ACTIN FILAMENT-MEMBRANE ATTACHMENT: ARE MEMBRANE PARTICLES INVOLVED?

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ABSTRACT

The association of actin filaments with membranes is an important feature in the motility of nonmuscle cells. We investigated the role of membrane particles in the attachment of actin filaments to membranes in those systems in which the attachment site can be identified. Freeze fractures through the end-on attachment site of the acrosomal filament bundles in *Mytilus* (mussel) and *Limulus* (horseshoe crab) sperm and the attachment site of the microvillar filament bundles in the brush border of intestinal epithelial cells were examined. There are no particles on the P face of the membrane at these sites in the sperm systems and generally none at these sites in microvilli. In microvilli, the actin filaments are also attached along their lengths to the membrane by bridges. When the isolated brush border is incubated in high concentrations of Mg^{++} (15 mM), the actin filaments form paracrystals and, as a result, the bridges are in register (330 Å period). Under these conditions, alignment of the particles on the P face of the membrane into circumferential bands also occurs. However, these bands are generally separated by 800–900 Å, indicating that all the bridges cannot be directly attached to membrane particles. Thus membrane particles are not directly involved in the attachment of actin filaments to membranes.

Actin filaments play an important role in many motile events in nonmuscle cells. In most cases the filaments are found in close association with membranes, generally the plasma membrane. Examples include cytokinesis in amphibian eggs (28), echinoderm eggs (35), and HeLa cells (34); amoeboid motion (10, 29); cytoplasmic streaming in *Nitella* (24, 17); motility of cultured fibroblasts (8, 15, 26); motility of microvilli (19, 21); phagocytosis (2, 5, 31); morphogenetic movements of embryonic epithelia (37); clot retraction (4, 44, 45); wound healing (16); lectin-induced capping (1); and the generation of the acrosomal process in echinoderm sperm (41), in *Limulus* sperm (38), and in *Mytilus* sperm (39). Since most of the above examples include movements related to the cell surface, clearly one function of the association of actin filaments with membranes is to couple the motile event with the cell surface. By anchoring one of the contractile proteins, generation of force and thus useful work could ensue.

This association of actin filaments with membranes would also account for two characteristics of actin filaments which must be considered in determining how the actin with or without other proteins provides movement in a nonmuscle cell. First, actin filaments are often located in specific regions in cells, e.g., they are abundant in the

cleavage furrow but sparse outside it, or they are present in microvilli but rare in the rest of the cytoplasm; and secondly, in order to provide directional movement, the actin filaments must be attached to a membrane with a precisely determined polarity. A mechanism for locating actin filaments in specific regions of cells with the requisite polarity could be achieved by having sites for the nucleated polymerization of actin filaments associated with specific regions on a membrane. Membrane-associated polymerization has now been documented for several systems. In microvilli, for example, an examination of stages in the reextension of microvilli after pressure-induced disassembly revealed that the actin filaments assemble from a dense material associated with the limiting membrane and from there elongate (40). Similar observation have been made by studying stages in *Mytilus* (18) and in *Limulus* (11) spermiogenesis, systems which are now known to contain actin filaments (38, 39). In these developing spermatids the filaments first appear attached to the acrosomal vacuolar membrane and with time they elongate. Many other cases undoubtedly exist as well, but, in order to distinguish between assembly of filaments from a membrane and secondary attachment of filaments to a membrane, one must follow stages in the assembly process—studies that have not yet been reported for other systems.

In microvilli and in *Mytilus* sperm, the polarity of the actin filaments relative to the membrane has been determined (21, 39). In both systems, all the filaments have the same polarity; the arrowhead complexes which are formed by the addition of myosin fragment, S_1, all point away from the membrane as if the membrane were replacing the Z line. Thus, the assembly of the filaments and the determination of polarity of the filaments may revolve around the same mechanism—controlled nucleation from specific sites on a membrane. Preliminary observations on the polarity of the filaments have been reported in several other systems which include the amoeba (30), platelets (4), and *Thyone* sperm (41). The polarity of the filaments relative to the membrane in these systems seems identical to that demonstrated in microvilli and in *Mytilus* sperm, yet it is not known whether the filaments are nucleated from the membrane or whether the filaments become attached to the membrane after assembly.

There is very little information available concerning the nature of the attachment of actin filaments to membranes from the point of view of the membrane. The simplest approach to this problem is to examine the membrane structure with the technique of freeze fracture. We have used this technique to determine whether membrane particles are involved in the attachment of actin filaments to the membrane. If particles are involved, the attachment of the actin filaments to membranes is by integral membrane proteins or proteins situated at least in part within the lipid bilayer. We selected for examination the only systems known in which the possible involvement of membrane particles in the attachment of actin filaments to membranes can *be easily* investigated. The morphology of these systems, as determined by thin sectioning, allows us to know precisely where actin filaments are attached to membranes. This is critical because the freeze-fracture technique which is used to resolve membrane particles cannot be used directly to visualize the involved filaments. For example, even though actin filaments are connected to isolated membrane preparations, i.e., the *Acanthamoeba* membrane (29), after freeze-fracturing it is extremely difficult to see whether an individual filament is attached to a particle or not. Thus, we have examined only those systems where we can easily and definitively answer the question as to whether filaments are attached to particles or not. Fortuitously, these systems include the only systems known in which the polarity of the filaments and their assembly on membrane sites have been unequivocally determined, i.e. the microvilli from intestinal epithelial cells and the acrosomal filament bundles in *Mytilus* sperm. We have also examined the acrosomal bundle in *Limulus* sperm. In microvilli and in *Limulus* and *Mytilus* sperm, actin filament bundles are attached to the membranes at one end. These attachment sites can be easily located in freeze-fracture replicas as the membrane fits overs the ends of the filament bundles like a glove so that the tip of each finger in the glove is obvious.

Actin filaments are also attached to the membrane all along their lengths in microvilli by cross bridges (21, 22). These bridges become periodic as the result of incubation in high concentrations of Mg^{++}. This is presumably caused by the formation of actin paracrystals *in situ* within the microvilli. If particles are involved in the attachment of the cross bridges to the membrane, one would expect the alignment of the cross bridges in the cytoplasm to result in a corresponding alignment of particles in the membrane, indicating cross bridge-particle attachment.

