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*Topical Review*

# Interaction of the Cytoskeleton with the Plasma Membrane

Verena Niggli* and Max M. Burger**
Department of Biochemistry, Biocenter of the University of Basel, CH-4056 Basel, Switzerland

## Introduction

The cellular cytoskeleton, consisting of actin filaments, microtubules and intermediate filaments, is involved in fundamental processes such as cell division, cell migration, shape change, cell adhesion, cell-cell interaction and possibly also in the transfer of information from the plasma membrane to the nucleus, thereby affecting gene expression [18, 186]. To exert these functions, the filaments must be anchored in the plasma membrane. Lateral and also end-on linkage of filaments to the plasma membrane have indeed been observed in electron-microscope studies, and have been confirmed by biochemical analyses [63, 64, 86, 113, 157].

A survey of the literature published in the last ten years on this topic reveals the complexity and variability of the structure of cytoskeleton-membrane anchorage sites in different cell types and for the different filament systems. Except for erythrocytes [15] the exact molecular mechanism and the regulation of the anchorage have not yet been clarified. For this reason, a clear cut proof for the involvement of cytoskeleton-membrane attachment, e.g. in cell migration and adhesion, has not yet been provided, but indirect evidence is abundant. In migrating cells actin filaments are arranged as a network of short fibers, concomitant with a diffuse distribution of the plasma membrane fibronectin receptor and of putative actin membrane linker proteins such as vinculin [54]. In adhesive cells, the actin filaments are bundled and end in focal contacts, which are areas of closest contact between cells and the substrate, where the fibronectin receptor and vinculin are specifically located. Motile and adhesive cells thus exhibit characteristic differences in actin filament organization, concomitant with changes in the structure of filament-membrane attachment sites, reflecting probably their involvement in motility and adhesion. In migrating cells actin-membrane interactions may be of low affinity and quickly reversible, whereas in adhesive cells relatively strong, stable linkages may be formed.

This review will focus on recent evidence on the molecular mechanism of cytoskeleton membrane linkage obtained with biochemical techniques. The literature survey for this review was completed in April, 1987. The abundant microscope and cell biology studies will not be considered (for an excellent recent review *see* M. Schliwa, 1986 [157]). As mentioned above, investigations of the mechanism of cytoskeleton-membrane interactions are most advanced in the red blood cells, which have proved to be an interesting model system. Several cytoskeletal proteins previously thought to be unique to these cells have recently been shown to be widely distributed in different cell types. Some of them may even be universal linker proteins. As this relevant topic has been extensively dealt with in several recent reviews, it will not be discussed in detail in this article. Interested readers should consult, e.g., Refs. 15, 113, 157. In addition, the topic of cytoskeleton-organelle linkage, important for instance in organelle transport, will be excluded since little is known on this topic [157, 178].

Several strategies have been adopted for the

* *Present address:* Institute of Pathology, University of Bern, Freiburgstr. 30, 3010 Bern, Switzerland.

** *Present address:* Friedrich Miescher Institute, CH-4002 Basel, Switzerland.

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study of cytoskeleton-membrane linkage. Retention of specific components (proteins and lipids) in the detergent-resistant cytoskeleton of cells, as well as the interaction of cytoskeletal components with purified plasma membranes, have been investigated. To get more insight into the molecular mechanisms of these interactions, purified components of the cytoskeleton, as well as purified plasma membrane proteins or lipids, have been recombined in vitro under controlled conditions. Results and concepts that have emerged from such studies will be discussed in the following sections.

## Present State of the Biochemical Evidence on Cytoskeleton-Membrane Interactions

### The Cytoskeleton as a Triton X-100 Insoluble Framework

A rapid method for the enrichment of the cellular cytoskeleton involves the extraction of cells with the detergent Triton X-100 [158, 192]. The Triton-insoluble residue remaining after centrifugation of the solubilized cells at 12,000 × *g* consists mainly of actin, myosin, actin-associated proteins, intermediate filaments and microtubules [158], but some glycoproteins and nucleic acids are also co-sedimenting [63]. Most of the membrane proteins and lipids are solubilized by this treatment. Such frameworks have also been obtained by detergent extraction of purified plasma membranes [116]. Co-isolation of specific membrane components with the Triton-insoluble cytoskeleton is often taken as an indication that these components interact in situ with the cytoskeleton. However, this method has pitfalls, as individual proteins just may not be soluble under these conditions and form large pelletable aggregates [91]. Specific lipids retained in the cytoskeleton may be less soluble in the detergent chosen to isolate the frameworks, compared to extracted lipids. Alternatively, components may be trapped unspecifically within the cytoskeletal network. Retention of membrane components in the cytoskeleton may also result from inadequate membrane lysis, as illustrated by findings of M.B. Zucker and N.C. Masiello [194]. These authors claim that the increase in the percentage of cellular proteins and lipids retained in Triton X-100-insoluble residues of platelets induced by thrombin can be attributed to insufficient lysis of aggregated platelets. Indeed, they observed macroscopic aggregates in these residues. Moreover, they could detect membrane-like structures in electron micrographs of platelet frameworks.

Thus the co-isolation of components with the Triton X-100-insoluble cytoskeleton is at best an indication, but never a conclusive proof, for a specific interaction of proteins or lipids with the cytoskeleton, which has to be confirmed with other techniques.

