Journal of Supramolecular Structure 9:373–389 (1978)
Transmembrane Signaling 357–373

Transmembrane Interactions and the Mechanisms of Transport of Proteins Across Membranes

S. J. Singer, J. F. Ash, Lilly Y. W. Bourguignon, Michael H. Heggeness, and Daniel Louvard

Department of Biology, University of California at San Diego, La Jolla, California 92093

We have made observations, by double fluorescence staining of the same cell, of the distributions of surface receptors, and of intracellular actin and myosin, on cultured normal fibroblasts and other flat cells, and on lymphocytes and other rounded cells. The binding of multivalent ligands (a lectin or specific antibodies) to a cell surface receptor on flat cells clusters the cell receptors into small patches, which line up directly over the actin- and myosin-containing stress fibers inside the cell. Similar ligands binding to rounded cells can cause their surface receptors to be collected into caps on the surface, and these caps are invariably found to be associated with concentrations of actin and myosin under the capped membrane. Although these ligand-induced surface phenomena appear to be different on flat and rounded cells, we propose that in both cases clusters of receptors become linked across the membrane to actin- and myosin-containing structures. In flat cells these structures are very long stress fibers; therefore, when clusters of receptors become linked to these fibers, the clusters are immobilized. In round cells, membrane-associated actin- and myosin-containing structures are apparently much less extensive than in flat cells; therefore, clusters of receptors linked to these structures are still mobile in the plane of the membrane. We suggest that in this case the clusters are then actively collected into a cap by an analogue of the muscle sliding filament mechanism.

To explain the transmembrane linkage, we propose that actin is associated with the plasma membrane as a peripheral protein which is directly or indirectly bound to an integral protein (or proteins) X of the membrane. Individual molecules of any receptor are not bound to X, but after they are specifically clustered into patches, a patch of receptors then becomes bound to S and hence to actin/myosin.

Lilly Bourguignon is now at the Department of Biology, Wayne State University, Detroit, MI 48282.

Received June 9, 1978; accepted August 14, 1978.

0091-7419/78/0903-0373$03.20

Patching and capping of specific receptors on rounded cells is often accompanied by a specific endocytosis of the ligand-receptor complexes. This represents one common transport mechanism of a protein (the ligand) across the plasma membrane. The possibility is discussed that this type of endocytosis is mediated by a transmembrane linkage of the clustered receptor to actin/myosin. Another mechanism of endocytosis involves the "coated pit" structures that are observed by electron microscopy of plasma membranes. The possible relationships between an actin/myosin and a coated pit mechanism of endocytosis are explored.

Key words: surface receptors, capping, endocytosis, actin, myosin

There are many important phenomena in cell biology in which a substance outside a cell induces specific chemical changes inside the cell. The substance outside (referred to as the ligand) usually initiates these changes by binding noncovalently to specific receptor molecules in the cell surface, but the precise sequence of molecular events that follows upon such ligand binding is in no case thoroughly understood. Indeed, this sequence is probably different in different cases. In some cases it is clear that the ligand molecule, or at least a portion of it, must enter the cell in order for the effect of the ligand to be produced, but in other cases ligand entry is presently thought not to be required. The subject of transmembrane signaling becomes even more wide-ranging if one generalizes from the effects on cells of external molecular ligands to include the signals transmitted by such physical factors as light photons, pressure changes, changes in membrane resting potentials, or other interacting cells. If there are some unifying mechanistic principles that are relevant to all of these diverse phenomena, they are not evident at present. This review is therefore deliberately limited in scope. It deals mainly with some recent studies from our laboratory which provide direct evidence that the binding of ligands to their receptors in the membrane can in many cases induce a transmembrane linkage of the receptors to actin- and myosin-containing structures on the other side of the membrane. Such linkage formation may trigger other processes, such as patching, capping, and endocytosis of the ligand-bound receptors. The possible relevance of these mechanisms to transmembrane signaling phenomena such as mitogen and hormone stimulation is discussed.

TRANSMEMBRANE INTERACTIONS INVOLVING ACTOMYOSIN STRUCTURES

Ligand-Induced Transmembrane Linkages — Flat Cells

The detailed experimental procedures we have employed in the experiments discussed throughout this paper, including all reagents and specificity controls, have been described in the original publications that are cited at the appropriate places below and will not be discussed here. Suffice it to say that all of our recent experiments have involved the simultaneous double fluorescence staining of a specific surface component and an intracellular mechanochemical component (actin, myosin, tubulin, etc) on the same cell. For surface components we have used fluorescence-labeled lectins or immunofluorescent reagents on intact cells, while for intracellular components on fibroblasts fixed and lightly detergent-treated cells were stained with immunofluorescent reagents to localize all components but actin. The latter was detected by a fluorescence method based on the specific binding of heavy meromyosin to F-actin [22].

Our studies [5, 7] have revealed a previously unrecognized phenomenon, namely, that when a multivalent ligand binds to its specific receptors on the surfaces of normal fibroblasts and other flat well-spread cells in monolayer culture, and induces the cross-linking of the receptor molecules into small clusters in the fluid membrane, there follows a lining up of the surface clusters with actin- and myosin-containing stress fibers underneath the membrane. The experiments that demonstrated this phenomenon are illustrated by the following example.

Normal human fibroblasts in monolayer culture have much of their cytoplasmic actin and myosin arranged in long thick fiber bundles [23, 29, 50], the so-called stress fibers (Fig. 1a, c). The unperturbed distribution of many cell surface components, such as the β_2-microglobulin (which is part of the histocompatibility antigen molecule (HLA) [45]), is nearly uniform (Fig. 1b) over the surface (however, see below), but if two layers of antibodies (bovine anti-human β_2-microglobulin followed by rabbit anti-bovine IgG) are allowed to cross-link and cluster the β_2-microglobulin for 20 min at 37°, that protein is redistributed into small patches that are lined up directly over the stress fibers (Fig. 1d; compare with 1c). These patches are still at the cell surface and are not endocytosed because they are accessible to a third layer of fluorescent-labeled goat anti-rabbit IgG. It seems reasonable to conclude that the lining up of the clusters of β_2-microglobulin indicates that the histocompatibility antigen is linked across the membrane directly or indirectly to the actin/myosin components underneath.

The addition of neither 10^{-5} M colchicine nor 20 mM NaN_3 (in glucose-free medium) to the cells prior to the antibody treatments alters the rate or extent of formation of the transmembrane linkage, and hence it appears that microtubules are not involved in the phenomenon, and that not much, if any, cell energy is required.

Closely similar results have been obtained with several other surface components and their specific multivalent antibody or lectin ligands [5, 7]. These include the cell surface enzyme aminopeptidase [4, 30] and Na^+,K^+-ATPase (adenosine triphosphatase), and their respective antibodies; and receptors for the lectins concanavalin A (Con A) and wheat germ agglutinin (WGA). Several different normal fibroblasts have been used from human, rat, and mouse sources. In each case, clustering of the specific surface component causes it to form patches that are superimposable on the stress fibers found in the same focal plane. We infer therefore that the patches are in each case linked to the stress fibers.