The results presented here clearly demonstrate that particles are not directly involved in the attachment of actin filaments to the membrane. A preliminary report of this work was presented at the 1975 meeting of the American Society for Cell Biology in Puerto Rico (20).

MATERIALS AND METHODS

Obtaining Sperm

The mussel *Mytilus edulis*, and the horsehoe crab, *Limulus polyphemus*, were collected by the supply department of the Marine Biological Laboratory in Woods Hole, Mass. The animals were maintained in instant ocean tanks. Sperm were obtained from *Limulus* as described by Tilney (38). Sperm were obtained from *Mytilus* by cutting out the gonad, mincing it with scissors in sea water and straining the solution through cheesecloth. The sperm were pelleted at 3,000 *g* at 0°C for 5 min.

Preparation of Sperm for Freeze-Fracture

Sperm were suspended in sea water at 0°C which contained 1% glutaraldehyde at pH 8.0 (Electron Microscope Sciences, Fort Washington, Pa.). Fixation was allowed to proceed for 10 min, at which time the sperm were pelleted (8,000 *g* for 5 min). The total time for fixation was 15 min, which included the centrifuge step. We found this to be critical, as longer fixation times resulted in fractures which cleaved only through the plasma membrane or the nuclear envelopes—cross fractures through the nucleus or the acrosomal vacuole or through the portion of the acrosomal vacuole membrane which lies directly anterior to the nucleus occurring only rarely. Since it is fractures through the latter that are pertinent to this study, the fixation time must be strictly controlled. After fixation the sperm were glycerinated in a graded series of glycerol solutions of 5%, 10%, 20%, 30% at 4°C. The sperm were incubated for 15–30 min in each solution and pelleted after each step. Because of the viscosity of the glycerol solution, at the last step the sperm were pelleted at 50,000 *g* for 30 min. The resulting pellet was a paste that was easily handled for freeze-fracturing.

Isolation and Preparation of the Brush Border for Freeze-Fracture

Brush borders were isolated by the procedure described in detail by Mooseker and Tilney (21). Preparations of isolated brush borders were incubated in a medium containing high Mg^{++} (15 mM $MgCl_2$, 75 mM KCl, 1 mM EGTA, 10 mM Imidazole, pH 7.3, 0.1 mg/ml soybean trypsin inhibitor [Sigma Chemical Co., St. Louis, Mo.]) at 0°C, for 30 min, in order to induce the alignment of the microvillar actin filaments into paracrystalline arrays (21). Control preparations were incubated in the same solution with 1 mM $MgCl_2$ substituted for 15 mM $MgCl_2$. The brush borders were pelleted (800 *g* for 5 min) and fixed for 15 min in 1% glutaraldehyde in 0.1 M phosphate buffer at pH 7.0. They were then transferred through a graded series of glycerol solutions of 10%, 20%, 30% for 10 min each. The brush borders were pelleted at high speed (50,000 *g* for 30 min) after incubation in 30% glycerol. All steps were carried out at 4°C.

Techniques of Freeze-Fracture

The pellets of sperm or brush borders in 30% glycerol were transferred to specimen holders (Denton Vacuum Co., Cherry Hill, N. J.). They were rapidly frozen in Freon 22 cooled with liquid N_2. The specimens were fractured in a Denton freeze-fracture apparatus at −115°C. Some specimens were etched for 1 min at −100°C. The replicas were then digested with Chlorox and transferred to grids and examined with a Philips 200 electron microscope.

Preparation of Samples for Thin Sectioning

Limulus sperm were fixed and processed for thin sectioning as outlined by Tilney (38). *Mytilus* sperm were fixed in 1% glutaraldehyde in sea water at pH 8.0 for no more than 30 min, washed briefly in sea water, and postfixed at 4°C in 1% OsO_4 in 0.1 M phosphate buffer at pH 6.0 for 45 min. They were dehydrated rapidly in acetone and embedded in Araldite. The brush borders were prepared for thin sectioning as described by Mooseker and Tilney (21). The thin sections were stained with uranyl acetate and lead citrate and examined with a Philips 200 electron microscope.

RESULTS

End-on Attachment of Actin Filaments To Membranes

MYTILUS SPERM: The basic morphology of *Mytilus* sperm has been described by Niijima and Dan (23) and Bourcart et al. (6) and the development by Longo and Dornfeld (18). Located in the center of this sperm is a bundle of filaments (39). Since the basic morphology is known, we will repeat here only those features which are pertinent to an understanding of the attachment of the actin filaments to membranes.

The acrosomal vacuole in *Mytilus* sperm is a hollow cone that sits on top of the nucleus (Figs. 1–3). The actin filament bundle extends basally from the membrane that covers the inner surface of the apex of the cone through a canal in the center of the nucleus towards the flagellum. While in the nucleus, it is separated from the chromatin

