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Detection and Ultrastructural Localization of Human Smooth Muscle Myosin-Like Molecules in Human Non-Muscle Cells by Specific Antibodies

(non-muscle cell myosin/ferritin-antibodies/membrane fluidity/erythrocytes)

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ABSTRACT **Spectrin, a protein complex which is peripherally attached to the cytoplasmic surface of the human erythrocyte membrane, cannot be detected (by complement fixation with anti-spectrin antibodies) in homogenates of several different human non-muscle cells studied. On the other hand, a protein antigenically identical or similar to human smooth muscle myosin was detected (by complement fixation with antibodies to uterine smooth muscle myosin) in these cells. In the case of human fibroblast line WI38, this smooth muscle myosin-like component was shown (by ferritin-antibody experiments in electron microscopy) to be at least partly associated with the cytoplasmic surface of the plasma membrane of the cell. It is proposed that the spectrin complex of the erythrocyte membrane and the smooth muscle myosin-like component of the fibroblast membrane play similar roles in regulating the translational mobilities of integral proteins in their respective membranes.**

It is generally accepted that biological membranes are two-dimensional solutions of proteins [more specifically, "integral" proteins (1, 2)] in a lipid bilayer, as described by the fluid mosaic model of membrane structure (3). It has been shown with a variety of membranes that these integral proteins can undergo rapid translational motion within the fluid lipid bilayer in the plane of the membrane. On the other hand, in some cases such rapid translational motion of integral proteins seems to be inhibited. The membrane of the adult human erythrocyte is an example (for review, see ref. 4). For this intact cell, it has been postulated (2, 4) that the protein complex called spectrin (5), which is attached to the cytoplasmic surface of the erythrocyte membrane (6), is responsible for the immobilization of integral proteins in the membrane (see *Discussion* below). We wished to determine whether spectrin was also associated with other types of mammalian cell plasma membranes, and if not, whether some other similar protein system was. To this end, we have undertaken an immunochemical and immunocytological study with several human non-muscle cells, utilizing antibodies prepared to human erythrocyte spectrin and to human uterine smooth muscle myosin. The first results of this study, reported here, show that spectrin is not antigenically detectable in the non-erythrocyte cells examined, but that a component that cross-reacts antigenically with smooth muscle myosin is found associated with these cells. In the one case (a human fibroblast cell line) so far examined by immunoferritin staining in the electron microscope, part of this smooth muscle myosin-like component was found in association with the cytoplasmic surface of the plasma membrane of the cell.

MATERIALS AND METHODS

Cells and Cell Homogenates. Human WI 38 fibroblasts were provided by Dr. Paul Price and were grown and harvested by trypsin treatment as described previously (7). HeLa cells grown in spinner culture were supplied by Dr. John J. Holland. Human peripheral blood lymphocytes from normal donors were provided by Dr. John Mendelsohn and were isolated free of erythrocytes (8). Platelet concentrates were purchased from the San Diego Blood Bank and were freed of residual erythrocytes by repeated differential centrifugation.

Homogenates of WI 38 fibroblasts, HeLa cells, and lymphocytes were prepared for complement fixation studies as follows: the harvested cells were washed three times by centrifugation with 0.15 M NaCl and resuspended in 0.5 ml of cold 0.2 M sucrose/20 μM Tris·HCl buffer (pH 7.4). The cold cell suspension was homogenized for 1 min in a Virtis model 45 blade homogenizer at 50% of top speed. After three cycles of freeze–thawing the samples were assayed for protein content by Lowry analysis and for antigenic activity by quantitative microcomplement fixation (9). For these experiments, platelets were disrupted by osmotic shock after slow equilibration with 40% glycerol (10), and human erythrocyte ghosts were prepared from fresh normal blood by a published method (11) and then were briefly sonicated.