The question arises, are all of these surface components linked across the membrane to actin/myosin in the unperturbed state, or do they become linked only after they are clustered? We do not have direct evidence bearing on this point, but our view is that the linkage occurs only after a receptor is clustered, as is discussed below. In this connection, it is interesting that the initial distributions of the surface antigens we have studied do not appear to be uniform. To determine the unperturbed distribution of β_2-microglobulin, for example, cells were fixed with 3% formaldehyde for 20 min and were then stained with the antibody reagents. Under these conditions, careful observation of the surface fluorescence shows that it is not structureless, but rather finely punctate and arranged in linear arrays (Fig. 1b). When these original surface arrays are carefully compared to the arrays of stress fibers in the same cell and the same focal plane, however, the two arrays appear to be *interdigitated* rather than *superimposable.* This is in contrast to the situation *after* clustering of the receptors has occurred (Fig. 1d) when the linear arrays of receptor patches are found to be *superimposable* on the stress fibers. On the other hand, with fluorescein-labeled Con A the initial distribution of Con A receptors is completely uniform within the resolution of the fluorescence miscroscope [5]. The possible significance of these observations of initial distributions is elaborated subsequently.

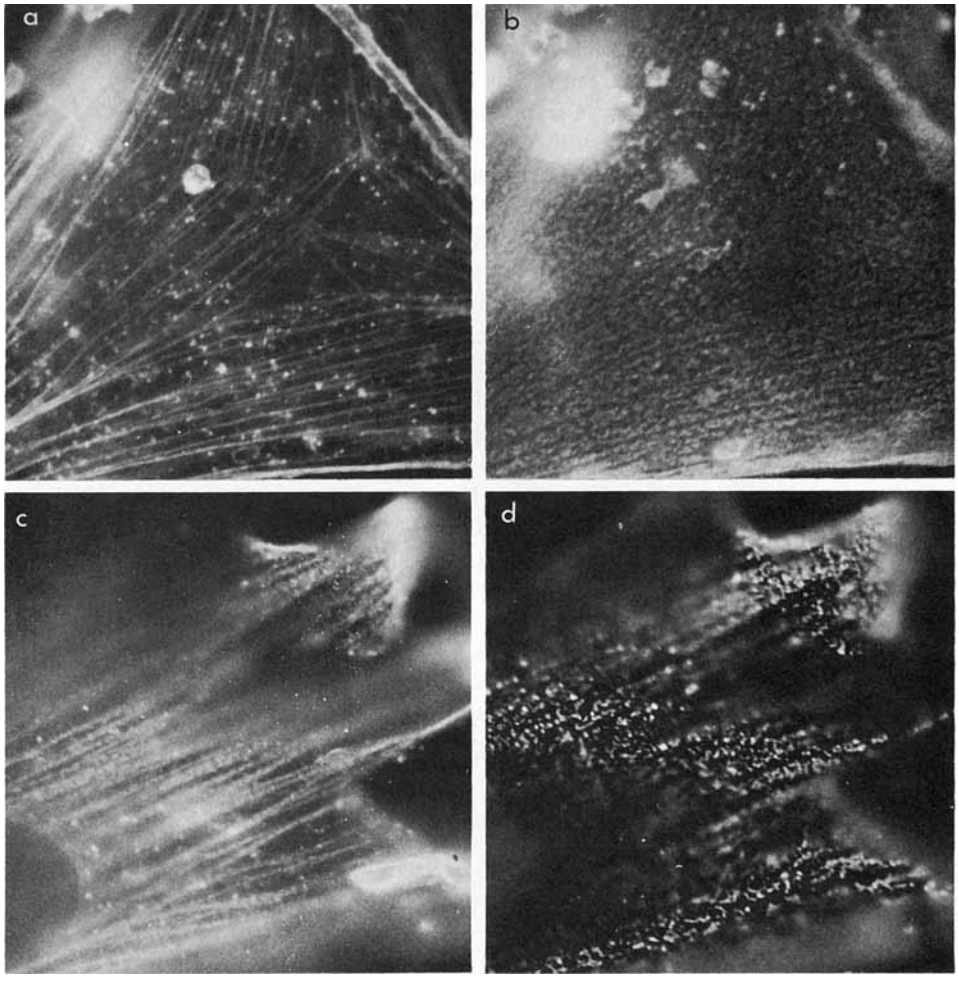


Fig. 1. WI38 human fibroblasts stained for surface β_2-microglobulin with indirect rhodamine immunofluorescence (b and d) and for intracellular actin by a specific fluorescein labeling procedure (a and c). The initial distribution of the β_2-microglobulin (b) is shown for the same cell whose intracellular actin is organized as shown in (a). The β_2-microglobulin is present in a finely punctate distribution which is linearly arrayed in parallel to the linear arrays of actin-containing stress fibers. Careful comparison of the two kinds of linear arrays, however, shows them to be interdigitated in projection. After the antibody reagents have been allowed to cluster the surface β_2-microglobulin, that component is now found (d) in small patches which are linearly arrayed. These arrays are superimposable on the linear arrays of the actin-containing stress fibers inside the same cell (c). See Ash et al [7].

Ligand-Induced Transmembrane Linkages — Rounded Cells: The Capping Phenomenon

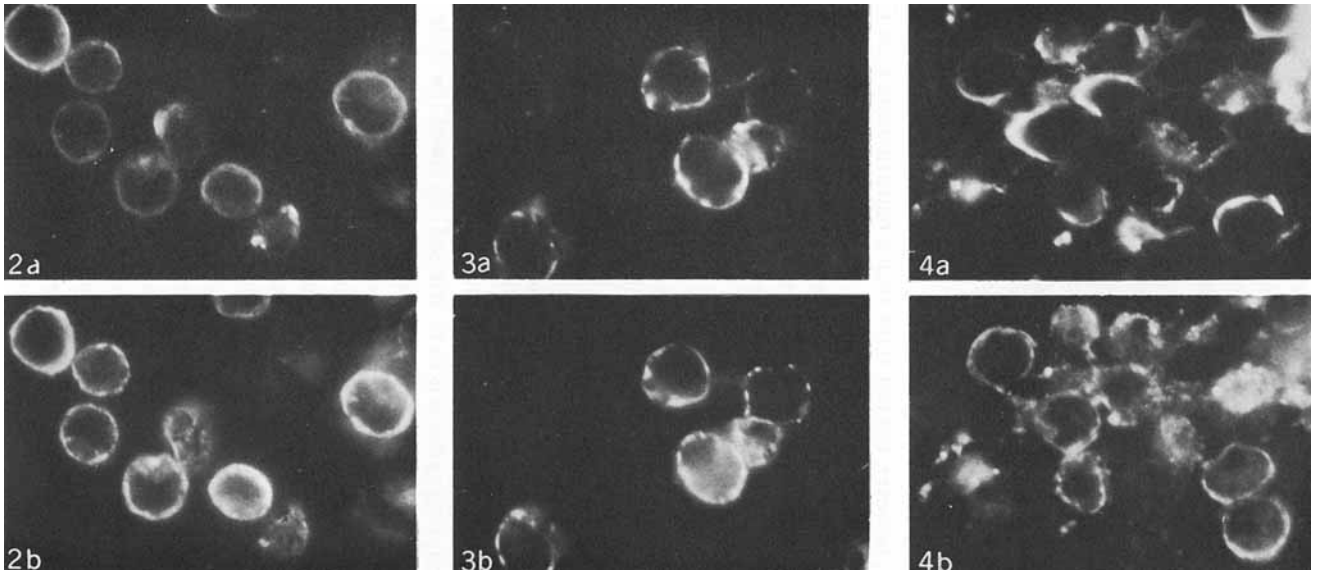
It has been known and widely demonstrated for some years now that the addition of suitable multivalent ligands for surface components of any cell in suspension (with the notable exception of mature mammalian erythrocytes) will cause, at 37°C, the collection of the ligand-receptor complex into several large patches or a single cap on the cell surface This capping takes a few minutes to an hour, depending on the systems involved. It is often, but not necessarily, accompanied by the endocytosis of the patched or capped

portions of the cell membrane (see below). The endocytosed vesicles may fuse with other intracellular vesicles (lysosomes) and may be subjected to some limited degradation and recycling processes.