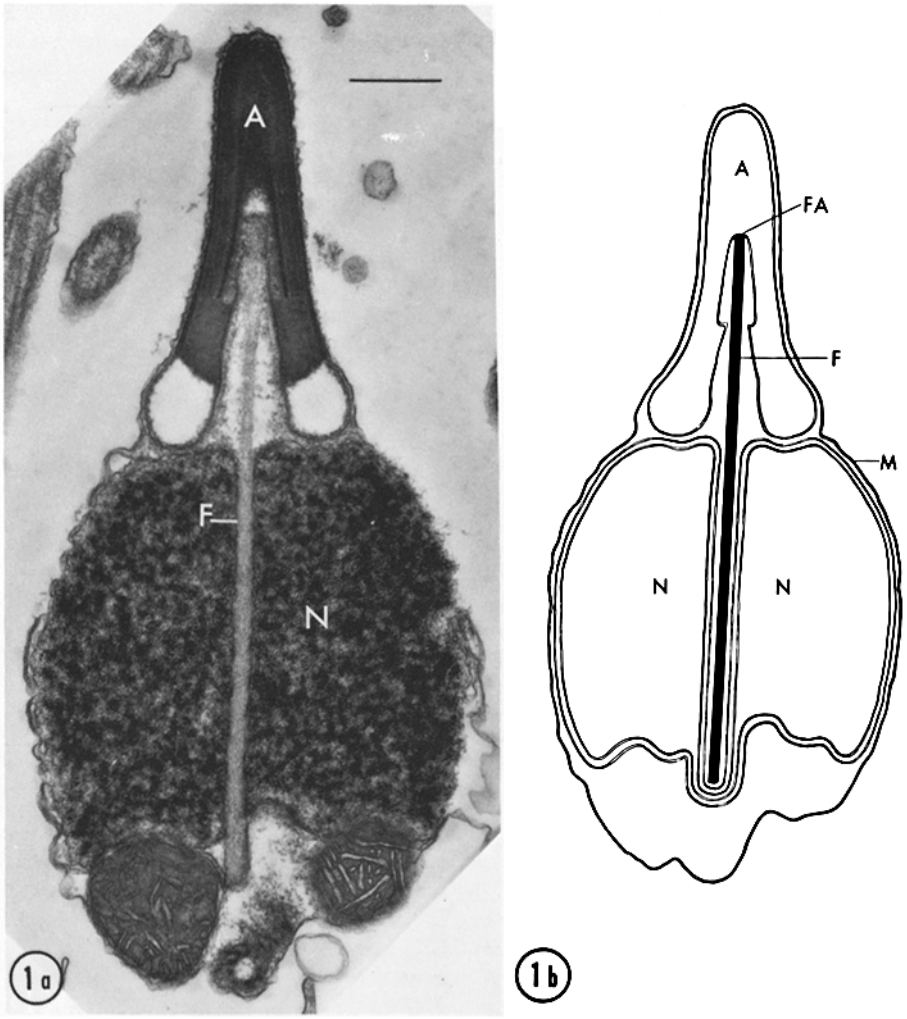

FIGURE 1 (*a*) Longitudinal section through a *Mytilus* spermatozoon. Of particular interest is the bundle of actin filaments (*F*) which extends from the membrane limiting the acrosomal vacuole (*A*) through a canal in the center of the nucleus (*N*) to the mitochondrial region. Scale is 0.5 μm. × 23,000. (*b*) Drawing of *Mytilus* sperm illustrating the positions of the membranes which limit the acrosomal vacuole (*A*), the nucleus (*N*), and the cell (the plasma membrane (*M*)). The filament bundle (*F*) is attached to the acrosomal vacuole membrane at the position labeled *FA*. In order to visualize the P surface of this membrane, the fracture must pass through the plasma membrane, then through the acrosomal vacuole.

by the nuclear envelope. This morphology is easy to understand by studying spermiogenesis. Longo and Dornfeld (18) demonstrated that the filaments first appear attached to the under surface of the acrosomal vacuole and then elongate posteriorly. As they extend, they push their way into the nucleus as one pushes one's finger into a soft rubber balloon. Since the filament bundle in mature sperm attaches to the membrane at the apex of the hollow cone, this point of attachment is easily recognizable in thin sections or by freeze-fracturing even though the filaments cannot be seen. Since the acrosomal vacuole exists as a cone, it is clear that in order to visualize the cytoplasmic or P face (we are using here the jargon of Branton et al., [7]) or that leaflet that is associated with the actin filaments, we must first pass through the plasma membrane, then through the acrosomal vacuole membrane which limits the outer surface of the cone, and finally through the outer or E face

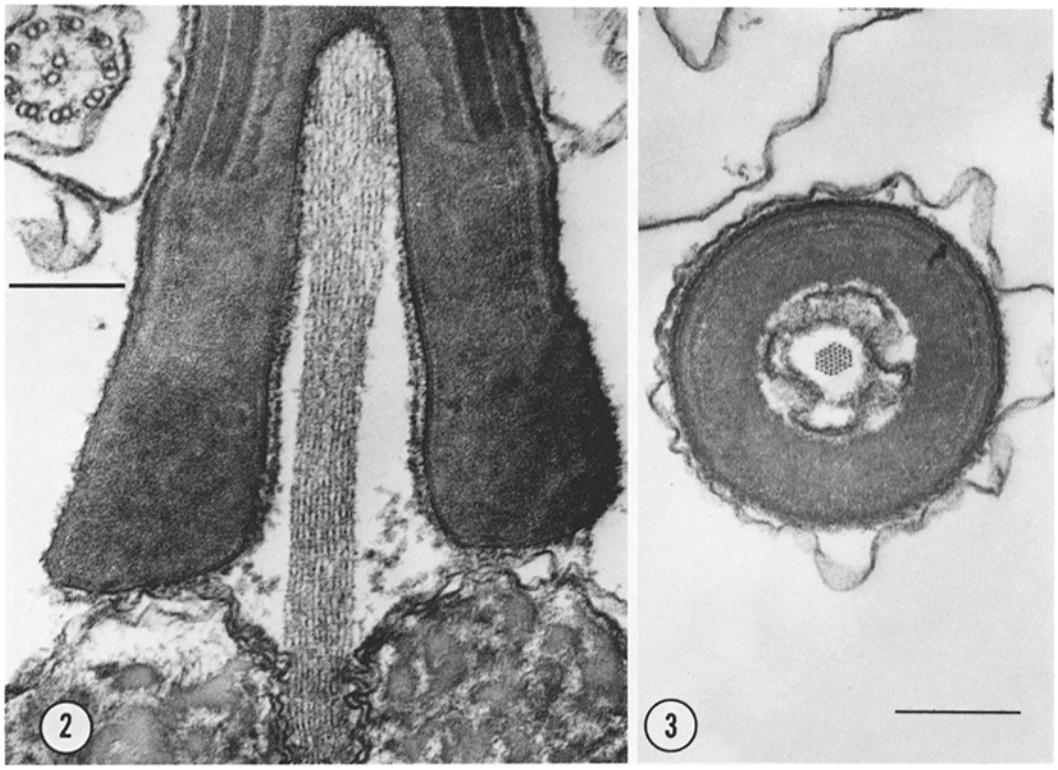

FIGURE 2 Thin section through the anterior end of a *Mytilus* spermatozoon. Note that the actin filament bundle is attached to the acrosomal vacuole membrane. Scale is 0.2 μm. × 81,000.