For electron microscopy studies, WI 38 cells and erythrocytes were made permeable to ferritin-antibody conjugates by using a milder method so as to preserve a significant degree of morphological integrity. These cells were first treated with 1 mM $ZnCl_2$ (12) for 15 min at room temperature, and then were gently homogenized in a Dounce homogenizer. Centrifugation at 2000 $\times$ *g* for 10 min produced a pellet which was washed twice with 10 mM Tris·HCl/0.15 M NaCl (pH 7.5). After two cycles of freeze–thawing, the pellets were labeled with ferritin-antibody as follows: 250 μg of homogenate pellet protein was incubated with 250 μg of the γ-globulin fraction of anti-myosin antisera or with 100 μg of anti-spectrin antibodies purified by affinity chromatography, for 30 min at room temperature. Following two washes with isotonic saline, the pellets were then suspended in 0.2 ml of isotonic buffer containing 100 μg of a ferritin-conjugate of affinity-purified goat antibodies to rabbit IgG. Following another two washes, the cells were fixed with 2% glutaraldehyde, post-fixed with 2% OsO_4, and then processed and embedded in Epon 812. Sections were stained with 2% uranyl acetate and examined with a Phillips EM 300 electron microscope.

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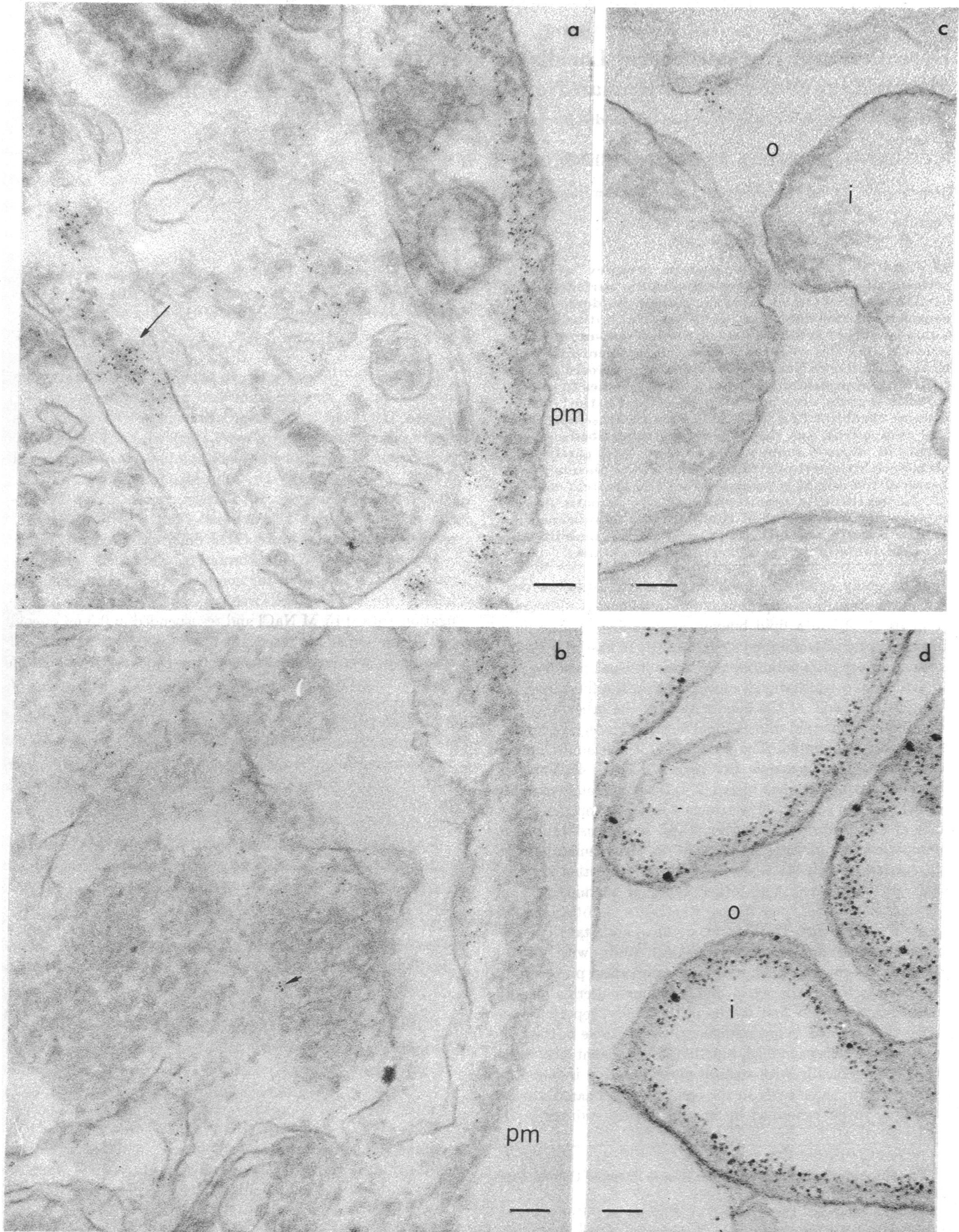