Let us first focus attention upon the early stages of this process, following upon the binding of the ligand to the cell surface. By double immunofluorescence experiments, we have followed the redistribution of any one of several different surface components, together with some intracellular mechanochemical protein, in the same cell [10, 11]. In every case we have examined so far involving a large number of combinations of ligand, surface component, and cell, capping was always accompanied by a redistribution and concentration of actin and myosin under the cap (the "subcap"). An example of this is the capping of mouse splenic T cells by fluorescein-conjugated Con A (Fl Con A). Cells stained at 0° with Fl Con A showed a uniform surface distribution of fluorescence (Fig. 2a), while those portions of the intracellular myosin (Fig. 2b) and actin (not shown) that appeared to be membrane-associated were also uniformly distributed on the inner surface of the membrane. However, when the temperature was raised to 37° for 15 min, the Fl Con A induced cap formation (Figs. 5a, 6a, 7a, 8a). Accompanying this process, there was a concentration of myosin (Fig. 5b, 6b) and of actin (Figs. 7b, 8b) immediately under the Con A caps on the same cells. Tubulin, however, did not show such a redistribution after Con A capping (Figs 4a, b). (In these experiments, microtubules had presumably been disaggregated by the initial exposure of the cells to 0° for 30 min.) This serves as an important control to eliminate the possibility that the intracellular redistributions of actin and myosin that we have observed are due simply to a displacement of the entire cytoplasm of these small lymphocytes under the capped region of the membrane.

Equally striking were the results we obtained when patching, rather than capping, of receptors was induced. At an earlier stage in the capping process, the ligand-receptor complexes were collected into many small patches that were distributed all over the cell surface. If NaN_3 was present, the process was arrested at this patching stage. But, in the absence of NaN_3, the small patches were subsequently collected into the few large patches or caps. We have shown [10, 11] that upon formation of such small patches actin and myosin are always found concentrated into "subpatches" immediately under the patches. This is shown for the Fl Con A–T cell system in Figure 3a, b. The similarity of these observations for a wide variety of different capping systems is to be stressed. At about the same time as our work was in process, reports appeared of similar findings in individual instances, for example, with the Ig receptor on mouse splenic B lymphocytes [21, 41] or the Con A receptors on two different cell types [35, 47]. But, the *generality* of the finding that patches and caps of such a wide range of surface components are in every case associated with subpatches and subcaps, respectively, containing actin and myosin, requires some special characteristics of a satisfactory molecular mechanism of the capping phenomenon.

These experiments warrant some additional technical attention. Our capping and patching experiments were carried out with a technique that was intended to preserve the ultrastructural integrity of the cell [11]. The conventional method is to fix cells with formaldehyde and then render them permeable by acetone treatment. Such a procedure may produce a great deal of ultrastructural damage and perhaps artifactual redistributions [49]. The method we used was a gentle formaldehyde fixation followed by infusion of the cells with 50% sucrose, freezing, and sectioning in the frozen state [48]. The thawed sections, 1.0 μ thick or less, containing the capped cells sliced through to expose their intracellular antigens, were then stained for actin or myosin. Our procedure may, however, not completely fix all intracellular antigens, and it is possible that our observations of the

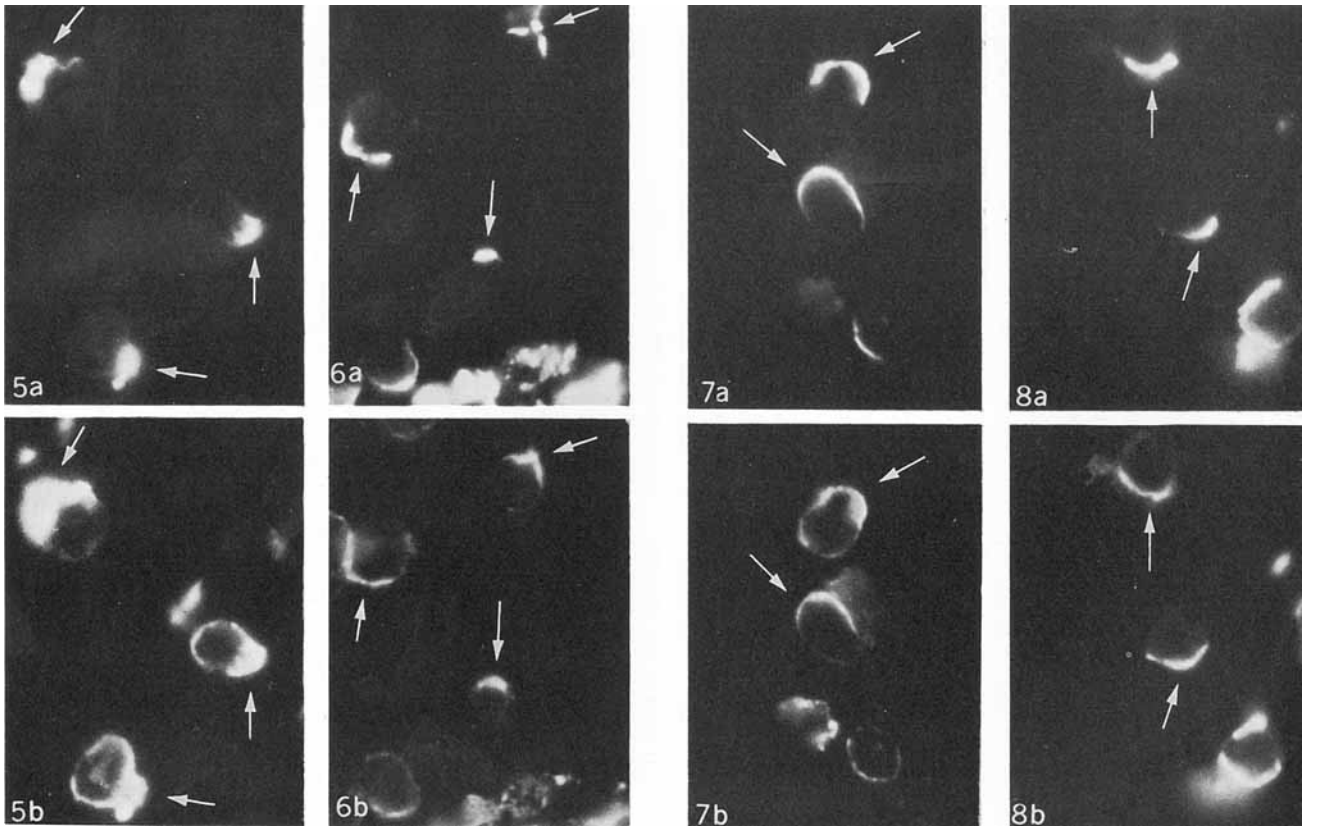


Figs. 2–8. Mouse splenic T cells treated with fluorescein-labeled Con A and intracellularly stained on the same cells with specific rhodamine-labeling procedures for actin, myosin, or tubulin.

