17. Clarke, G. N., and R. Yamagimachi. 1978. Actin in mammalian sperm heads. *J. Exp. Zool.* 205:125–132.

18. Fawcett, D. W. 1975. The mammalian spermatozoon. *Dev. Biol.* 44:394–436.

19. Feramisco, J. R., and K. Burridge. 1980. A rapid purification of α-actinin, fimbrin and a 130,000-dalton protein from smooth muscle. *J. Biol. Chem.* 255:1194–1199.

20. Flaherty, S. P., W. G. Breed, and V. Sarafis. 1983. Localization of actin in the sperm head of the plains mouse, *Pseudonys australis. J. Exp. Zool.* 225:491–500.

21. Forer, A. 1982. Actin localization within cells by electron microscopy. *Methods Cell Biol.* 25:131–142.

22. Franke, W. W., C. Grund, and E. Schmid. 1979. Intermediate-sized filaments present in sertoli cells are of vimentin type. *Eur. J. Cell Biol.* 19:269–275.

23. Friend, D. S. 1982. Plasma-membrane diversity in a highly polarized cell. *J. Cell Biol.* 93:243–249.

24. Gard, D. L., and E. Lazarides. 1982. Analysis of desmin and vimentin phosphopeptides in cultured avian myogenic cells and their modulation by 8-bromo-adenosine 3'5'-cyclic monophosphate. *Proc. Natl. Acad. Sci. USA.* 79:6912–6916.

25. Gaunt, S. J., C. S. Brown, and R. Jones. 1983. Identification of mobile and fixed antigens on the plasma membrane of rat spermatozoa using monoclonal antibodies. *Exp. Cell Res.* 144:275–284.

26. Glenney, J. R., P. Glenney, M. Osborn, and K. Weber. 1982. An F-actin- and calmodulin-binding protein from isolated intestinal brush borders has a morphology related to spectrin. *Cell.* 28:843–854.

27. Glenney, J. R., P. Glenney, and K. Weber. 1982. Erythroid spectrin, brain fodrin, and intestinal brush border proteins (TW 260/240) are related molecules containing a common calmodulin-binding subunit bound to a variant cell type-specific subunit. *Proc. Natl. Acad. Sci. USA.* 19:4002–4005.

28. Goldstein, I. J., and C. E. Hayes. 1978. The lectins: carbohydrate-binding proteins of plant and animals. *Adv. Carbohydr. Chem. Biochem.* 35:127–340.

29. Goodman, S. R., I. S. Zagon, and R. R. Kulikowski. 1981. Identification of a spectrin-like protein in nonerythroid cells. *Proc. Natl. Acad. Sci. USA.* 78:7570–7574.

30. Gwatkin, R. B. L. 1976. Fertilization. *Cell Surf. Rev.* 1:1–54.

31. Hiller, G., and K. Weber. 1977. Spectrin is absent in various tissue cultured cells. *Nature (Lond.).* 266:181–183.

32. Hirokawa, N., R. E. Cheney, and M. Williard. 1983. Location of a protein of the fodrin-spectrin-TW260/240 family in the mouse intestinal brush border. *Cell.* 32:953–965.

33. Jones, H. P., M. M. Bradford, R. A. McRorie, and M. J. Cormier. 1978. High levels of a calcium-dependent modulator protein in spermatozoa and its similarity to brain modulator protein. *Biochem. Biophys. Res. Commun.* 82:1264–1272.

34. Jones, H. P., R. W. Lenz, B. A. Palevitz, and M. J. Cormier. 1980. Calmodulin localization in mammalian spermatozoa. *Proc. Natl. Acad. Sci. USA.* 77:2772–2776.

35. Kakiuchi, S., and S. Sobue. 1983. Control of the cytoskeleton by calmodulin and calmodulin-binding proteins. *Trends Biochem. Sci.* 4:59–62.

36. Kakiuchi, S., K. Sobue, K. Kanda, K. Morimoto, S. Tsukita, S. Tsukita, H. Ishikawa, and M. Kurokawa. 1982. Correlative biochemical and morphological studies of brain calspectin: a spectrin-like calmodulin-binding protein. *Biomed. Res.* 3:400–410.

37. Koehler, J. K. 1978. The mammalian sperm surface: studies with specific labeling techniques. *Int. Rev. Cytol.* 54:73–108.