FIGURE 3 Transverse section through the acrosomal region of a *Mytilus* spermatozoon. Note the precise hexagonal packing of the actin filaments in the bundle. Scale is 0.25 μm. × 66,000.

of the inner acrosomal vacuole membrane (see Fig. 1*b*). We will then be examining the cytoplasmic (P) face of the membrane limiting the inner surface of the acrosomal vacuole. This is the membrane to which the actin filaments attach. The cytoplasmic face of this membrane is particle-free including the site of actin filament attachment as revealed by transverse fractures through the apex of the inner vacuole membrane (Fig. 4). The fine granularity seen upon close examination is present on all membranes and is a characteristic of the platinum replica rather than the biological topography of the membrane. Unlike the P face of the inner acrosomal vacuole membrane, the other membranes in *Mytilus* sperm all contain particles, as, for example, the P or E faces of the plasma membrane or the E face of the outer vacuole membrane.

LIMULUS SPERM: The overall morphology of *Limulus* sperm is similar to that of *Mytilus* sperm. A bundle of filaments extends from the membrane limiting the lower surface of the acrosomal vacuole through a canal in the nucleus; this bundle extends even farther and forms a coil at the base of the sperm (3, 11, 38) (Fig. 5).

In an excellent paper on spermiogenesis, Fahrenbach (11) demonstrated that a short bundle of filaments can first be seen extending posteriorly from the acrosomal vacuole membrane. These filaments then elongate; while doing so, they "push" the nuclear membrane in front of them. As with *Mytilus*, the acrosomal vacuole takes the form of a shallow, yet hollow, cone which sits on the anterior surface of the nucleus. The filaments then extend from the apex of the lower surface of the cone, a fact which allows us to readily localize by freeze-fracturing the association point of the actin filaments with the membrane. In order to expose the membrane to which the actin filaments are attached, the P face of the inner acrosomal vacuole membrane, the fracture must pass through the center of the sperm. Fractures exposing this

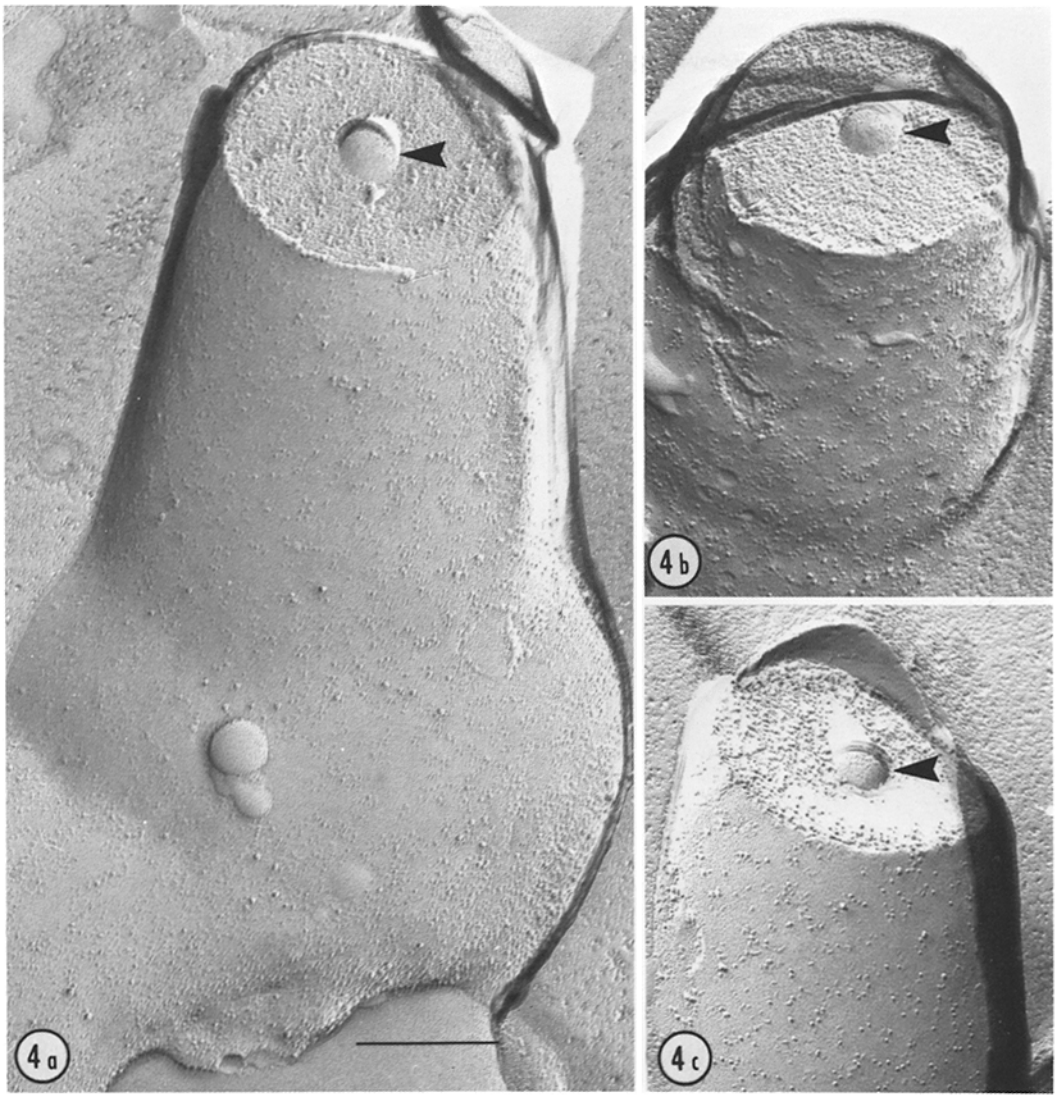

FIGURE 4 Freeze-fractures of *Mytilus* sperm revealing the P face of the membrane at the site of the attachment of the actin filaments to the membrane. This region is indicated by the arrows. Scale is 0.25 μm. (*a*) × 75,000; (*b*) × 75,000; (*c*) × 75,000.

face of the membrane at the apex of the cone, the site of filament attachment, are particle free (Figs. 6 and 7). There are particles on the other membrane surfaces and, in fact, a few particles on the P surface of the inner acrosomal membrane except at the point of attachment of the filaments, indicating that the lack of particles at the site of attachment is not due to insufficient platinum deposition or to an improper shadow angle (Fig. 6).