Fig. 2. a) Initial surface distribution of F Con A. b) Initial intracellular distribution of myosin in the same field as (a).

Fig. 3. a) Patching of Con A receptors, produced by treating cells with F Con A for 30 min at 0°C and then incubating at 37° for 2 min. b) Intracellular myosin in the same field as (a). Note the correspondence of myosin "subpatches" with the F Con A patches.

Fig. 4. a) Capping of Con A receptors, produced by treating cells with F Con A for 30 min at 0°C and then incubating at 37° for 15 min. b) Intracellular tubulin in the same field as (a).



Figs. 5, 6. a) Capping of Con A receptors, as in Fig. 4a. b) Intracellular myosin in the same field as (a). The oriented arrows direct attention to the Con A caps and the corresponding myosin "subcaps" on the same cells.

Figs. 7, 8. a) Capping of Con A receptors, as in Fig. 4a. b) Intracellular actin in the same field as (a). The oriented arrows point to the Con A caps and the actin "subcaps" on the same cells.

redistributions of actin, for example, were enhanced by a partial loss of unfixed intracellular actin. That part of the membrane-bound actin associated with the receptor patches and caps might be more resistant to such loss than the rest (and probably the large majority) of the actin.

The following features of patching and capping must be explained:

a. Our results suggest that actin, myosin, and perhaps other mechanochemical proteins as well, appear to be actively involved in the patching and capping of many surface components. On the other hand, in general, microtubules do not appear to be directly involved in capping. Although the inhibition of capping of Con A does depend upon the presence of intact microtubules [20], this is a special case. There is no satisfactory evidence that microtubules are required for capping in any system. In general, if microtubules in intact cells are disrupted by cold shock or by treatment with colchicine at concentrations of 10^{-5} M, the capping of most surface receptors is unaffected. In one study carried out with immunofluorescence techniques [47], however, it was claimed that both tubulin and actin were concentrated under the cap. This observation could, however, be artifactual; it could result from a shift of the small amount of cytoplasm in a cell into the uropod that is formed upon capping, as mentioned above. It is essential to eliminate such a possibility by a control experiment such as that shown in Fig. 4.

b. In most cases so far studied, components that are molecularly independent in the membrane cap independently. For example, it was shown early on [46] that the Ig receptor or the H-2 histocompatibility antigen was each capped by its specific antibody reagents without significantly affecting the surface distribution of the other. Many similar results of independent capping have been obtained since then. This suggests that the initial clustering of a membrane component by its multivalent ligands triggers the selective process which ultimately results in the capping of that component and no others.

A Proposed Mechanism for the Formation of Transmembrane Linkages

The following elements together constitute a molecular mechanism for the transmembrane linkage phenomenon:

a. Intracellular actin is assumed, in part, to be bound to the cytoplasmic surface of the plasma membrane and in part distributed in the cytoplasm. Its association with the membrane is most likely as a peripheral protein [43, 44]. That is, actin is ordinarily a water-soluble protein, and therefore its binding to a membrane very likely requires the presence of one or more integral proteins X, embedded in the membrane and protruding from the cytoplasmic surface to provide the specific attachment sites for actin molecules. (Alternatively, actin may be attached to other peripheral proteins such as α-actinin [31] with one of the latter bound to X.)

b. The clustering of any of a wide variety of different surface components by its specific antibody or lectin reagents leads to a binding of those clusters to X in the plane of the fluid mosaic membrane; in this manner, the clustering of a surface component results in the specific association of that component and no other with actin and myosin across the membrane (Fig. 9). Isolated surface components that are not clustered are generally not linked to X, nor to actin and myosin. (If, on the contrary, most isolated surface components were linked to X in the unperturbed membrane, and hence to actin and myosin, then one would not expect the capping of individual membrane components to occur without incorporating other components into the cap; see following section.)

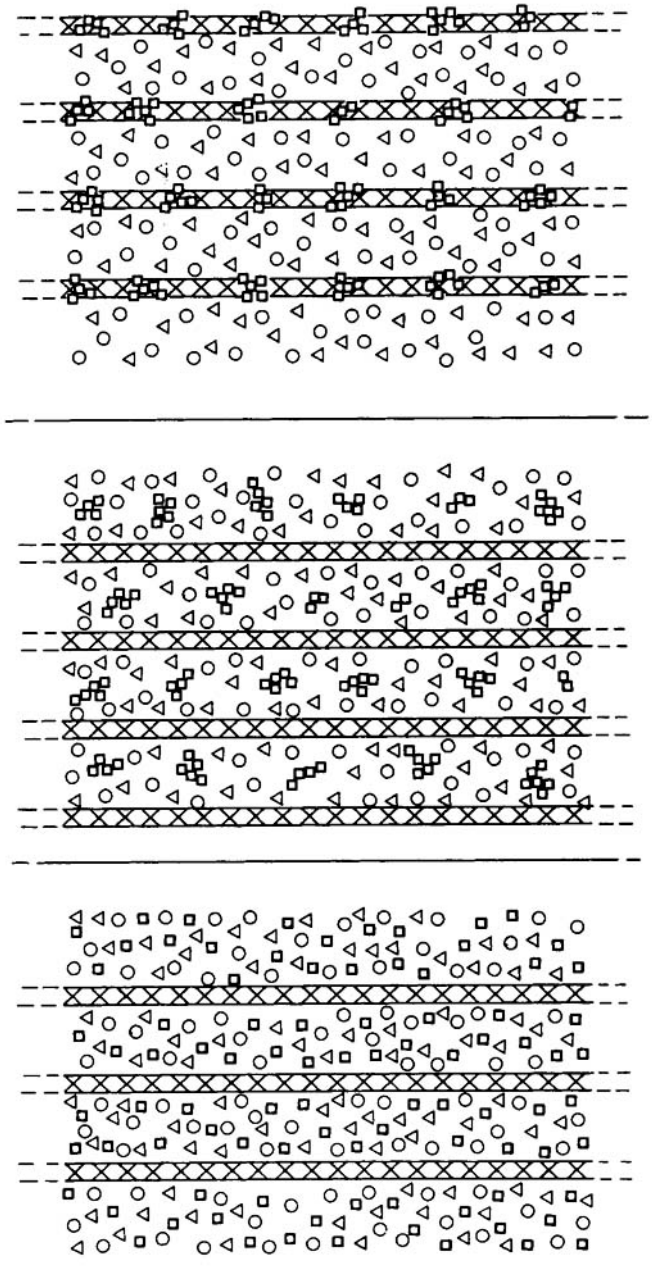


Fig. 9. Proposed schema of transmembrane linkage phenomena on flat cells such as are demonstrated in Fig. 1. The view is down onto the surface of a flattened fibroblast. The symbols □, ○, and △ represent three different kinds of independent receptors in the plane of the membrane. The parallel bars represent the actin- and myosin-containing stress fibers lying underneath the membrane. The symbol X represents the integral protein which is presumed to be attached on the cytoplasmic surface of the membrane directly or indirectly to actin in the stress fibers. In the initial state (left) the receptors are depicted as randomly distributed in the plane of the membrane, except that they are excluded from the membrane above the fibers. When antibody reagents specific for the □ receptor are incubated with the cells at 37°, they first cluster the □ receptor molecules (center), and these clusters then associate laterally with X molecules (right). The initial state corresponds to Figure 1a, b, the transmembrane linkage condition to Figure 1c, d.