38. Koehler, J. K., E. D. Nudelman, and S. Hakomori. 1980. A collagen-binding protein on the surface of ejaculated rabbit spermatozoa. *J. Cell Biol.* 86:529–536.

39. Köhler, G., and C. Milstein. 1976. Derivation of specific antibody producing tissue culture and tumor lines by cell fusion. *Eur. J. Immunol.* 6:511–519.

40. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–683.

41. Lazarides, E., and W. J. Nelson. 1982. Expression of spectrin in nonerythroid cells. *Cell.* 31:505–508.

42. Lehto, V.-P. 1983. 140,000 dalton surface glycoprotein. A plasma membrane component of the detergent-resistant cytoskeletal preparations of cultured human fibroblasts. *Exp. Cell Res.* 143:271–286.

43. Lehto, V.-P., T. Hovi, T. Vartio, R. A. Badley, and I. Virtanen. 1982. Reorganization of cytoskeletal and contractile elements during transition of human monocytes into adherent macrophages. *Lab. Invest.* 47:391–399.

44. Lehto, V.-P., T. Vartio, R. A. Badley, and I. Virtanen. 1983. Characterization of a detergent-resistant surface lamina in cultured human fibroblasts. *Exp. Cell Res.* 143:287–294.

45. Lehto, V.-P., and I. Virtanen. 1983. Immunolocalization of a novel, cytoskeleton-associated polypeptide of M_r 230,000 daltons (p230). *J. Cell Biol.* 96:703–716.

46. Lehto, V.-P., I. Virtanen, R. Paasivuo, R. Ralston, and K. Alitalo. 1983. The p36 substrate of tyrosine-specific protein cinases colocalizes with nonerythrocyte α-spectrin antigen, p230, in surface lamina of cultured fibroblasts. *EMBO J.* 2:1701–1706.

47. Lehtonen, E., V.-P. Lehto, R. Paasivuo, and I. Virtanen. 1983. Parietal and visceral endoderm differ in their expression of intermediate filaments. *EMBO (Eur. Mol. Biol. Org.) J.* 2:1023–1028.

48. Lenz, R. W., and M. J. Cormier. 1982. Effects of calmodulin-binding drugs on the guinea pig spermatozoon acrosome reaction and the use of these drugs as vaginal contraceptive agents in rabbits. *Ann. NY Acad. Sci.* 383:85–95.

49. Levine, J., and M. Willard. 1981. Fodrin: axonally transported polypeptides associated

with the internal periphery of many cells. *J. Cell Biol.* 90:631–643.

50. Loor, F. 1981. Cell surface-cell cortex transmembranous interactions with special reference to lymphocyte functions. *Cell Surf. Rev.* 7:255–335.

51. Means, A. R., J. S. Tash, J. G. Chafouleas, L. Lagace, and V. Guerrico. 1982. Regulation of the cytoskeleton by Ca^{2+}-calmodulin and cAMP. *Ann. NY Acad. Sci.* 383:69–81.

52. Myles, D. G., P. Primakoff, and A. R. Bellvé. 1981. Surface domains of the guinea pig sperm defined with monoclonal antibodies. *Cell.* 23:433–439.

53. Nicolson, G. L., N. Usui, R. Yanagimachi, H. Yanagimachi, and J. R. Smith. 1977. Lectin-binding sites on the plasma membranes of rabbit spermatozoa. *J. Cell Biol.* 74:950–962.

54. O'Connor, C. M., D. L. Gard, D. L. Asai, and E. Lazarides. 1981. Phosphorylation of intermediate filament proteins, desmin and vimentin in muscle cells. *Cold Spring Harbor Conf. Cell Proliferation.* 8:1157–1169.

55. Palfrey, H. C., W. Schiebler, and P. Greengard. 1982. A major calmodulin-binding protein common to various vertebrate tissues. *Proc. Natl. Acad. Sci. USA.* 79:3780–3784.

56. Peterson, R., L. Russel, D. Bundman, and M. Freund. 1978. Presence of microfilaments and tubular structures in boar spermatozoa after chemically inducing the acrosome reaction. *Biol. Reprod.* 19:459–466.

57. Pollard, T. D. 1981. Cytoplasmic contractile proteins. *J. Cell Biol.* 91 (3, Pt. 2): 156s–165s.

58. Primakoff, P., and D. G. Myles. 1983. A map of the guinea pig sperm surface constructed with monoclonal antibodies. *Dev. Biol.* 98:427–428.