THE BRUSH BORDER: Each microvillus in the brush border of intestinal epithelial cells contains a bundle of 20–30 actin filaments (15, 21, 42) (Fig. 8). Each filament bundle is embedded in a dense matrix at the tip of the microvillar membrane. Antibodies prepared against α-actinin, the main protein of the Z line of skeletal muscle, react *in situ* with this dense matrix (33). Numerous particles are present on the P face of the microvillar membrane. The E face contains fewer particles (Fig. 9). Whereas these particles are randomly distributed along the length of the microvillar membrane, replicas of fractures through the tips

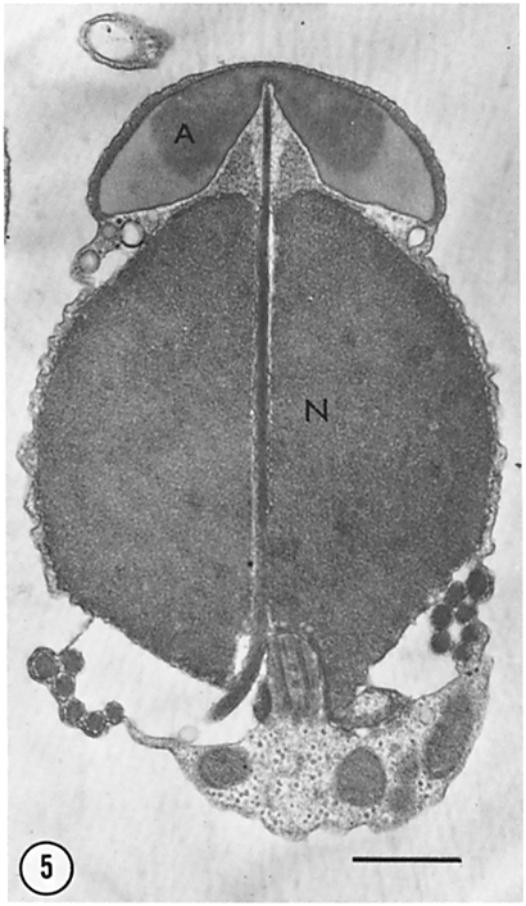

FIGURE 5 Longitudinal section through a *Limulus* spermatozoon. The actin filament bundle extends from the center of the acrosomal vacuole (*A*) through a canal in the center of the nucleus (*N*) to the basal end of the cell. The distribution of membrane is similar to that of *Mytilus* sperm. See Fig. 1*b*. From Tilney (38). Scale is 0.5 μm. × 29,000.

of the microvillar membrane usually show a conspicuous absence of particles on both the P and E faces (Fig. 10). This substantiates the earlier observations of Mukherjee and Staehelin (22) and the recent observations of Perrelet (27).

The Lateral Attachments between Actin Filaments and the Membrane

Mukherjee and Staehelin (22), using freeze-fracture techniques, demonstrated in intact intestinal epithelial cells the existence of cross bridges connecting the actin filament bundles along their lengths to the membrane. By incubating isolated brush borders in high Mg^{++} (15 mM), we demonstrated (21) that the cross bridges become periodic, repeating every 330 Å (Fig. 11). We assume that this is due to the induction of actin paracrystals *in situ*. This assumption is based on the fact that Mg^{++} induces actin filaments or thin filaments isolated from muscle to align (13) with all the crossover points of the actin helices in transverse register. If the actin filaments are aligned into a paracrystalline array, then the periodicity of the bridges must indicate that each bridge is attached at precise positions along the actin helix. (A full description of these conclusions has been reported by Mooseker and Tilney [21]). We have made use of the Mg^{++}-induced periodicity of the cross bridges to determine whether these bridges connect the actin filaments to particles in the plane of the membrane. If a set of membrane particles were coupled to the cross bridges, then the alignment of the bridges into periodic register as a result of Mg^{++} incubation should also result in the alignment of a set of membrane particles. This would probably appear as bands of particles on the cytoplasmic fracture face separated by a distance related to the bridge period in the cytoplasm, i.e., either equal to or a harmonic of the 330 Å spacing.

Examination of brush borders treated with high Mg^{++} indicate a marked redistribution of membrane particles. This is particularly evident in the P face where there are a large number of particles. The particles often aggregate into circumferential bands along the lengths of the microvilli. These bands of particles are spaced at fairly regular intervals repeating every 860 Å ± 110 Å. Generally, one sees less variation in band spacing within a single microvillus than if one compares the band spacing in two different microvilli. There is a large variation in the number of particles per band and, as a result, in band width. It is important to note that *all* the particles are aggregated; rarely do we see particles in the bare zone present between bands. The spacing of particles (860 Å) is obviously much greater than the spacing of the bridges (330 Å).

DISCUSSION

End-On Attachments of Actin Filaments to Membranes

The results presented in this report demonstrate that membrane particles are not involved in the