The binding of ligand-bound clusters of membrane components, but not their single molecules, to X may be analogous to the binding of antigen-bound clusters of immunoglobulins, but not their individual molecules, to the Clq component of the complement system [32].

At this point, we have no basis to estimate the stoichiometry of the binding reaction of receptor clusters to X. It is important to note the possibility, however, that small clusters, contaning only a few receptor molecules, might already be capable of binding to X. If this were so, it would mean that even a small degree of receptor clustering might lead to a selective association of that receptor with the actin/myosin contractile machinery.

One conceptual difficulty with the proposed mechanism is the requirement that clusters of many different kinds of membrane components must all be capable of binding to X. It should first be appreciated that some such apparently unlikely phenomenon must be invoked to explain the generality of the transmembrane linkage formation. Perhaps it will turn out that many different membrane components have some presently unsuspected common structural features that permit them all, when clustered, to bind to X.

We are actively seeking direct evidence of the existence of the putative protein X. It is interesting, however, that the initial distributions on the surfaces of normal fibroblasts of the three integral membrane proteins β_2-microglobulin (Fig. 1), aminopeptidase, and the Na^+, K^+-ATPase, are not completely uniform, but these proteins appear to be excluded from the regions of the fibroblast membrane directly over the stress fibers, whereas after these proteins are clustered, the patches line up directly over the fibers. It is possible that the presence of substantial amounts of X in the membrane above the stress fibers is responsible for this exclusion effect (Fig. 9). The fact that, by contrast, the initial surface distribution of Con A is indeed uniform within the resolution of the microscope could then be explained if X were a Con A receptor.

A Proposed Mechanism for Capping

This discussion allows us to formulate a molecular mechanism for capping [10], which is illustrated in highly schematic form in Fig. 10. Different integral membrane components are initially uniformly distributed over the surface of the cell (upper left panel). The binding of a ligand to the specific receptor R induces a clustering of R in the fluid membrane (upper right panel). The clusters of R meet and bind to X and thus become linked to small aggregates containing actin and myosin (lower right panel). Such complexes are, however, still mobile in the plane of the membrane. At some stage in this process, a signal is transmitted across the clusters which ultimately produces a redistribution of Ca^{++} in the vicinity of the clusters, such that when the actin/myosin-linked clusters meet by diffusion in the plane of the membrane, an actin/myosin sliding filament mechanism similar to that of muscle is activated which collects (lower left panel) the clusters of R into patches and caps. The energy known to be required for capping would then be the ATP which provides the energy for the sliding filament mechanism. Other independent receptors in the membrane are not affected by this process, because they do not become linked to actin/myosin.

The course of the active collection of receptor patches into caps is likely to be more complex than is suggested by the scheme in Fig. 10. The fluorescent subcaps containing actin/myosin are large enough to extend some considerable distance into the cytoplasm from the surface of the membrane, and electron micrographs of capped regions of cells [1, 40] show that substantial accumulations of filamentous structures occur in the cytoplasm under the cap. It seems likely, therefore, that during the operation of the proposed

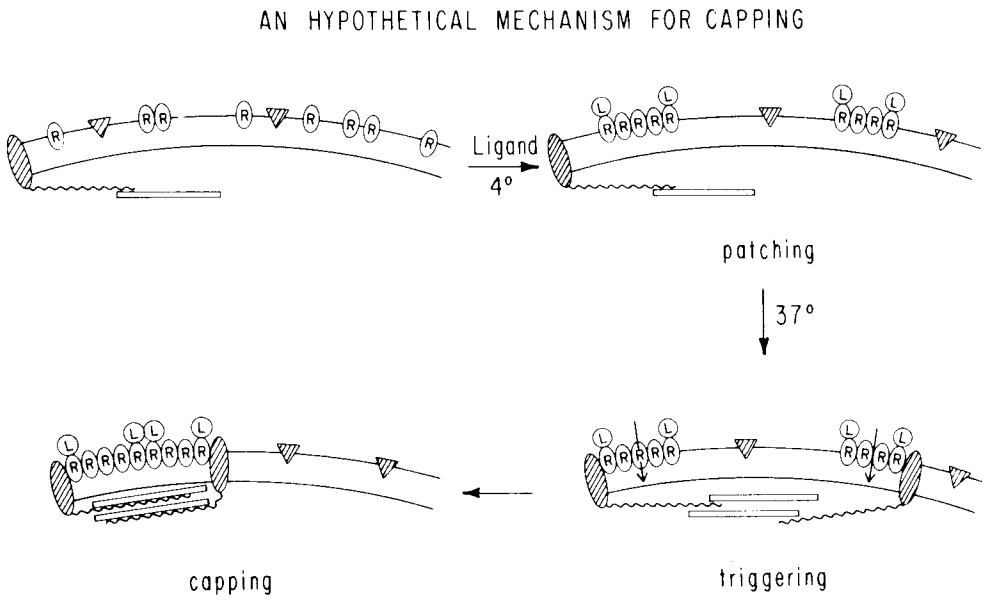


Fig. 10. Schematic representation of a mechanism of capping on rounded cells. Two kinds of receptors are indicated by ▼ and ®. The striped oval component is equivalent to X in Figure 9, the proposed integral protein to which some intracellular actin filaments and myosin rods are directly or indirectly attached. In the initial state (left, top) the receptors are dispersed in the membrane, not associated with the protein X. When a ligand (L) specific for the receptor ® is added to the cells, it induces a specific clustering of ® molecules, not affecting the distribution of ▼ molecules (right, top). Upon warming to 37°, the clusters of ® associated with X in the plane of the membrane (right, lower), during which process a signal (arrow) is transmitted which ultimately produces a transient local increase in Ca^{++} concentration. This allows the actin/myosin associated with individual patches to undergo a sliding filament interaction which collects the patches of ○ specifically into a cap (left, lower). The last step, cap formation, may also involve the recruitment of intracellular actin and myosin not originally associated with the membrane (not shown). The last step is the part of the process requiring energy, and is presumed to be the step that is inhibited by the presence of NaN_3 during the process.

sliding filament mechanism for capping, there is recruited some actin and myosin that were initially in the cytoplasm, as well as their initially membrane-bound forms.

An active capping mechanism involving actin and myosin was suggested by Schreiner and Unanue [40] for the specific case of the capping of the Ig receptor on B lymphocytes, but these authors consider that the capping of other surface molecules proceeds by a different mechanism. Our proposal, however, provides a general mechanism for all capping phenomena, one which is significantly different from others [12, 18, 19] that have been suggested previously.