Clustering of a variety of surface receptors by multivalent ligands appears to result in their increased association with the Triton-insoluble cytoskeleton, as summarized by B.S. Jacobson [86]. How these receptors interact with the cytoskeleton, and how this interaction is induced, is not clear. Possibly, clustering of receptors may convert single, low affinity actin-binding sites into multivalent high-affinity sites. One of the systems studied in more detail is a complex of two major surface membrane proteins of platelets (GP IIb and GP III) that is induced to co-pellet with the Triton-insoluble cytoskeleton by treatment of the cells with concanavalin A, a process independent of metabolic energy [133]. This complex very likely corresponds to the platelet fibrinogen receptor (*see* below). Interestingly, the treatment of enriched plasma membrane fractions of platelets with Concanavalin A also results in the association of these glycoproteins with an actin-rich Triton-insoluble matrix. That this association is most likely not an artifact of the Triton extraction is indicated by crosslinking experiments [133]. Using bifunctional reducible protein crosslinkers on Concanavalin A-treated membranes it could be shown that this lectin induces the formation of Triton-insoluble complexes, consisting of 180,000–200,000-Da proteins, GP IIb and III, and actin. Such complexes also appear to exist in membranes not treated with Concanavalin A, although there the amounts of crosslinked GP IIb/III are greatly reduced, whereas the amounts of the other proteins and actin are comparable. Very likely the 180,000–200,000-Da proteins are not directly involved in the actin-receptor linkage. Based on these results, the authors suggest that a small fraction of the GP IIb/III complex is already associated with the cytoskeleton in the absence of the lectin. The latter would then cluster mobile receptors around these immobilized molecules.

Recently a method has been developed to show convincingly that proteins which co-purify with the cytoskeleton indeed interact specifically with the latter [39]. Triton X-100 treated cells are applied to sucrose-density gradients. The drug phalloidin specifically stabilizes microfilaments and effects a shift of the microfilaments further into the gradient. Components that are very tightly associated with the microfilaments not only co-migrate on gradients with actin, but are also shifted by phalloidin. Using this technique, it has been demonstrated that a large

fraction of the ecto-enzyme 5'-nucleotidase is associated with actin in the presence and in the absence of phalloidin, presenting evidence for a specific interaction with the cytoskeleton [39]. Interestingly, this enzyme may interact both with extracellular matrix proteins and with the cytoskeleton, suggesting its involvement in cell adhesion [53].

P. Burn and coworkers [32] observed the presence of specific lipids in the frameworks of platelets. They found that Triton X-100-insoluble frameworks of thrombin-activated platelets that were previously incubated with [$^3$H]-palmitate contain significantly increased amounts of labeled lipids compared to resting platelets. The main labeled components retained in the cytoskeleton of palmitic acid-labeled platelets could be identified as palmitic acid, diacylglycerol and some phospholipids. Moreover, diacylglycerol and palmitic acid could be shown to bind very tightly to one component of the cytoskeleton, namely $\alpha$-actinin. These results suggest that the retention of specific lipids in the frameworks of thrombin-stimulated platelets is not just due to insufficient lysis of aggregated platelets [194], or to differential solubilization of various lipids by Triton X-100. Rather, this finding can be attributed to an interaction of specific lipids with $\alpha$-actinin (*see* below). To conclude, the retention of both membrane proteins and specific lipids in Triton X-100-resistant frameworks may not be fortuitous, but rather indicative of tight in situ complexes. However, this has to be confirmed by valid control experiments.

## Interaction of the Cytoskeleton with Isolated Plasma Membranes

Another biochemical approach, complementary to the analysis of Triton X-100-insoluble residues, involves the purification of plasma membranes, followed by the analysis of co-purifying cytoskeletal proteins and by the study of the interaction of exogenously added cytoskeletal proteins with plasma membranes. In such experiments controls have to be carried out to ensure that co-purifying cytoskeletal components are not just trapped unspecifically inside plasma membrane vesicles. In addition, such preparations are usually contaminated to some degree by organelle membranes possibly containing cytoskeletal proteins. Concerning the rebinding studies, it has to be considered that the sidedness of the membrane vesicles, and therefore the accessibility of putative binding sites, varies in different cell systems and with different isolation techniques. Moreover, removal of endogenous cytoskeletal proteins from membranes without denaturation of binding sites may be difficult.

### *Interaction of Plasma Membranes with Actin*

Several different techniques have been employed to investigate such interactions [107]:

—Co-sedimentation of exogenously added F-actin with plasma membranes under conditions where F-actin alone does not pellet. The disadvantages of this method are a high background and a poor sensitivity.

—Low shear viscometry of F-actin. With this assay, increased crosslinking of F-actin by bi- or multivalent ligands can be detected, as well as the effect of bundling factors, which decrease F-actin viscosity. This assay is very sensitive, but obviously not useful for univalent F-actin binding components.

—E.J. Luna and co-workers [107] have introduced a new technique, using fluorescein-labeled F-actin bound to antifluorescein antibodies covalently coupled to Sephacryl-S-100 beads. Thus, F-actin can be easily and quickly separated from free plasma membranes by low speed centrifugation. One disadvantage of this method is that the plasma membranes first have to be sonicated in order to make them penetrable to the bead matrix. Otherwise, this is a sensitive, relatively quantitative assay, adaptable also for the isolation of putative detergent-solubilized actin binding membrane proteins.

—In order to identify possible actin membrane receptors, incubation of SDS polyacrylamide gels or nitrocellulose transblots of gels with radioactively labeled actin is often used. However, the results have to be interpreted with caution, as discussed by G. Isenberg and co-workers [156]. Weak unspecific binding of actin to many proteins occurs. Only high affinity binding, which can be inhibited by the addition of excess unlabeled actin, is significant. Moreover, a negative result is not conclusive, as irreversible denaturation by SDS, or separation of subunits, may destroy the actin-binding activity.

A very promising system for the study of actin-plasma membrane interactions and their regulation is *Dictyostelium discoideum*, a cellular slime mold amoebae. E.J. Luna and co-workers [106, 107, 159] have analyzed the interaction of actin with purified plasma membranes of this organism extensively, using the methods outlined above. They present convincing evidence for a specific, saturable high-affinity interaction. Moreover, these authors have used the F-actin affinity beads as an affinity matrix

to extract at least 12 integral plasma membrane proteins from a detergent solubilisate, possible candidates for integral transmembrane actin receptors. In agreement with these authors, saturable, high-affinity binding of short glutaraldehyde crosslinked actin filaments to *Dictyostelium* plasma membranes has been described [171]. A 24,000-Da protein has been purified from these membranes, which is only soluble in high concentrations of deoxycholate. This protein binds short actin filaments in a gel-overlay assay, a process which can be inhibited by excess cold actin [171]. In a comparable approach, two putative actin receptors have been identified, one possibly identical with the 24,000-Da protein mentioned above [156]. Moreover, polyclonal antibodies raised against *Dictyostelium* membrane proteins inhibit the actin-membrane interaction significantly [29]. Thus, convincing evidence exists for specific actin protein receptors being present in *Dictyostelium* plasma membranes. Purification of these receptors and generation of antibodies against them should shed more light on their molecular function, localization and distribution.