FIG. 1. (*Legend appears at bottom of the next page.*)

Details concerning the preparation and characterization of human erythrocyte spectrin and human uterine smooth muscle myosin, the preparation of the rabbit and goat antisera, and the isolation, characterization, and ferritin-conjugation of the antibodies, are presented elsewhere (13;§).

RESULTS

The human erythrocyte spectrin and human uterine myosin used as antigens were pure proteins according to electrophoresis in 1% sodium dodecyl sulfate/3.25% polyacrylamide gels. Ouchterlony double diffusion analysis showed that only a single sharp precipitin line formed between the anti-myosin antiserum and both the purified myosin and a crude soluble 0.5 M KCl extract of the WI 38 fibroblasts. Furthermore, absorption of this antiserum with the purified uterine myosin removed all reactivity to the crude soluble extract of the fibroblasts. Similarly, the anti-spectrin antibodies showed mono-specificity in their reaction with spectrin.

The results of complement fixation assays for myosin and spectrin in cell homogenates of WI 38 fibroblasts, peripheral lymphocytes from normal donors, HeLa cells grown in spinner culture and erythrocyte ghost membranes are summarized in Table 1. All cells examined, with the notable exception of the erythrocyte, demonstrated smooth muscle myosin-like antigenic activity comprising 1–3% of the total cell protein. The inner membrane surface of the erythrocyte was clearly available for reaction, since anti-spectrin antibodies reacted with spectrin, known to be localized exclusively on the interior membrane face (6). Additionally, exogenous uterine myosin added to the erythrocyte ghosts prior to sonication gave a positive complement fixation, indicating that myosin is not denatured during preparation of the ghosts.

Spectrin, on the other hand, was not detected in significant amounts in the non-erythrocyte cell types examined. Spectrin added exogenously to the fibroblasts prior to sonication reacted positively, indicating that if any endogenous activity was present, it was not lost due to autolysis prior to assay.

Fig. 1a shows an Epon section of a partially homogenized $ZnCl_2$-treated WI 38 fibroblast cell treated with three equivalents (determined by complement fixation) of rabbit antibodies to smooth muscle myosin and, after excess antibodies were washed away, treated with ferritin-conjugated goat antibodies to rabbit IgG. Homogenates prepared in this manner consisted of disrupted cells and organelles but, in addition, relatively intact cells which were permeable to ferritin-antibody conjugates. Extensive and fairly uniform labeling was found directly underlying the inner plasma membrane (pm) surface of such a cell. In addition to the labeling patterns shown, ferritin-antibody was also found attached to the outer surfaces of some (but not all) smooth membrane vesicles found in the cellular interior. No nuclear labeling was found. No labeling of the outer surface of the plasma membrane was ever seen. As a specificity control, antiserum preabsorbed with purified myosin did not show labeling in any cell regions.

§ M. Sheetz, R. G. Painter, and S. J. Singer, manuscript in preparation.

TABLE 1. *Reaction of disrupted human cells and proteins with anti-myosin and anti-spectrin**

Disrupted Cell	Concentration of protein required for maximum complement fixation†‡ (μg/ml)		Percent of total protein	
	Anti-myosin	Anti-spectrin	Anti-myosin§	Anti-spectrin
WI 38 fibroblast	25	NR(100)	1.8	<0.1
Lymphocytes	35	NR(140)	1.3	<0.07
HeLa (spinner)	25	NR(200)	1.8	<0.05
Platelets	20	NR(263)	2.3	<0.04
Erythrocyte ghosts (sonicated)	NR(200)	6.7	<0.05	9.0
Myosin	0.45	NR(3.9)	100	—
Spectrin	NR(5)	0.60	—	100

* Some of these experiments were performed by Gloria Ma during a laboratory project.