Differences in Surface Properties of Normal and Transformed Fibroblasts

One satisfying aspect of the proposals discussed so far is that they provide a unifying mechanism to explain the differences in surface properties of flat and round cells. For both types of cells, it is proposed that the clustering of a surface component by its specific ligand leads to a transmembrane linkage to actin/myosin-containing structures under the membrane. The only difference is that in the flat cell, the actin and myosin are organized in extended stress fibers and the formation of the transmembrane linkage then

immobilizes the clusters. In the round cell, the actin and myosin are much less highly organized. The transmembrane linkage results in the formation of complexes which are still mobile and can be actively collected into patches and caps by the sliding together of actin and myosin filaments.

This scheme provides a reasonable explanation for one aspect of the different surface properties of normal and transformed fibroblasts in culture [6]. It has been suggested by many investigators [27, 33, 34, 38] that the surface components of normal fibroblasts are relatively immobile in the plane of the membrane, whereas the same components are quite mobile in the membrane of the transformed cell. This difference may be important in explaining the more ready lectin agglutinability of transformed as compared to normal fibroblasts [15, 26]. Many explanations have been offered for this apparent mobility difference; this is not the appropriate place, however, to discuss them. Our explanation for this difference is as follows: Transformed fibroblasts are rounded cells in which the actin and myosin are no longer present in extended stress fibers but are much less highly organized [6]. Therefore, whereas the lectins used to detect the mobility of their membrane receptors induce an immobilization of these receptors on the normal fibroblasts, on the contrary, they induce patch formation on the transformed cells, by the mechanisms discussed above. The perception of these experimental results would therefore be that surface components were apparently immobile on normal fibroblasts but mobile on transformed ones. If this view is correct, and these surface mobility properties of normal and malignant fibroblasts mainly reflect the differences in organization of the actin and myosin in these cells, the interesting question then becomes, How does the transformation process cause a breakdown of the stress fibers present in normal fibroblasts? That is a subject for consideration elsewhere.

Cell Surface Distribution of Matrix Proteins

Transmembrane linkages to actin/myosin structures underneath the membrane may also be important in organizing those peripheral proteins that are present on the external surfaces of cells, including the LETS protein [25, 28], collagen [9], and other matrix proteins. It has been known, for example, that the LETS protein is organized in short, fibrous bundles on the surfaces of fibroblasts in culture [49]. By double fluorescence staining for the LETS protein and for actin [24], it can be seen (Fig. 11a, b) that these LETS protein bundles on fibroblast surfaces are arranged in discontinuous linear segments that are superimposed on the stress fibers. By studying the reappearance of LETS protein and stress fibers of normal fibroblasts that had been trypsinized [24], we have shown that the stress fibers reappeared earlier than surface LETS protein and presumably, therefore, that the stress fibers determined the subsequent arrangement of LETS on the cell surface.

The simplest explanation of these findings that is consistent with the results and proposals discussed above is that the fibrous bundles of LETS protein (or some other matrix component attached to LETS) bind as a multivalent ligand to some integral protein receptor in the fibroblast surface membrane. Just as with other multivalent ligands, this binding produces a clustering of the specific receptor in the plane of the membrane, which results in the binding of the receptor cluster to X, and hence to actin/myosin on the other side of the membrane.

MECHANISMS OF PROTEIN TRANSPORT ACROSS MEMBRANES

A very important and widely occurring phenomenon in cell biology is the transport of large protein molecules across apparently intact membranes. Individual examples demon-

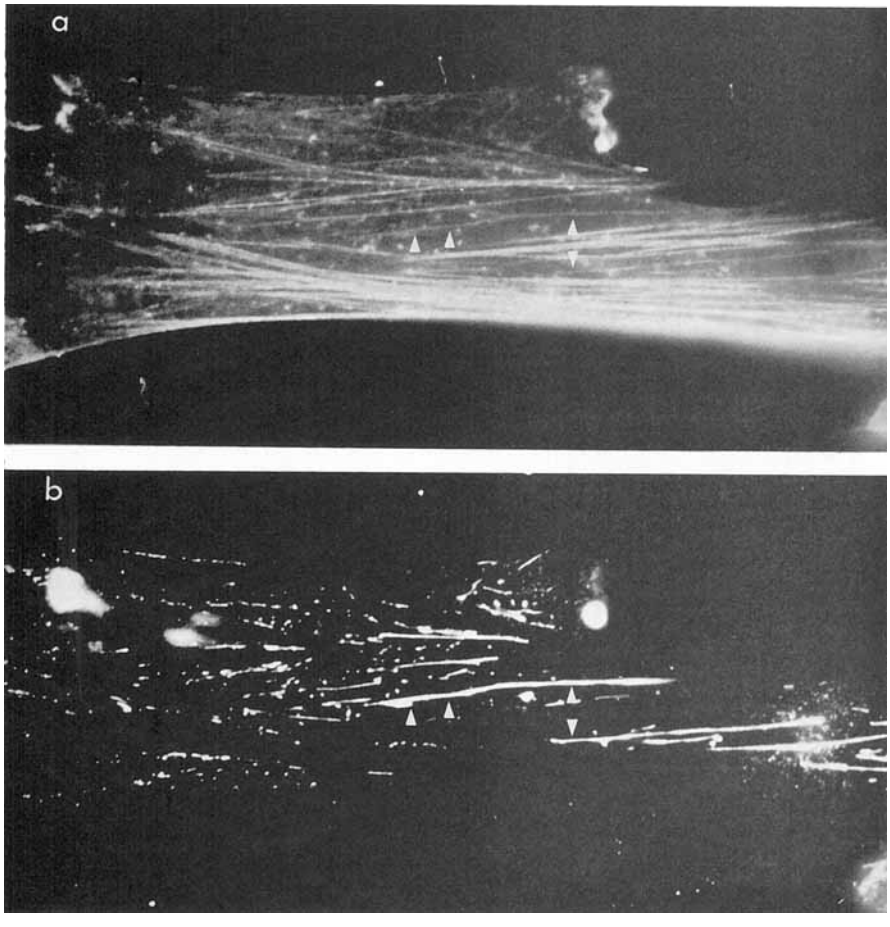


Fig. 11. WI38 human fibroblasts, cultured for 24 h after trypsinization and replating, fixed with 3% formaldehyde and surface stained by rhodamine indirect immunofluorescence (using antibodies specific to cold insoluble globulin) for LETS protein (b), and then permeabilized with detergent treatment and stained for intracellular actin by a specific fluorescein labeling procedure (a). Arrowheads point to surface LETS fibrils and intracellular actin-containing stress fibers that are clearly superimposable. See Heggeness et al [24].

strating the diverse nature of such transport include: serum lipoproteins that must gain access into cells in order to regulate cholesterol biosynthesis [13]; the soluble matrix proteins of mitochondria, which are synthesized in the cytoplasm of cells and must get across the inner membrane of the mitochondria [8]; and toxin molecules such as diphtheria toxin, a part of which must get into cells to shut off protein synthesis [36]. In certain other cases, whether a protein must gain entry into cells to carry out its function is not clear. It is already evident that more than one molecular mechanism is used to transport different proteins across membranes, and it is very likely that other mechanisms will be discovered in the near future. In what follows, this general problem is considered.