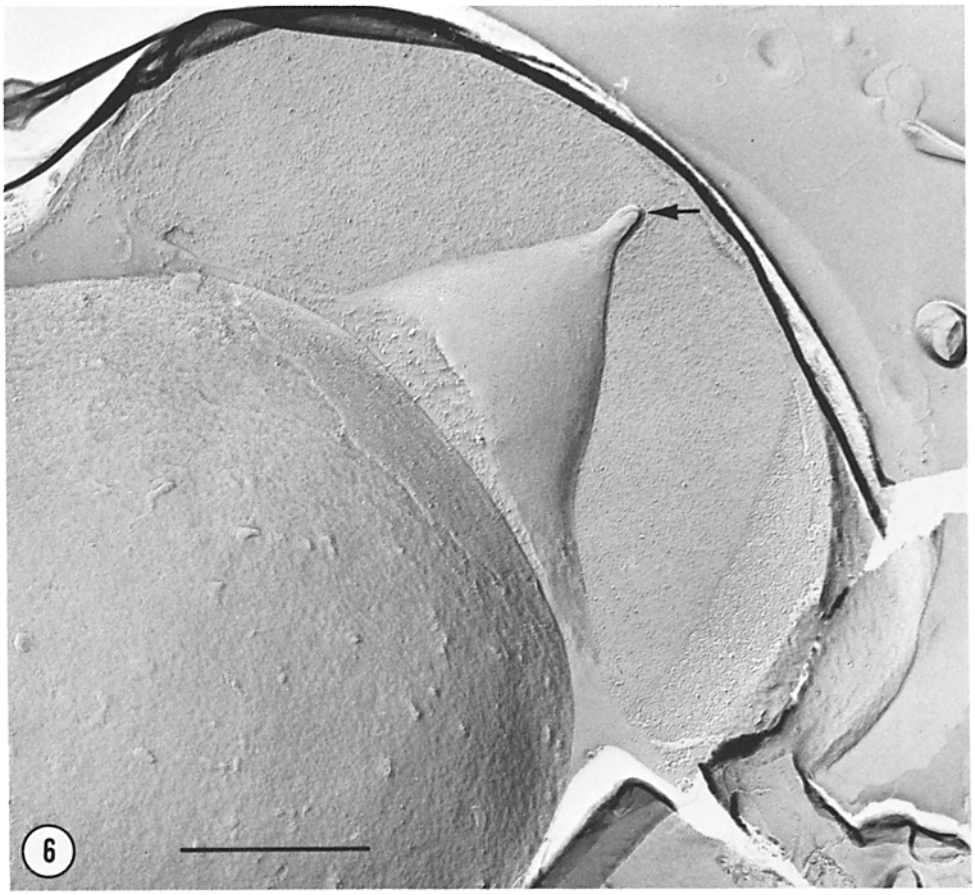

FIGURE 6 Freeze-fracture exposing the P face of the acrosomal vacuole membrane in a *Limulus* spermatozoon. The site of actin filament-membrane attachment is indicated by the arrow. Scale is 0.5 μm. × 49,000.

end-on attachment of actin filaments to membranes.

The noninvolvement of particles in the attachment is not surprising, given what we can deduce from the available evidence concerning the molecular weight of the proteins represented by membrane particles. It is not clear what the minimum molecular weight of a particle is, although it is probable that a particle represents a component which exceeds 100,000 daltons. There are three reports which are relevant here; one on rhodopsin, a second on the particles in the gap junction, and a third on glycophorin. The main difficulty in interpreting the available data is to know what proportion of these proteins is visible in replicas of membranes and how many copies of the proteins comprise each particle (see Segarest et al., [36]). Chen and Hubble (9) demonstrated that purified rhodopsin can be reassociated with lipid bilayers. When these bilayers are fractured, particles are present showning that the rhodopsin is located in the bilayer, a conclusion strengthened by the recent work of Sardet et al. on the association of detergents with rhodopsin (32). Rhodopsin is thought by most investigators to exist in membranes as a dimer or tetramer, suggesting that a minimum mol wt for a rhodopsin particle is about 100,000 daltons. There is evidence for a similar minimum mol wt (120,000 daltons) for the particles in the gap junction. Each particle appears to be made up of six subunits of the protein, connexin, whose monomeric mol wt is about 20,000 (12). The experiments of Segarest et al. (36) have addressed this problem more directly. They calculated that each particle formed by adding tryptic fragments of glycophorin contained 16–20 frag-

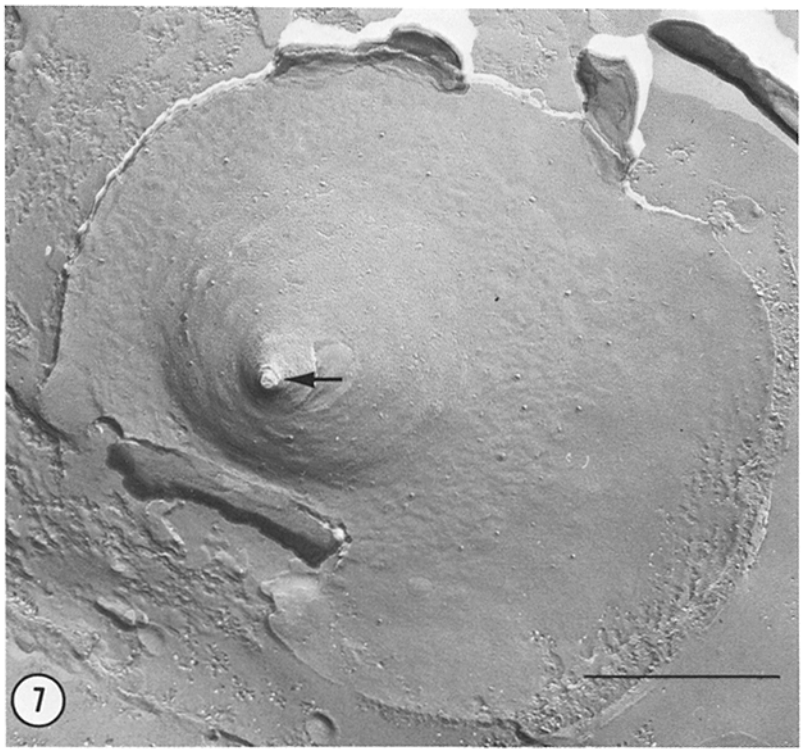

FIGURE 7 Freeze-fracture through the acrosomal vacuole membrane in a *Limulus* spermatozoon at the site of attachment of the actin filament bundle. The site of interest is indicated by the arrow. Scale is 0.5 μm. × 51,000.

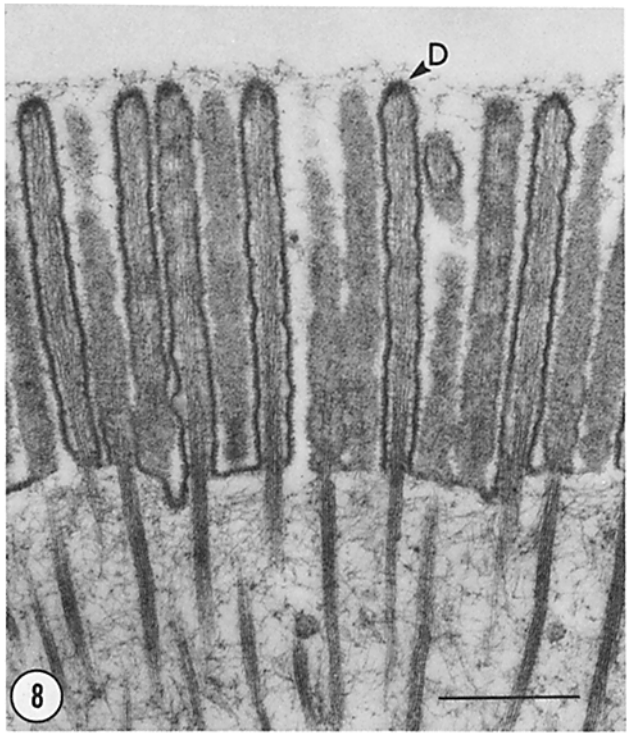

FIGURE 8 Thin section through the brush border of an intestinal epithelial cell. The actin filament bundles are inserted into a dense matrix (*D*) at the tips of each microvillus (from Mooseker and Tilney [21]). Scale is 0.5 μm. × 37,000.