Plasma membranes of mammalian cells have also been shown to increase the low shear viscosity of F-actin, as for example rat liver plasma membranes [174], human platelet membranes [51] and adrenal medulla plasma membranes [9]. Binding studies of F-actin to liver plasma membranes do not yield easily interpretable results. Binding is not saturable, and electron microscopic analysis indicates that very few actin filaments bind directly to the membranes, suggesting that an actin crosslinking protein co-purifies with the membranes [174].

Platelet plasma membrane preparations contain actin, which appears to be tightly associated as it cannot be extracted by potassium iodide, which depolymerizes F-actin [51]. In contrast, a protein with a relative molecular mass comparable to that of α-actinin, which is retained in the cytoskeleton, is mainly removed by this treatment, implying that it is not an important factor in actin membrane attachment [51]. A similar finding was made by K. Burridge and L. McCullough [36]. These authors studied the nature of the interaction of endogenous α-actinin and actin with purified plasma membranes from HeLa cells. α-actinin was detected and quantified with a specific antibody. Under specific extraction conditions most of the α-actinin could be removed, whereas very little actin was released. The authors conclude that α-actinin is very likely not involved in actin membrane linkage.

Of course, the possibility cannot be excluded that a minor subpopulation of α-actinin is retained in the membranes and acts as a linker. α-actinin would thus have a dual role, both as an actin crosslinker and (possibly after post-translational modification) as an actin-membrane linker. Alternatively, several functionally exchangeable proteins could be involved in this linkage. As already mentioned, α-actinin appears to interact specifically with rare membrane lipids [32].

In erythrocytes much more is known on the mechanism of actin-membrane linkage. There spectrin, via linkage to ankyrin, binds short actin filaments to the membrane [15]. Interestingly, this specific function seems to be restricted to erythrocyte spectrin. Intestinal brush-border spectrin binds to inside-out erythrocyte plasma membrane vesicles with low affinity, in a nonsaturable manner, in contrast to erythrocyte spectrin, which binds with high affinity in a saturable manner [82]. The main binding site for erythrocyte spectrin corresponds to ankyrin, and indeed brush border spectrin does not interact with ankyrin in vitro [82]. These findings illustrate the point that immunologically related cytoskeletal proteins have different functions in different cell types: spectrin in brush border acts mainly as a filament crosslinker rather than a membrane linker. Yet another function has been proposed for brain spectrin (fodrin). According to a recent report [165], isolated synaptosomal plasma membranes contain fodrin. Evidence is presented for a functional control of the glutamate receptor by fodrin, as proteolytic degradation of plasma membrane-associated fodrin results in a doubling of glutamate binding sites. Specific antifodrin antibodies and their Fab fragments block both fodrin degradation and the increase in glutamate binding. The molecular mechanism of this regulation is not yet resolved, but the regulation of membrane protein function by cytoskeletal proteins may be of general importance.

To summarize, promising progress has been made in the study of *Dictyostelium* plasma membrane actin interactions. Plasma membranes of mammalian cells also contain endogenous cytoskeletal proteins and affect F-actin viscosity, but due to the multitude of F-actin associated proteins it is not yet clear how the actin-membrane linkage is mediated in the individual cases, except for the erythrocyte system [15].

### *The Interaction of Plasma Membranes with Intermediate Filaments*

In contrast to the actin filament system, much less is known of the attachment of intermediate filaments to membranes. On the electron microscopic level, the association between intermediate filaments and the cell membrane is well documented in

epithelial cells where keratin filaments interact with desmosomal junctions [157] but the molecular mechanism is not known. In cultured fibroblasts the intermediate filament network appears to be closely associated with the extracellular matrix protein fibronectin, based on electron-microscope and also some biochemical evidence [75].

Fibronectin co-purifies with vimentin under conditions where the former should be soluble. Such an interaction would presumably occur via a membrane protein [75]. Evidence has been presented based both on electron-microscope studies as well as on a biochemical system for an association of vimentin with lens fiber plasma membranes [144]. Vimentin, newly synthesized in a reticulocyte cell free system, associates rapidly with exogenously added plasma membranes. The interaction appears to be very strong and possibly hydrophobic, as washing of the membranes with urea does not remove vimentin. Moreover, peripheral proteins do not seem to be involved as pretreatment of the membranes with urea does not affect vimentin binding. This interaction appears to be different from the association of lens vimentin with inside-out erythrocyte vesicles [66]. In this system, ankyrin quite clearly acts as the main vimentin acceptor site, as binding is abolished by treatment of the membrane by trypsin and by specific anti-ankyrin antibodies. Moreover, ankyrin could be shown to interact with vimentin in vitro, as detected by rate zonal centrifugation of a mixture of these proteins, and by coprecipitation of both proteins by an anti-ankyrin antibody [67]. Both the binding of vimentin to membranes and to ankyrin appears to involve its amino-terminal head domain. This domain is also implicated in the formation of vimentin filaments, suggesting that intermediate filaments do not originate from membrane-bound vimentin as its head domain is occupied. Rather, intermediate filaments may contact the membranes with the head domains of their terminal subunits [67]. Recently, intermediate filament-associated proteins (IFAPs) have been described that could also serve as membrane anchors, for instance at the nuclear surface [71]. Thus, different mechanisms of vimentin-membrane linkage may be operative in different cell types and in different cellular locations.