59. Repasky, E. A., B. L. Granger, and E. Lazarides. 1982. Widespread occurrence of avian spectrin in nonerythroid cells. *Cell.* 29:821–833.

60. Russel, L. D., R. N. Peterson, T. A. Russel, and W. Hunt. 1983. Electrophoretic map of boar sperm plasma membrane polypeptides and localization and fractionation of specific polypeptide subclasses. *Biol. Reprod.* 28:393–413.

61. Sadoz, D., P. Gounon, E. Karsenti, and M.-E. Sauron. 1982. Immunocytochemical localization of tubulin, actin, and myosin in axonemes of ciliated cells from quail oviduct. *Proc. Natl. Acad. Sci. USA.* 79:3198–3202.

62. Sanger, J. W., and J. M. Sanger. 1975. Polymerization of sperm actin in the presence of cytochalasin-B. *J. Exp. Zool.* 193:441–447.

63. Schollmeyer, J. V., and M. J. Wheelock. 1982. Role of Ca^{2+}-activated protease in the acrosome reaction of sperm. *J. Cell Biol.* 79:160a. (Abst.)

64. Shapiro, B. M., R. W. Schackmann, and C. A. Gabel. 1981. Molecular approaches to the study of fertilization. *Annu. Rev. Biochem.* 50:815–843.

65. Sobue, K., K. Kanda, M. Inui, K. Morimoto, and S. Kakiuchi. 1982. Actin polymerization induced by calspectin, a calmodulin-binding spectrin-like protein. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 148:221–225.

66. Spruill, W. A., A. L. Steiner, L. L. Tres, and A. L. Kierszenbaum. 1983. Follicle-stimulating hormone-dependent phosphorylation of vimentin in cultures of rat Sertoli cells. *Proc. Natl. Acad. Sci. USA.* 80:993–997.

67. Steck, T. L., R. S. Weinstein, J. H. Straus, and D. F. H. Wallach. 1970. Inside-out red cell membrane vesicles: preparation and purification. *Science (Wash. DC).* 168:255–257.

68. Strauch, A. R., E. J. Luna, and J. R. LaFountain, Jr. 1980. Biochemical analysis of actin in crane-fly gonial cells: evidence for actin in spermatocytes and spermatids—but not sperm. *J. Cell Biol.* 86:315–325.

69. Tamblyn, T. M. 1980. Identification of actin in boar epididymal spermatozoa. *Biol. Reprod.* 22:727–734.

70. Tash, J. S., L. Lagace, D. R. Lynch, S. M. Cox, B. R. Brinkley, and A. R. Means. 1981. Role of cAMP-dependent protein phosphorylation in microtubule assembly and function. *Cold Spring Harbor Conf. Cell Proliferation.* 8:1171–1185.

71. Tilney, L. G. 1976. The polymerization of actin. II. How non-filamentous actin becomes nonrandomly distributed in sperm: evidence for the association of this actin with membranes. *J. Cell Biol.* 69:51–72.

72. Tilney, L. G. 1976. The polymerization of actin. III. Aggregates of nonfilamentous actin and its associated proteins: a storage form of actin. *J. Cell Biol.* 69:73–89.

73. Tilney, L. G., S. Hatano, H. Ishikawa, and M. Mooseker. 1973. The polymerization of actin: its role in the generation of the acrosomal process of certain echinoderm sperm. *J. Cell Biol.* 59:109–126.

74. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

75. Virtanen, I., V.-P. Lehto, E. Lehtonen, and R. A. Badley. 1980. Organization of intermediate filaments in cultured fibroblasts upon disruption of microtubules by cold treatment. *Eur. J. Cell Biol.* 23:80–84.

76. Virtanen, I., V.-P. Lehto, E. Lehtonen, T. Vartio, S. Stenman, P. Kurki, O. Wager, J. V. Small, D. Dahl, and R. A. Badley. 1981. Expression of intermediate filaments in cultured cells. *J. Cell Sci.* 50:45–63.

77. Weber, K., and M. Osborn. 1982. Microtubule and intermediate filament networks in cells viewed by immunofluorescence microscopy. *Cell Surf. Rev.* 7:2–55.

78. Weber, K., M. Osborn, W. W. Franke, E. Seib, U. Scheer, and W. Herth. 1977. Identification of microtubular structures in diverse plant and animal cells by immunological cross-reaction revealed in immunofluorescence microscopy using antibody against tubulin from porcine brain. *Cytobiologie.* 15:285–302.