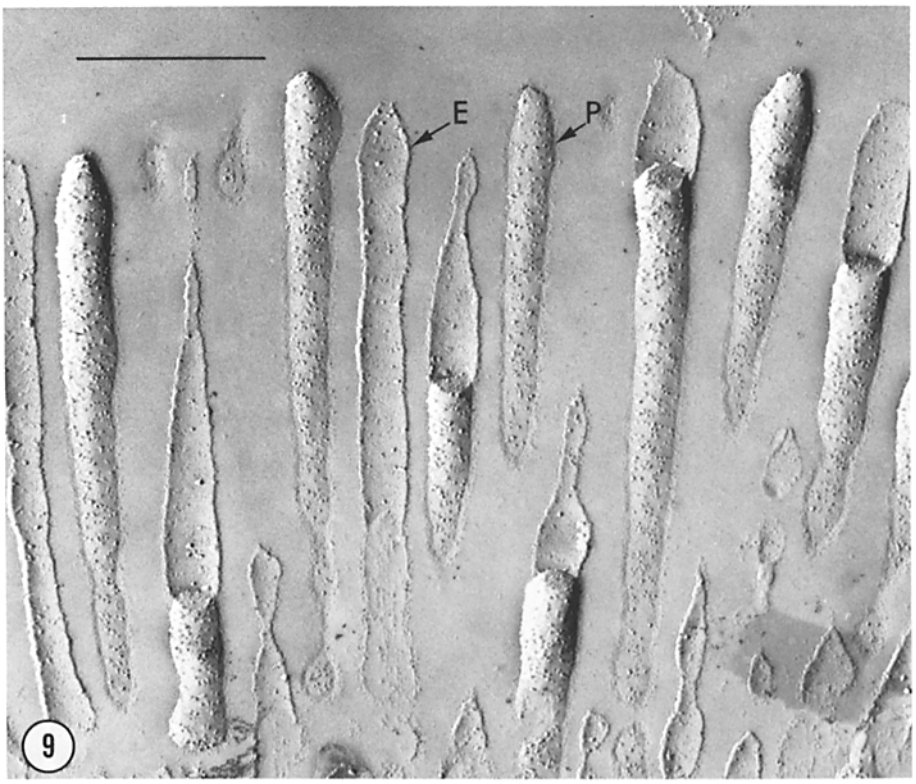

FIGURE 9 Freeze-fracture through microvilli of an isolated brush border. The *P* and *E* faces are indicated. Scale is 0.5 μm. × 48,000.

ments. The molecular weight of these fragments determined from the recent sequence data by Tomita and Marchesi (43) is about 6,000–7,000 daltons. Thus, the minimum mol wt of these "artificial" membrane particles is 90,000–120,000. From these three reports we conclude that if actin filaments are associated with integral membrane proteins, the size of these proteins is likely to be less than 100,000 daltons, perhaps considerably less.

There remain three alternative mechanisms for the association of actin filaments with membranes. This association could be brought about (*a*) by integral membrane proteins which are not resolved by the freeze-fracture technique, (*b*) solely by association of actin filaments with peripheral membrane proteins that do not enter the bilayer, or (*c*) by association with peripheral membrane proteins which in turn are coupled to integral membrane components which are not resolved by the freeze-fracture technique. In microvilli, we already know that a peripheral membrane component, the dense matrix, which is composed at least in part of an α-actinin-like protein, is involved in the attachment of actin filaments to the microvillar membrane.

From the work of Tilney and Cardell (40), we know that this dense matrix is also involved in the nucleated assembly of microvillar actin filaments. The important question is, then, how positional information is stored in the membrane so that specific association of the dense material can occur which, in turn, allows for nucleated assembly of actin filaments from the membrane. The only available information that suggests that the membrane could have such positional information is that the region of the membrane associated with the dense tip material is biochemically different. This region of the membrane resists solubilization with the detergent, Triton X-100 (see Fig. 7, Mooseker and Tilney [21]). Differences in membrane solubility are thought to be conferred by variations in the kinds or amounts of proteins associated with the membrane rather than by differences in the lipid composition of the membrane (14). It is, of course, impossible to know