### *Interaction of Plasma Membranes with Tubulin*

There is both structural and functional evidence for the interaction of microtubules with membranes. A very striking example is represented by the unicellular algae *Distigma proteus*, where ordered arrays of microtubules along the plasma membrane can be observed in negative stain preparations [122, 123]. These arrays are maintained in isolated plasma membrane fragments. After removal of endogenous tubulin by calcium-induced depolymerization, exogenously added tubulin induced to polymerize in the presence of membranes is localized in the same area previously occupied by the endogenous protein, indicating the presence of specific binding sites. Some indirect evidence suggests that membrane receptors and channels may be functionally regulated by microtubules [8].

Studies of the endogenous tubulin content of purified plasma membranes and of the interaction of exogenous tubulin with plasma membranes reveal a rather complex system: not only evidence for a specific interaction of tubulin with isolated membranes has been found, but in addition purified plasma membranes contain very tightly bound tubulin-like molecules, which can only be removed by detergents (membrane tubulin). Such tightly bound tubulin has for example been identified in rat and chick brain synaptosomal membranes [11, 23, 74], synaptosomal vesicles from bovine cerebral cortex [193], platelet plasma membranes [170], plasma membranes and mitochondria from liver cells, and in adrenal medulla secretory granules [20]. Membrane tubulin was found to be comparable to soluble tubulin by the following criteria: identical molecular mass, comparable peptide maps, high-affinity binding of colchicine and vinblastine, crossreactivity with anti-tubulin antibodies. According to Ref. 23, membrane tubulin solubilized by the detergent Nonidet P40 can be carried through several cycles of polymerization and depolymerization. However, some structural differences between soluble and membrane-bound tubulin appear to exist. It has been suggested that membrane tubulin may be a glycoprotein, whereas soluble tubulin does not carry any sugar moieties [57]. The $\alpha$-chain of membrane-bound tubulin in brain has been reported to lack a post-translationally added tyrosine at the C-terminus, whereas about 14% of the soluble tubulin is modified in this unusual way [124]. There is also some evidence that $\alpha$-tubulin is bound more tightly to membranes than $\beta$-tubulin, both in plasma membranes of synaptosomes and in synaptic vesicles [74, 193]. How is membrane tubulin retained in the bilayer? The data on its solubility in different detergents are somewhat controversial. At least part of the membrane tubulin of brain plasma membranes [23], liver plasma membranes [20] and platelet plasma membranes [170] can be released by 0.2–0.4% Nonidet P40. In contrast, other researchers [193, 11] find that membrane tubulin from synaptosomal plasma membranes can only be solubilized by deoxycholate or SDS. For instance, such discrep-

ancies could be explained by contamination of the plasma membrane preparation by intact synaptosomes. Detergent treatment would then release trapped cytosolic tubulin, as discussed in Ref. 11. Certainly part of the membrane tubulin appears to be very tightly bound, comparable to an integral membrane protein.

The question of whether membrane tubulin consists of cold insoluble tubulin or of tubulin aggregates that adhere to the membrane in a nonfunctional way has been addressed [11]. Indeed, cold insoluble tubulin aggregates are not dissolved by detergent treatment. However, such tubulin aggregates, added to isolated synaptosomes and carried through the isolation procedure, are mainly found in the cytoplasmic fraction. Less than 0.5% co-isolates with the plasma membranes [11]. It is thus not very likely that membrane tubulin corresponds to cold-denatured cytosolic tubulin aggregates. However, the exact nature of its interaction with membranes, and its function, remain unknown. The finding that membrane tubulin is more heat resistant than cytosolic tubulin further suggests that it is indeed located in another environment, e.g. the lipid bilayer, compared to cytosolic tubulin [22, 170].

An attractive hypothesis views membrane tubulin as a receptor for cytoplasmic tubulin, initiating its polymerization at the membrane. Indeed, evidence has been presented for saturable, reversible, specific high-affinity binding of exogenous tubulin to purified plasma membranes and mitochondria of rat liver, as well as to secretory vesicles of adrenal medulla [20]. Tubulin is not just trapped inside plasma membrane vesicles, as it reacts quantitatively with antibodies even after binding to membranes. The authors propose that this binding represents assembly of tubulin at the membrane, initiated by a specific receptor, possibly membrane tubulin. In agreement with this hypothesis, erythrocyte plasma membrane vesicles that lack membrane tubulin do bind much less exogenous tubulin.

To conclude, some evidence exists for the interaction of tubulin with purified plasma membranes, but the molecular mechanism has not yet been clarified. Tubulin-like membrane proteins may be involved.

## Plasma Membrane Components Possibly Involved in the Linkage of the Cytoskeleton to the Plasma Membrane

An alternative approach, besides the study of frameworks and of purified plasma membranes, concerns the isolation and purification of cytoskeleton-associated proteins and the investigation of their putative linker function in vitro, be it by the study of their interactions with lipids or membrane proteins or with actin. The final goal would be the reconstitution of actin membrane linkage in vitro. Three mechanisms of linkage can be postulated [157]:

1) A direct interaction of the cytoskeleton with specific transmembrane protein receptors, which could be in contact with extracellular matrix proteins.
2) A direct insertion of filament proteins into the lipid bilayer.
3) An association of the cytoskeleton with peripheral proteins, which themselves either insert directly into the lipid bilayer or interact with integral membrane proteins, or both.

Biochemical evidence for all three types of interaction will be presented in the following sections.

### Overview (Tables 1 and 2)

In Table 1 an overview is given on putative actin-membrane linkers. Integral membrane proteins and proteins that may interact reversibly with membranes are listed separately. Table 2 summarizes the few proteins that have been implicated in intermediate filament and tubulin membrane linkage. The biochemical studies of the latter interactions are less advanced, compared to the microfilament system. It cannot be excluded that, in contrast to the microfilament system, tubulin and the intermediate filaments may also insert directly, without linker proteins, into the bilayer, as will be discussed later. Interestingly, spectrin and ankyrin have been implicated as universal linker proteins, connecting different filament systems and acting also as membrane linkers for all three types of filaments.