† Homogenate or protein (0.2 ml) at the stated concentration was allowed to react overnight with 0.2 ml of anti-myosin antiserum (diluted 1/400) or anti-spectrin antibodies (diluted to 1 μg/ml) and 0.2 ml of complement, all in a total volume of 1.2 ml. The minimum concentration of material required for 90% fixation of complement is listed.

‡ NR, no reaction; the figures in parentheses indicate the highest concentration tested. In the case of no detectable reaction the maximum possible concentration which could have been detected was 0.1 μg/ml in both cases.

§ Assuming 100% cross-reaction of the antigen with the uterine smooth muscle myosin.

When anti-spectrin antibodies were utilized, no specific labeling was found in any cell area even though ferritin conjugates had clearly penetrated into the cell interior (Fig. 1b).

Erythrocytes prepared identically show a pattern of labeling with the two antibodies which is opposite of that of the fibroblasts. Fig. 1c shows a complete lack of labeling with anti-myosin antibodies (30 out of 30 ghosts examined were negative) while anti-spectrin antibodies, as expected, gave heavy labeling (Fig. 1d) of the inner plasma membrane surface (16 out of 30 ghosts examined were so labeled, the unlabeled ghosts presumably having become resealed before antibody treatment).

DISCUSSION

These results demonstrate that spectrin, a protein complex attached to the cytoplasmic surface of the human erythrocyte membrane, cannot be detected antigenically in the several different human non-muscle cell lines studied. On the other hand, there is a protein component present in all of these non-muscle cells that cross-reacts antigenically with human uterine smooth muscle myosin, but is not detectable in the erythrocyte. In the case of the human fibroblast line WI 38, ferritin-

FIG. 1 (*on preceding page*). Electron micrographs of (a) $ZnCl_2$-treated and homogenized WI 38 fibroblast labeled with rabbit anti-myosin antibodies followed by ferritin-conjugated goat antibodies to rabbit IgG. Ferritin is seen uniformly underlying the plasma membrane (pm) as well as free in the "cytoplasm" (arrow); (b) $ZnCl_2$-treated and homogenized WI 38 fibroblast treated with rabbit anti-spectrin antibodies followed by ferritin-anti-rabbit IgG antibodies. No significant labeling is seen even though ferritin conjugates did enter the cell interior (arrow); (c) $ZnCl_2$-treated and homogenized erythrocyte treated with the anti-myosin antibodies and the ferritin-anti-IgG antibodies. No significant labeling is seen; and, (d) $ZnCl_2$-treated and homogenized erythrocyte treated with the anti-spectrin antibodies and the ferritin-anti-IgG antibodies. Heavy labeling of the inner (i) but *not* the outer (o) surface is seen. Bar denotes 0.1 μm.

antibody staining (Fig. 1) has shown that part of the smooth muscle myosin-like component is associated with the plasma membrane of the cell in a manner closely resembling the way that spectrin is attached to erythrocyte membranes; namely, in a dense filamentous array extending about 500–800 Å in from the cytoplasmic surface of the membrane. Whether the smooth muscle myosin-like component detected by complement fixation in homogenates of the peripheral blood lymphocytes, HeLa cells, and platelets is also membrane-associated has not yet been determined.