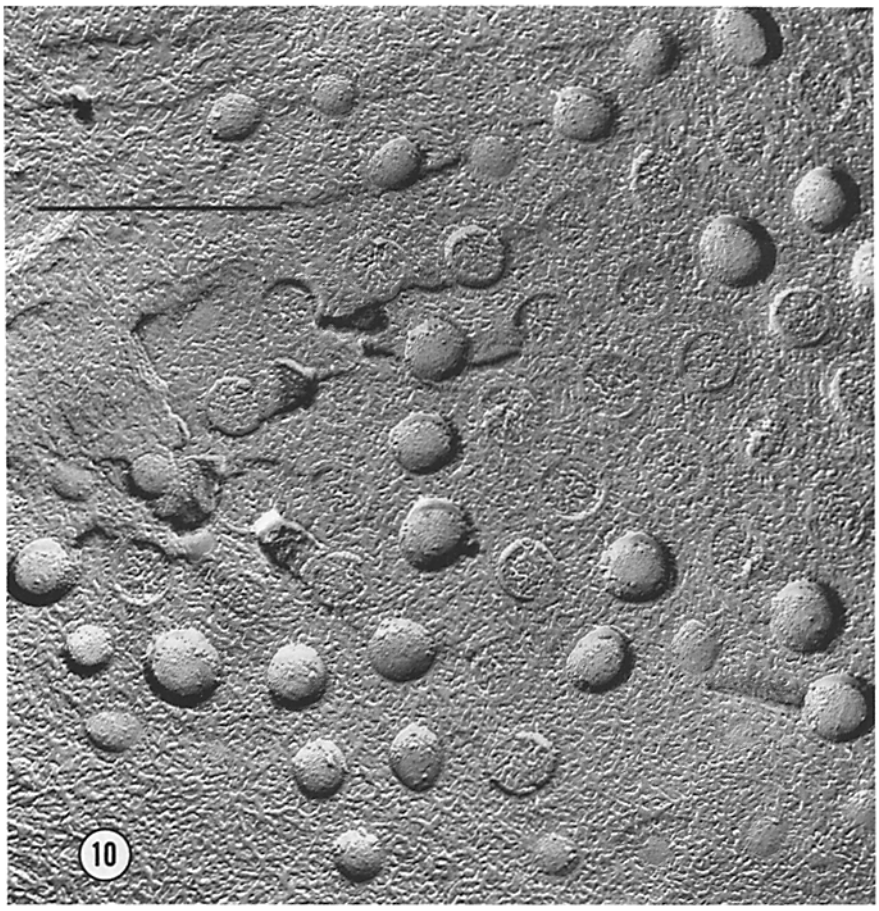

FIGURE 10 Freeze-fracture through the tips of microvilli; the P faces are exposed. Scale is 0.5 μm. × 66,000.

whether the membrane components responsible for this solubility difference were present *before* the association of the dense material with the membrane.

Lateral Attachment of Actin Filaments to Membranes by Bridges

The above considerations also apply to the connections of the cross bridges to the microvillar membrane. Although particle redistribution does occur as the result of incubation in high Mg^{++}, the center-to-center separation of the bands of particles is about 850 Å, not the 300–400 Å one would expect if the aggregation were caused by alignment of the bridges in the cytoplasm. From the differences in these periodicities, it seems unlikely that all of the bridges would attach to particles because many must contact an area of the membrane between adjacent bands of particles. Nor would one expect all the particles in the membrane to be involved in cross bridge attachment because some of the particles must be related to the many transport systems that are found in the brush border membrane (after all, its function is that of an absorbing epithelium), yet all the particles are aggregated. It is interesting to note, therefore, that in replicas of embryonic intestinal tissue where transport functions have not yet fully developed, the microvillar membranes are particle free. These microvilli do contain a core of actin filaments as in the adult (D. Burgess and J.-P. Revel,

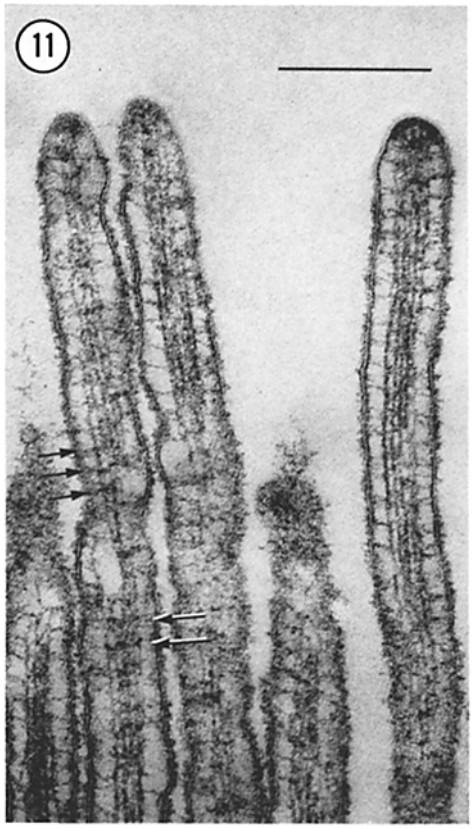

FIGURE 11 Microvilli of an isolated brush border in a solution containing 15 mM $MgCl_2$. Note the cross bridges connecting the actin filament bundles to the membrane. Lateral striations are present on one of the filament bundles (arrows). From Mooseker and Tilney (21). Scale is 0.2 μm. × 100,000.

personal communication). Thus, membrane particles do not seem to be *directly* involved in cross bridge attachment of actin filaments to membranes.

When we submitted this manuscript for publication, one of the reviewers suggested to us a model in which the bridges could be associated with the particles. Although the sum of our evidence suggests that such a model is unlikely, we cannot eliminate it from consideration. Thus, we would like to present evidence for and against it. Since microvilli are 1,000 Å in cross section and since thin sections for electron microscopy are routinely 400–500 Å thick, a longitudinally sectioned microvillus would contain bridges from more than one actin filament. Let us assume that, after treatment with magnesium, the bridges spiral around the core filaments, and that adjacent spirals are separated by 900 Å. Thus, if we consider just one filament, the nearest distance between adjacent bridges attached to that filament would be 900 Å. Since thin sections could contain 3–4 filaments in depth, we might see a 330 Å spacing by observing the bridges connected to several superimposed filaments. For this model, therefore, the particles in the membrane should be grouped into a helical pattern resulting in a barber-pole appearance in freeze-fracture images. Inspection of the micrographs, in fact, reveals that the particles often do seem to be grouped in a barber-pole array. These observations, then, would support a model in which the bridges would be coupled to particles.

There are several observations which argue against such an interpretation. First, as is the case in Fig. 11, we have observed lateral striations on the filament bundles that appear to be continuous with the cross bridges and that have the same periodicity as the bridges (330 Å) (see arrows). These striations are best attributed to cross and/or oblique sections of bridges extending in an out of the plane of section. These striations do not cross the microvillar filament bundles at the acute angle one would expect if the bridges were spirally wound around the filament bundle.

Secondly, there are large differences not only in the number of particles in the various bands but also in the pitch of the bands (Fig. 12). In some bands, the pitch is negligible; in others, it seems to reverse in the same microvillus (see Fig. 12). And, finally, in oblique fractures, which are deeply etched, we do find bridges, but often these bridges do not contact particles on the "P" face of the membrane. On the basis of these three observations, we feel that such a model is unlikely.

The aggregation of the particles might be simply an effect of the magnesium on the system, but we are left with the puzzling observation that the particles aggregate into regular bands, not the random clumps as is true of particle aggregation in other systems (i.e., the erythrocyte membrane). One explanation for this is that the observed particle aggregation may be brought about as a result of the magnesium treatment, and the aggregation into regular bands may be the result of a damming effect brought about by structural components within the membrane that are not resolved by the freeze-fracture technique.

FIGURE 12 Freeze-fracture of brush border incubated in 15 mM $MgCl_2$. Scale is 0.5 μm. × 68,000.

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