A variety of cell surface proteins have been characterized, which may be involved in cell adhesion, by selecting, e.g., for monoclonal antibodies that inhibit cell adhesion to the substrate or that react with proteins selectively located at sites of actin-membrane linkage [129, 150]. Only those proteins have been listed in Table 1 for which additional evidence exists, indicating that they are connected to the actin filament system.

### Integral Membrane Proteins as Putative Cytoskeleton-Receptors

Proteins belonging to this class, which interact not only with the cytoskeleton, but also with extracellular matrix components, are of special interest. For instance, their existence could explain the observations that the extracellular matrix can influence the

organization of the cytoskeleton [78] and that depolymerization of actin filaments by cytochalasin B leads to the loss of fibronectin from the cell surface [2]. Such proteins could transfer information from the extracellular space to the cytoskeleton, thereby affecting cell function and motility. Direct evidence for the existence of such transmembrane receptor proteins is as yet scarce, but several promising candidates have been recently identified. A few other putative plasma membrane receptor molecules, not in contact with extracellular components, will also be discussed in this section.

*The Fibronectin Receptor*

This receptor has recently been purified and characterized from both mammalian and avian tissues. Different approaches have led to its isolation. One approach, applied to the avian system, involved the search for monoclonal antibodies that inhibit cell adhesion. For instance, the monoclonal antibodies JG22 [76] and CSAT [125] strongly inhibit the attachment of a variety of chick cells to fibronectin and laminin substrata [42, 81]. Both antibodies react with the same antigen in chick cells [80]. This antigen has been purified from chick embryos solubilized in detergent by using an antibody-affinity column, followed by release of the bound material with diethylamine, 50 mM, at pH 11.5 [96]. Interestingly, the antigen, three glycoproteins of about 140,000 Da in reducing SDS gels, still retains some of its functional activity after this rather harsh treatment, as it could be shown to interact in vitro both with laminin and with fibronectin. This binding specificity correlates with the inhibitory effect of CSAT on the adhesion of cells to both fibronectin and laminin. The technique used to demonstrate this interaction is equilibrium gel filtration. The gel filtration column is pre-equilibrated with or without the ligand, and changes in the elution pattern of the receptor reflect a specific interaction. This method is useful for the study of rapidly dissociating complexes [81]. A $K_d$ of $10^{-6}$ M for the fibronectin- and of $2 \times 10^{-6}$ M for the laminin-receptor interaction was determined [81]. The receptor occurs in a variety of chick cells (except erythrocytes): fibroblasts, epithelial cells, muscle cells, liver cells and lymphocytes. It is localized in close contacts at the ventral side of fibroblasts and at the periphery of focal contacts; it co-aligns with portions of stress fibers and is found at the basolateral side of epithelial cells [41, 49].

A different approach has been used to purify a fibronectin receptor from mammalian cells (e.g. from human osteosarcoma cells), as described by R. Pytela and co-workers [143]. These authors used an affinity column containing the covalently bound cell binding fragment of fibronectin. The receptor was then eluted specifically from this column by a hexapeptide containing the Arg-Gly-Asp- sequence which is crucial for the interaction of cells with fibronectin (153). The purified mammalian receptor differs in some respects from the avian protein. For instance the mammalian receptor consists of only two instead of three subunits. Reconstituted into liposomes, it interacts specifically with fibronectin, and not with laminin. Although no affinity constants for the interaction of the mammalian receptor with fibronectin have yet been determined, it appears to bind much more strongly than the avian receptor, as the latter could not be isolated by the affinity chromatography technique described above [1]. As discussed by C.A. Buck and co-workers [30] different specific receptors appear to be involved in the attachment of mammalian cells to the various extracellular matrix proteins, whereas the avian protein may serve as a receptor for a multitude of different matrix proteins. It is also possible that CSAT extracts a mixture of different receptors, whereas with the fibronectin affinity column only the fibronectin receptor is isolated. The mammalian receptor may be localized in the same areas as the avian protein, as shown for a putative fibronectin receptor in 3T3 fibroblasts [69]. Recently the complete sequence of cDNA clones encoding subunit 3 of the CSAT antigen has been determined [173]. In addition, *c*DNA sequences have been obtained for the $\alpha$-subunit of the mammalian fibronectin receptor [7]. Both proteins contain a typical membrane-spanning region, confirming that they are, indeed, integral membrane proteins. The complete sequence analysis of both proteins will yield more information on their structural and functional homologies and differences.

A question of key interest is the possible interaction of these purified receptors with cytoskeletal components. Recently evidence has been provided that at least the avian receptor interacts in vitro with talin, a focal contact protein [80]. This interaction appears to be rather weak, as it could only be shown using equilibrium gel filtration, but not, for example, with sucrose density gradients. A dissociation constant of 0.7 $\mu$M for the interaction of talin with the purified, detergent-solubilized receptor has been determined [80]. The receptor has a binding site for talin that is different from the site for fibronectin, as it can bind both proteins simultaneously, shown by a different migration of the receptor in a column equilibrated with talin alone or with talin and fibronectin. Moreover, the peptide Arg-Gly-Asp inhibits only the interaction of the receptor with fibronectin, not that with talin. How is