Ligand-Induced Clustering and Endocytosis of Ligand-Receptor Complexes

As has been mentioned above, multivalent ligand-induced patching and capping of specific surface components on rounded cells are often accompanied by endocytosis of

the patched regions of the membrane. For example, after antibody-induced clustering of the Ig receptors on mouse splenic B cells, most of the receptors are swept from the cell surface by endocytosis [46]. However, some surface components of round cells can be patched or capped with endocytosis occurring only very slowly or not at all. It appears, therefore, that for systems of this type, clustering of the surface component is a necessary, but not sufficient, condition for endocytosis. It is possible either that clustering events lead to an inhibition of the endocytosis of the clusters in some cases and not in others, or that some additional factors are required beyond clustering to induce endocytosis, and that these act in some cases but not in others. It is worth noting at this stage that when receptors in the surfaces of normal fibroblasts are clustered and linked to the stress fibers on the underside of the membrane, and are thereby "immobilized," if observations are made with unfixed cells over the next several hours the surface fluorescence is found to diminish gradually. It is likely that this is due to the slow endocytosis of these immobilized patches on these flat cells.

It is tempting to suggest that the transmembrane linkage of clusters of receptors to actin/myosin is a critical, but not sufficient, step in the endocytosis of the ligand-receptor complexes. The actin/myosin might provide the contractile machinery, not only to patch or cap the receptor clusters to which they were linked, but also to produce the mechanical forces required for the invagination and vesiculation of the clustered regions. On the other hand, there may be a variety of mechanisms to produce membrane invagination and endocytosis, and actin/myosin involvement may not be necessary for at least some of these mechanisms to operate.

It may be useful to think of endocytosis generally in terms of the "bilayer couple" hypothesis [42]. This hypothesis is based on the fact that usually different lipid and protein components are present in the two half-layers of a membrane bilayer, and as a result, the two half-layers can to some extent be independently affected by a particular perturbation. Thus, a local invagination of a membrane can be viewed as a local relative increase of the area occupied by the inner half-layer compared to the outer half-layer of the bilayer. One can conceive of a variety of molecular mechanisms to achieve such relative area changes. Some integral membrane proteins may have a molecular shape and disposition in the membrane such that, upon being clustered together by a multivalent ligand, they could produce such a relative area change locally in the two half-layers of the membrane. In such a case, no factor other than the clustering would need to be invoked to account for the invagination of the membrane. On the other hand, the clustering of a membrane protein could have secondary consequences: It might induce a local redistribution of membrane lipids, or it might activate a local phospholipase or other enzyme activity, which might in turn lead to the relative area changes required for the local invagination of the membrane. It is also possible that the actin/myosin that becomes associated on one face of the membrane with receptor clusters is directly responsible for a relative increase in the local area of the inner compared to the outer half-layers of the membrane.

At present, too little is known about the molecular mechanisms of endocytosis to warrant further speculation. The main point is that it is an experimental fact that multivalent ligand-induced clustering of specific membrane components not only results in a transmembrane linkage of the clusters to actin/myosin, but often also leads to endocytosis of the clustered regions of the membrane. Whether these two results of clustering are connected to one another, or are independent, is not clear. Such clustering-induced endocytosis is apparently a major pathway allowing the transport of many different proteins across the plasma membrane of a cell.

Coated Pits and Endocytosis

Because this subject is discussed in some detail elsewhere in this volume [14], it is only briefly reviewed here. It is now clear that there is another mechanism whereby eukaryotic cells endocytose ligand-receptor complexes besides the ligand-induced clustering mechanism. Some specific receptors, such as the low-density lipoprotein (LDL) receptor on human fibroblasts [3, 13], are largely if not exclusively present in already clustered structures called coated pits [39]. These are clusters of membrane proteins which are morphologically recognized in the electron microscope by additional densities on both surfaces. They comprise generally only a few percent of the surface area of a plasma membrane of which they form a contiguous part.

On normal human fibroblasts it has been shown [2] with ferritin-conjugated LDL (Fer-LDL) that the bulk of LDL receptors are in coated pits and that many, if not all, coated pits contain the LDL receptor. On the other hand, in mutant cells carrying a defective LDL receptor [3], none of that receptor is in coated pits, yet the numbers and sizes of the coated pits do not appear to be significantly altered on these mutant cells. These results suggest that each coated pit on a normal fibroblast contains a mixture of receptor molecules, those specific for LDL as well as those for other as yet unknown ligands.

The binding of Fer-LDL to LDL receptors in these coated pits leads to a rapid endocytosis of the ligand-bound coated pits, followed by a physiologically critical processing of the endocytotic vesicles [13]. The mechanism for this endocytosis is not yet understood, but presumably it does not involve a ligand-induced clustering of the LDL receptors since these are already clustered. The suggestion has been made that the protein clathrin, which has been found associated with brain synaptosomes [37], is present in coated pits on fibroblasts and other cells, but this has yet to be established by direct immunoferritin staining of the coated pits with antibodies specific for clathrin.

There are many interesting questions remaining about the nature, composition, formation, and function of coated pits. Until such time as they are all shown to be distinctive structures (eg, all containing clathrin or some other characteristic proteins), the possibility must be considered that at least some structures that appear to be coated pits in electron microscopy are, unbeknownst to us, produced by a ligand-induced clustering mechanism such as has been discussed above. It is possible that the medium in which cells are cultured contains factors or ligands which bind to specific receptors on cell surfaces and induce them to cluster into small patches. Alternatively certain receptors that are molecularly dispersed in a membrane may be modified metabolically so that they now tend to aggregate into small patches. However they may be formed, such small patches may then bind to X, and thus become associated with actin/myosin on the cytoplasmic surface of the membrane. For some reason the subsequent activation of the mechanism to collect such patches into a cap (Fig. 10) may not occur, and the patches may remain at the surface until the appropriate ligand binds to the receptor and stimulates the endocytosis of the complex. Examination of these cells might therefore reveal the presence of such preformed clusters with extra densities (due to actin/myosin) on their cytoplasmic surfaces which might be morphologically quite similar in appearance to coated pits.

Another possibility is that receptors which are collected into patches by their multivalent ligands, and become linked with actin/myosin as a result, may now become associated with the coated pits. The endocytosis of the patches might then occur together with the coated pits. It is clear that there are a number of questions that must be answered about the composition and function of coated pits and their possible relationship to ligand-induced surface clusters, in order to clarify the role of these interesting membrane structures.

Cell Activation and Endocytosis

The question has often been asked, Do ligands that stimulate cells to divide or otherwise alter their metabolism have to get inside cells in order to carry out their functions? Such ligands include mitogens, growth factors, antigens, and hormones. Experimentally, this question resolves into two parts: a) Can these molecules get inside cells? and b) If so, is such entry essential to their activities? Mitogens and (T cell-independent) antigens are generally multivalent ligands that can certainly enter cells by the ligand-induced clustering and endocytosis mechanism which has been discussed above. More recently, it has been shown that several polypeptide hormones, after binding to specific receptors in cell surfaces, are very likely endocytosed. The best-studied case to date is that of epidermal growth factor (EGF), of molecular weight about 5,400, interacting with cultured fibroblasts [16, 17]. With ^{125}I-EGF, it was shown that its uptake by fibroblasts at 0°C was essentially completely confined to the exterior cell surface, but at 37° uptake was apparently followed by a rapid internalization and degradation of the EGF, with the release and excretion of ^{125}I-tyrosine. This degradation was inhibited by chloroquine, presumably indicating that lysosomal protease activities were responsible for the degradation.