Proteins resembling muscle actin and myosin have been demonstrated in, and isolated from, a wide range of eukaryotic non-muscle cells (for review, see ref. 14). These observations have led many investigators to propose a role for such intracellular actomyosin-like proteins in determining cell shape, motility, and other mechanochemical functions in non-muscle cells. The results in this paper extend these observations in two significant respects. (*1*) With one exception discussed below, a smooth muscle myosin-like component has not previously been directly associated ultrastructurally with the plasma membrane of a eukaryotic cell, although actin-like components have been (14–18). (*2*) Immunochemical criteria have been used for the myosin-like component, rather than the usual ATPase activity criteria. This is important for the following reason. While different actin-like proteins, from different cell types and different species, all appear to be closely similar to one another and to muscle actin, there may be a large family of myosin-like proteins with similar enzymatic activities, but which are otherwise structurally distinct. For example, the myosins of striated and smooth muscles show little or no antigenic cross-reactivity (19), and similarly, it is reported that the myosin-like component of platelet thrombosthenin does not cross-react with smooth muscle myosin (20). Even the myosins from rabbit skeletal and cardiac muscles differ in the amino-acid sequences of their heavy chains (21). It seems likely, therefore, that the functions of these different myosin-like proteins and their localization in cells may be different; immunochemical and immunocytological studies may be very useful in the further elucidation of the roles played by this complex family of proteins.

The relationship of our results to certain similar investigations should be pointed out. Kemp and coworkers (22), using antibodies to smooth muscle myosin prepared from chicken gizzard, claimed that embryonic chick liver and muscle cells exhibit a smooth muscle myosin-like antigen on their outer surfaces. With our antibodies and human fibroblast cells, we found no such outer surface localization (Fig. 1a) despite the fact that the antiserum had not been preabsorbed with any cell type. While this paper was in preparation, Willingham and colleagues (23) reported that a myosin-like protein extracted from mouse L929 fibroblast cells was present, according to immunohistochemical methods, on the outer surfaces of these cells. However, antibodies to this L cell myosin did *not* cross-react with mouse uterine smooth muscle myosin, and were, therefore, not comparable to the antibodies used in our studies. The latter results again emphasize the diversity of myosin-like proteins that are associated with non-muscle cells.

Our primary purpose in undertaking these studies was to explore the possible role of spectrin or related membrane-associated proteins in regulating the mobility of integral proteins in membranes. In our original formulation of the fluid mosaic model of membrane structure (3), it was suggested that in certain instances where the rapid translational motion of the integral proteins in the plane of the membrane seems to be inhibited, "some agent extrinsic to the membrane (either inside or outside the cell) interacts multiply with specific integral proteins" and thereby restricts the latter's mobility. This suggestion was made before it was appreciated that the intact human erythrocyte, that most commonly studied of all mammalian cells, possesses a membrane whose integral proteins are not freely mobile. The mobility of components in the plane of a membrane is demonstrable by several types of experiments, but most readily by the effects of specific antibodies or lectins on the distribution of components in the cell surface. When bivalent antibodies directed to surface antigens of a lymphocyte, for example, are bound to the lymphocyte cell surface at 37°, they induce a clustering of the antigen (into patches, and more extensively, into "caps"), followed by a pinocytosis of these clustered regions of membrane into the cell interior (24–26). But when adult human erythrocytes are similarly treated with specific antibodies directed to surface antigens, no clustering or pinocytosis is observed (26–29). On the other hand, *newborn* human erythrocytes (29), and pigeon erythrocytes (A. Ruoho, K. T. Tokuyasu, and S. J. Singer, unpublished observations) do show clustering and pinocytosis under the same conditions. The presence of large amounts of spectrin localized to the cytoplasmic surface of the adult human erythrocyte membrane (6) led us to suggest (2, 4) that spectrin is the "agent" or one of the "agents" involved in regulating the mobility of integral proteins in that membrane.

The properties of the spectrin-erythrocyte system have been studied in some detail, and are extensively discussed elsewhere§. For our present purposes, the following features of the system are relevant. (*1*) Spectrin is a protein complex which is bound peripherally (1, 2) to the cytoplasmic surface of the erythrocyte membrane. There is evidence (13) that this noncovalent binding is to the intramembranous particles of the membrane, presumably at sites where those particles protrude from the lipid bilayer. (*2*) Spectrin belongs to the myosin family, because we have shown§ that antibodies directed to human uterine smooth muscle myosin show a small but significant cross-reaction with human erythrocyte spectrin. (Under the conditions of the experiments listed in Table 1 or shown in Fig. 1, however, this cross-reaction is not detectable.) (*3*) The protein in band 5 (30) of the erythrocyte membrane is an actin, by its capacity to stimulate the ATPase activity of muscle myosin, and the "decoration" of its fibrous form with muscle heavy meromyosin§.