**Table 1.** Proteins putatively involved in microfilament-plasma membrane linkage

| Protein | Rel. molecular mass | Tissue distribution | Location | Ref. |
|---|---|---|---|---|
| *1. Integral membrane proteins* | | | | |
| Fibronectin/laminin receptor (avian) | Nonreducing conditions<br>160,000 (band 1)<br>135,000 (band 2)<br>110,000 (band 3)<br>Reducing conditions:<br>ca. 140,000<br>(bands 1–3) | Fibroblasts<br>Epithelial cells<br>Muscle cells<br>Lymphocytes | Close contacts at periphery of focal contacts<br>Colocalization with portions of stress fibers and fibronectin fibers<br>Adjacent to dense plaques in smooth muscle<br>Cell-cell contacts<br>Basolateral membrane of epithelial cells (clusters) | [1, 42, 81, 173] |
| Fibronectin receptor (mammalian) | Nonreducing conditions<br>$\alpha$-subunit:<br>ca. 140,000<br>$\beta$-subunit:<br>ca. 120,000<br>Reducing conditions:<br>$\alpha$- and $\beta$-subunits:<br>ca. 140,000 | Human osteosarcoma cells<br>Rat fibroblasts<br>Human placenta<br>Rat kidney cells | Plasma membrane | [7,143] |
| Fibrinogen/fibronectin/ vitronectin receptor (human) | Reducing conditions:<br>GP IIb<br>$\alpha$: 124,000<br>$\beta$: 20,000<br>($\beta$ is disulfide linked to $\alpha$)<br>GP IIIa<br>108,000 | Platelets | Plasma membrane | [40, 61, 133, 134, 142] |
| Laminin receptor | 68,000–72,000 | Murine fibrosarcoma cells<br>Murine melanoma cells<br>Myoblasts | Plasma membrane | [28, 114, 145, 187] |
| Heparan sulfate proteoglycan | $3–5 \times 10^5$ | Epithelial cells<br>Liver<br>Fibroblasts | Plasma membrane<br>Co-localizes with actin filaments | [37, 94, 104, 147, 148, 189] |
| Cytoskeleton associated glycoprotein (CAG) | 75,000–80,000 | Microvilli of rat mammary adenocarcinoma | Plasma membrane | [91] |
| A-CAM | 135,000 | Cardiac muscle<br>Eye lens<br>Brain<br>Cultured kidney cells | Fascia adherens (cardiac muscle)<br>Adherens junctions (epithelial cells) | [183, 184] |
| Rat liver actin binding protein | 50,000 | Normal rat liver<br>Liver tumor cells | Plasma membrane | [12] |
| 5′-nucleotidase | 79,000 | Chicken gizzard<br>Pig lymphocytes<br>Rat mammary adecarcinoma cells | Plasma membrane (ecto-enzyme) | [39, 53] |
| Band 3 | 90,000–100,000 | Erythrocytes | Plasma membrane | [105] |
| Glycophorin | 31,000 (dimer) | Erythrocytes | Plasma membrane | [113] |
| *2. Membrane associated proteins* | | | | |
| Ankyrin (goblin) | 200,000 (monomer) | Erythrocytes<br>Cardiac and skeletal muscle<br>Brain | Plasma membrane Z-line (muscle)<br>Along microtubules | [16, 17, 52, 126, 160, 179] |
| Spectrin | 2 subunits<br>220,000<br>240,000 | Erythrocytes | Plasma membrane | [160] |

**Table 1.** (Continued)

| Protein | Rel. molecular mass | Tissue distribution | Location | Ref. |
|---|---|---|---|---|
| Non-erythrocyte spectrin (fodrin) | 2 subunits<br>240,000<br>235,000-260,000 | Cultured cells<br>Muscle<br>Brain<br>Intestinal brush border | Plasma membrane<br>Costameres (skeletal muscle)<br>Terminal web of brush border | [73, 103] |
| Protein 4.1 | 2 subunits<br>80,000<br>78,000 | Erythrocytes<br>Platelets<br>Neutrophils<br>Lymphoid cells<br>Fibroblasts<br>Brain | Plasma membrane<br>Along stress fibers | [45, 72, 73, 166] |
| α-Actinin | 100,000 (dimer) | Cultured cells<br>Vertebrate muscle<br>Epithelial cells<br>Platelets<br>Brain<br>*Dictyostelium*<br>*Acanthamoeba* | Focal contacts<br>Along stress fibers<br>Secretory vesicles<br>Z-line (striated muscle)<br>Dense bodies and dense plaques (smooth muscle)<br>Fascia adherens of intercalated discs (cardiac muscle) | [34, 55, 88, 175, 32] |
| Talin | 215,000 | Cultured cells<br>Vertebrate muscle<br>Platelets | Focal contacts<br>Costameres (striated muscle)<br>Dense plaques (smooth muscle)<br>Intercalated disks (cardiac muscle) | [14, 33] |
| Vinculin | 130,000 (monomer) | Cultured cells<br>Vertebrate muscle<br>Brain<br>Epithelial cells<br>Platelets | Focal contacts<br>Costamers (striated muscle)<br>Dense plaques (smooth muscle)<br>Intercalated disks (cardiac muscle)<br>Adherens junctions of epithelia | [31, 59, 62, 65, 85, 127, 154, 175] |
| Metavinculin | 152,000 | Vertebrate muscle (most abundant in smooth muscle) | (*See* Vinculin) | [58, 70, 154, 163, 164] |
| 110,000-dalton protein of microvilli | 110,000 | Microvilli of intestinal epithelium | May link actin bundles laterally to the plasma membrane | [46, 47, 68] |

**Table 2.** Proteins putatively involved in tubulin or intermediate filament-membrane linkage

| Protein | Comments | Ref. |
|---|---|---|
| *A. Tubulin* | | |
| Microtubule-associated proteins (MAPs) | MAPs associate in vitro with liposomes and may mediate the association of tubulin with lipids | [90, 191] |
| Ankyrin | Ankyrin associates with microtubules in vitro and in situ | [16] |
| Fodrin | Fodrin bundles microtubules in vitro | [84] |
| *B. Intermediate filaments* | | |
| Spectrin | Spectrin associates with intermediate filaments in vitro and in situ | [100, 112] |
| Ankyrin | Ankyrin interacts with vimentin in vitro and mediates its linkage to erythrocyte plasma membrane | [66] |

the link from talin to the cytoskeleton mediated? Talin has previously been shown to interact in vitro with vinculin [35], and indeed, talin appears to be able to bind simultaneously to vinculin and the fibronectin receptor [80]. Vinculin or $\alpha$-actinin alone do not appear to interact directly with the receptor. Whether or not vinculin, bound to the receptor via talin, then constitutes a direct link to the actin filaments is unclear, as extensively purified vinculin has recently been shown not to bundle actin filaments nor to interact with actin, in contrast to previous reports [56, 132, 188]. $\alpha$-Actinin, which appears to interact with vinculin in vitro, may be the missing link [185]. Possibly the functional reconstitution of the whole complex from the isolated components can now be attempted. C.A. Buck and co-workers [30] have recently provided more information on the molecular mechanism of the interaction of the fibronectin receptor with its ligands. They could show that the receptor is only functional as an oligomeric complex. The individual subunits alone (either subunit 3 alone, or subunits 1 and 2 together) do not bind talin, fibronectin or laminin. Only the recombination of all three subunits again allows binding of these proteins.