If EGF and other low-molecular-weight polypeptide hormones and growth factors do indeed enter cells by endocytosis, it would be of great interest to determine whether they do so by a ligand-induced clustering mechanism, by a coated pit mechanism, or perhaps by still another as yet undiscovered means. If a ligand-induced clustering is involved, this would be unusual in that EGF and other low-molecular-weight hormones are most probably acting as univalent ligands, by contrast with multivalent antibodies and lectins. We have proposed earlier [44], however, that a univalent hormone, upon binding to its receptor, may induce cooperative interactions leading to an aggregation of ligand-bound and unbound specific receptors in the fluid membrane. If, on the other hand, such hormones had their receptors confined to coated pits, the univalence of the hormones would be no conceptual obstacle to their function.

All of this discussion of the possible entry of mitogens, growth factors, and hormones into cells leaves entirely open the question of whether such entry is essential to their function.

The question of the entry of diphtheria toxin and related proteins into cells is still another matter. It is known that a part of the diphtheria toxin molecule, the A fragment, must gain access to the EF-2 ribosomal protein inside cells to adenosylribosylate and inactivate it, thereby turning off protein synthesis [36]. It has often been proposed that such toxins enter cells by endocytosis after binding to a specific surface receptor, but the fact that exceedingly low concentrations of toxin can inactivate cells has led other investigators to doubt whether a ligand-induced clustering mechanism of endocytosis is involved, although such an objection would not equally well apply to a coated pit mechanism. The suggestion has been made [36] that some more direct mode of entry is involved, in which the toxin-receptor complex may in some manner function as a channel for the direct entry and release of the A fragment. If this were true, it would represent a new mechanism of protein transport across membranes in addition to the two endocytotic mechanisms discussed above.

ACKNOWLEDGMENTS

The original work from our laboratory that is discussed in this paper was supported by US Public Health Service grants GM-15971, AI-06659, and CA-22031 to S.J.S., who is an American Cancer Society Research Professor.

REFERENCES

1. Albertini DF, Anderson E: J Cell Biol 73:111, 1977.
2. Anderson RGW, Brown MS, Goldstein JL: Cell 10:351, 1977.
3. Anderson RGW, Goldstein JL, Brown MS: Nature (London) 270:695, 1977.
4. Aoyagi T, Suda H, Nagai M, Ogawa K, Suzuki J, Takeuchi T, Umezawa H: Biochim Biophys Acta 452:131, 1976.
5. Ash JF, Singer SJ: Proc Nat Acad Sci USA 73:4575, 1976.
6. Ash JF, Vogt PK, Singer SJ: Proc Nat Acad Sci USA 73:3603, 1976.
7. Ash JF, Louvard D, Singer SJ: Proc Nat Acad Sci USA 74:5584, 1977.
8. Ashwell M, Work TS: Ann Rev Biochem 39:251, 1970.
9. Bornstein P, Ash JF: Proc Nat Acad Sci USA 74:2480, 1977.
10. Bourguignon LYW, Singer SJ: Proc Nat Acad Sci USA 74:5031, 1977.
11. Bourguignon LYW, Tokuyasu KT, Singer SJ: J Cell Physiol 95:239, 1978.
12. Bretscher MS: Nature (London) 260:21, 1976.
13. Brown MS, Goldstein JL: Science 191:150, 1976.
14. Brown MS, Goldstein JL: J Supramol Struct (In press).
15. Burger MM: Proc Nat Acad Sci USA 62:994, 1969.
16. Carpenter G, Cohen S: J Cell Biol 71:159, 1976.
17. Carpenter G, Cohen S: J Cell Physiol 88:227, 1976.
18. de Petris S, Raff MC: Eur J Immunol 2:523, 1972.
19. de Petris S, Raff MC: "Locomotion Tissue Cells." Ciba Foundation Symposium No. 14, 27, 1973.
20. Edelman GM, Yahara I, Wang JL: Proc Nat Acad Sci USA 70:1442, 1973.
21. Gabbiani G, Chaponnier C, Zumbe C, Vassalli, P: Nature (London) 269:697, 1977.
22. Heggeness MH, Ash JF: J Cell Biol 73:783, 1977.
23. Heggeness MH, Wang K, Singer SJ: Proc Nat Acad Sci USA 74:3883, 1977.
24. Heggeness MH, Ash JF, Singer SJ: Ann NY Acad Sci 312:414, 1978.
25. Hynes RO: Biochim Biophys Acta 458:73, 1976.
26. Inbar M, Sachs L: Proc Nat Acad Sci USA 63:1418, 1969.
27. Inbar M, Sachs L: FEBS Lett 32:124, 1973.
28. Keski-Oja J, Vaheri A, Ruoslahti E: Int J Cancer 17:261, 1976.
29. Lazarides E, Weber K: Proc Nat Acad Sci USA 71:2268, 1974.
30. Louvard D, Semeriva M, Maroux S: J Mol Biol 106:1023, 1976.
31. Mooseker MS, Tilney LG: J Cell Biol 67:725, 1975.
32. Muller-Eberhard HJ: Harvey Lect 66:75, 1972.
33. Nicolson GL: Nature New Biol 243:218, 1973.
34. Noonan KD, Burger MM: J Cell Biol 59:134, 1973.
35. Oliver JM, Lalchandani R, Becker EL: J Reticuloend Soc 21:359, 1977.
36. Pappenheimer AM Jr: Ann Rev Biochem 46:69, 1977.
37. Pearse BMF: Proc Nat Acad Sci USA 73:1255, 1976.
38. Rosenblith JZ, Ukena TE, Yin HH, Berlin RD, Karnovsky MJ: Proc Nat Acad Sci USA 70:1625, 1973.
39. Roth TF, Porter KR: J Cell Biol 20:313, 1964.
40. Schreiner GF, Unanue ER: Adv Immunol 24:37, 1976.
41. Schreiner GF, Fujiwara K, Pollard TD, Unanue ER: J Exp Med 145:1393, 1977.
42. Sheetz MP, Singer SJ: Proc Nat Acad Sci USA 71:4457, 1974.
43. Singer SJ: Ann Rev Biochem 43:806, 1974.
44. Singer SJ, Nicolson GL: Science 175:720, 1972.
45. Springer TA, Strominger JL: Proc Nat Acad Sci USA 73:2481, 1976.
46. Taylor RB, Duffus PH, Raff MC, de Petris S: Nature New Biol 233:224, 1971.
47. Toh BH, Hard GC: Nature (London) 269:695, 1977.
48. Tokuyasu KT: J Cell Biol 57:551, 1973.
49. Wartiovaara J, Linder E, Ruoslahti E, Vaheri A: J Exp Med 140:1522, 1974.
50. Weber K, Groeschel-Stewart U: Proc Nat Acad Sci USA 71:4561, 1974.