This information that the spectrin complex has many of the properties of an actomyosin system, together with the similarities in membrane localization of spectrin in erythrocytes and the smooth muscle myosin-like protein in fibroblasts (Fig. 1), suggest that these two protein systems may have similar membrane-associated functions in their respective cells. There is evidence that lectin receptor proteins in the plasma membranes of normal fibroblasts show much more restricted translational mobilities than in the membranes of malignantly transformed fibroblasts, or of trypsinized normal fibroblasts (31–34). We, therefore, suggest that a complex of the smooth muscle myosin-like protein and actin is peripherally attached to integral proteins in the fibroblast plasma membrane, and regulates the mobilities of these integral proteins in the plane of the membrane (2, 4).

How might such membrane-associated, actomyosin-like

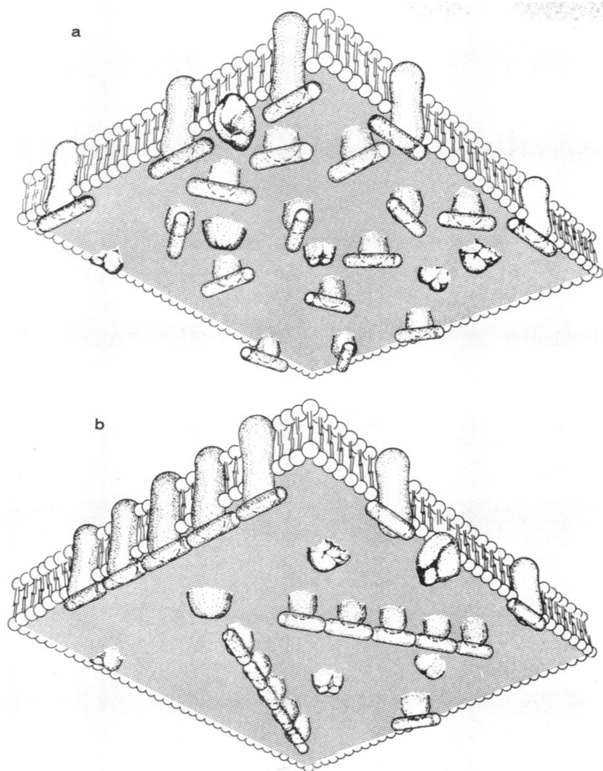

FIG. 2. A highly schematic representation showing how a peripheral protein, in going from (a) its disaggregated state to (b) its aggregated state, could markedly inhibit the translational mobilities of integral proteins in the plane of the membrane. (See *text* for further details).

systems function? Most of the critical information required to propose a detailed mechanism is not yet available. It may be useful to point out, however, that an appropriate peripheral protein system which undergoes reversible self-aggregation and disaggregation could regulate the mobility of integral membrane proteins. This concept is illustrated in highly schematic form in Fig. 2. Individual molecules of peripheral protein(s) are attached to single intramembranous particles. In the disaggregated state of the peripheral protein (Fig. 2a), the translational or rotational diffusion of the intramembranous particles in the plane of the membrane is not significantly impeded. In its aggregated state (Fig. 2b), however, the peripheral protein ties the particles together in arrays which cannot freely diffuse past one another in the plane of the membrane. The aggregation-disaggregation equilibrium of the peripheral protein system could be under metabolic control (e.g., dependent on intracellular ATP concentration). It is also clear that the gross mechanical properties of the cell, such as shape and motility, might be considerably different in the two states.

The scheme in Fig. 2 is intentionally simplified to present the basic ideas involved. The disaggregated state need not have the peripheral protein completely disaggregated; only a difference in the extent of its aggregation in the two states is required. Furthermore, a more complex aggregation-disaggregation process would operate if two or more components were involved, as would be the case in an actomyosin-like protein system. The suggestion of a membrane-associated, actomyosin-like system undergoing reversible aggregation and disaggregation may be related to electron microscopic observations (17, 18) which show much more extensive microfilament arrays inside normal fibroblasts than in their malignantly transformed counterparts, which correlates with the difference in mobilities of surface receptors in normal and transformed cells discussed earlier.

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