An interesting aspect of the avian fibronectin receptor is its phosphorylation by a tyrosine kinase [79]. Subunits 2 and 3 of the receptor appear to be phosphorylated predominantly on tyrosine in Rous Sarcoma Virus (RSV) transformed chicken embryo fibroblasts, whereas no phosphorylation can be detected in nontransformed cells [79]. Concomitant with phosphorylation, the localization of the receptor is changed from the vicinity of the focal contacts to a more diffuse distribution [79], comparable to its localization in migratory cells [54]. The latter finding has been confirmed by other authors [43, 69]. Tyrosine phosphorylation of the receptor may be the causal effect of its changed localization in transformed cells and is possibly connected to the disorganization of stress fibers occurring in transformed cells. This role has previously been attributed to vinculin, which is also increasingly phosphorylated on tyrosine in transformed cells. However, the latter event does not correlate well with microfilament organization in various viral mutants [151]. Talin, intriguingly, also appears to be increasingly phosphorylated on tyrosine in RSV transformed cells [136]. The functional role of protein phosphorylation in the formation of substrate-cell adhesions via the fibronectin receptor has yet to be shown, but the data discussed above point to its importance.

### *The Fibronectin/Fibrinogen/Vitronectin Receptor of Platelets*

This receptor has been purified by the same technique as the mammalian fibronectin receptor [61, 142], i.e., with an affinity column containing either a cell-binding fragment of fibronectin, or a hexapeptide with the Arg-Gly-Asp sequence. The receptor, reconstituted into liposomes, binds to microtiter plates coated with fibrinogen, fibronectin or vitronectin [142]. It thus has a broader specificity than the mammalian fibronectin receptor. The purified receptor consists of two subunits of 108,000 and 124,000 Da (with a light chain of 20,000 Da, disulfide linked to the latter) and has been shown to be identical with the previously described platelet glycoproteins GP IIb (124,000 Da) and GP IIIa (108,000 Da) [142]. Indeed, it could be shown that the purified complex GP II/IIIa, reconstituted into large liposomes, binds fibronectin directly with a $K_d$ of about $10^{-7}$ M [135]. This binding is inhibited by excess fibrinogen or fibronectin, as well as by a monoclonal antibody directed against the receptor and by the tetrapeptide Arg-Gly-Asp-Ser, comparable to the effect of these agents on fibronectin binding to stimulated platelets (except that there excess fibronectin does not inhibit).

Interestingly, there is also some evidence that the GP IIb/IIIa complex interacts with actin (*see also* a previous section). For example part of the receptor is retained in the Triton X-100-insoluble framework of resting platelets, and this fraction of the protein co-isolates with actin upon gel filtration or immunoprecipitation [134]. The population of receptor retained in the cytoskeleton also binds exogenously added F-actin, whereas the extractable receptor does not. Apparently the receptor can exist in at least two different biochemical states: one form binds strongly to actin, the other lacks this ability. It is not known whether the interaction with actin is direct or mediated by another protein (or lipid) and how this interaction is regulated. An interesting question is how thrombin induces interaction of the receptor with both fibrinogen and actin. It has been observed that thrombin stimulation enables platelets to bind to fibrinogen and fibronectin [190]. Moreover, an increased amount of GP IIb/IIIa is recovered in the frameworks of thrombin-stimulated platelets [140]. It is not clear why resting platelets do not bind fibrinogen and fibronectin. Changes in the structure of the receptor complex or its microenvironment have been implicated to occur during activation, but no conclusive proof exists yet. As detergent-solubilized receptor can be isolated as a heterodimer on a fibronectin affinity column [142], it is rather unlikely that clustering of the receptor is a prerequisite for fibronectin binding. It has been suggested that resting platelets contain functional fibrinogen receptors in an internal, but surface-connected, compartment, which is accessible to Fab-fragments of specific antibodies, but not to fibrinogen or intact antibodies [190]. Thrombin would then induce widening of the pores and possi-

bly redistribution of the receptor to the platelet surface. How thrombin increases the association of the receptor with the cytoskeleton is not yet known. Possibly, binding of fibronectin to the receptor affects its interaction with actin, or thrombin regulates the receptor via an indirect pathway, for instance by protein phosphorylation. Such a functional connection between the cytoskeleton and extracellular glycoproteins is probably important in platelet functions, such as fibrinogen-mediated aggregation and adherence of platelets to sites of vascular injury.

Proteins very similar to the GP IIb/IIIa complex have recently been described to be present in endothelial cells, smooth muscle cells and fibroblastic cells [40]. These surface glycoproteins crossreact with antibodies against the platelet receptor; they also exist as complexes and have similar molecular masses and isoelectric points. The GP IIb/IIIa complex could thus belong to a family of related surface proteins, involved in cell-cell and cell substrate interactions, widely distributed in mammalian cells.

*The Laminin Receptor*

The extracellular matrix protein laminin can mediate the attachment of a wide variety of epithelial and endothelial cells to type IV collagen. Laminin affects the orientation of the cellular cytoskeleton [172], suggesting the presence of a direct transmembrane link, comparable to fibronectin. In avian cells, the fibronectin receptor described above may play this role. In mammalian cells, specific laminin receptors have been isolated and characterized, for example from murine fibrosarcoma cells [110], murine melanoma cells [145] and from mouse thigh muscles and rat myoblasts [102]. In all cases the receptor was isolated with an affinity column containing covalently linked laminin, followed by release of tightly bound protein by either 0.2 M glycine, pH 3.5, or by increasing the concentration of NaCl from 0 to 0.5 M. In all cases the purified protein appeared to be still native, as it could be shown to bind laminin. A $K_d$ of $2 \times 10^{-9}$ M, similar to data obtained for the binding of laminin to plasma membranes or to whole cells, was determined for the murine fibrosarcoma and melanoma protein. The purified muscle cell receptor binds to some extent also to fibronectin and collagen type I, but the affinity to laminin is higher, and the protein cannot be isolated with a fibronectin affinity column [102].

It has to be noted that in these experiments the receptor is solubilized in detergent, which may affect the specificity of binding. Indeed, detergents may denature the receptor to some extent, as the protein reconstituted into liposomes is able to bind laminin at physiological ionic strength, whereas the receptor solubilized in detergent only interacts with laminin at low ionic strength [102]. Some evidence also exists for a direct interaction of the purified receptor with actin. In a centrifugation assay, F-actin increases the amount of receptor pelletable at about $150{,}000 \times g$ significantly [28]. The experiment is complicated by the variable amount of receptor pelleting already in the absence of F-actin, probably due to the low concentration of detergent used in these studies, which is not sufficient to completely solubilize the receptor. The labeled receptor also binds to F-actin, which is immobilized on nitrocellulose, and this interaction can be inhibited by excess unlabeled receptor. The mammalian laminin receptor may thus also qualify as a direct connection between the extracellular matrix and the cytoskeleton, but this function has to be confirmed for the protein purified from other cell types and under more native conditions, for instance with receptor reconstituted into liposomes. Interestingly the laminin receptor may be closely related to the enzyme 5'-nucleotidase from smooth muscle [114].

*A Membrane-Inserted Heparan Sulfate Proteoglycan*

A subpopulation of one type of extracellular matrix components, the heparan sulfate proteoglycans, constituents of basement membranes, may insert directly into the lipid bilayer. For example, a special class of such molecules, which can only be solubilized by detergents and which can be intercalated into the bilayer of lipid vesicles, have been found to be associated with rat liver plasma membranes [94], mouse mammary epithelial cells [147] and human lung fibroblasts [104]. Proteolysis of the proteoglycan inserted into liposomes releases a hydrophilic molecule of similar relative molecular mass to the intact molecule, indicating the presence of a lipophilic domain responsible for its insertion into the bilayer [147]. Some evidence has been presented for the in vitro interaction of this hydrophobic proteoglycan with actin [146], but these data have to be confirmed and extended to the in situ system. Support for a putative interaction of this extracellular matrix component with actin comes from data on its localization in epithelial cells. Such a cell surface proteoglycan appears to co-localize with actin filaments [148]. Moreover, a significant percentage of such proteoglycans is retained in the Triton-insoluble framework [37, 148]. Upon crosslinking of the cell surface molecule with antibodies, it aggregates and becomes more resistant to extraction with detergent [148]. This process is sensitive to cytochalasin D, implicating again a direct or indirect associa-

tion of a special class of proteoglycans with the cytoskeleton.

### *Microvilli*

The microvilli of intestinal cells represent a specialized system, consisting mainly of a bundle of axially arranged microfilaments anchored at the tip, and also laterally, to the microvilli membrane. Microvilli are one of the best studied model systems for actin-membrane interactions [121]. However, similar to erythrocytes, the results obtained for this specialized system cannot be extended to other systems as focal contacts or adherens junctions.

The Triton X-100 resistant framework of microvilli consists of several major proteins: actin, tropomyosin, the actin crosslinkers villin and fimbrin, a 110,000- and a 140,000-Da protein (both putative actin-membrane linkers), as well as calmodulin. There is good evidence that the bridges connecting the actin filament bundles laterally to the membrane, as visualized in electron micrographs, consist at least partially of a complex of the 110,000-Da protein (not identical with α-actinin) and calmodulin [157]. Controversial results have been published on the membrane-binding properties of the 110,000-Da protein. It has been claimed [68] that the 110,000-Da protein is directly inserted into the lipid bilayer. The evidence is based on the following observations. ATP is known to induce removal of the 100,000-Da protein, and of the lateral bridges, from the microvillus core filament. According to Ref. 68 the protein released by ATP appears to be highly aggregated, and can be pelleted by high-speed centrifugation. The aggregated protein can be solubilized with specific detergents or in distilled water, indicating hydrophobic interactions. Moreover, the 110,000-Da protein partitions into the detergent-rich phase upon phase separation of Triton X-114 (a technique devised by C. Bordier [27] to separate hydrophobic from hydrophilic proteins); it binds to liposomes and can be labeled by the lipophilic probe phenylisothiocyanate. These findings certainly implicate the presence of hydrophobic domains in the protein, but are not a definitive proof for its direct interaction with the lipid bilayer. For instance, small hydrophobic probes such as phenylisothiocyanate have been shown not only to label integral membrane proteins, but also accessible hydrophobic pockets of hydrophilic proteins [24]. Indeed, in contrast to these results, according to a subsequent report [46], the 110,000-Da protein partitions only into the detergent phase of Triton X-114 as a result of the isolation procedure using detergent as described in Ref. 68. Without detergent treatment the protein cannot be pelleted by high speed centrifugation and partitions into the aqueous phase. Moreover, the partitioning of the 110,000-Da protein into the detergent phase appears to be at least partly due rather to aggregation of the protein than to its hydrophobic properties. These data suggest that the 110,000-Da protein is in its native state hydrophilic, only peripherally associated with membranes, and that treatment with detergents leads to its denaturation, aggregation and exposure of hydrophobic regions. This interpretation is supported by the finding that the protein before detergent treatment interacts in vitro with actin, but loses this ability after detergent solubilization. Therefore, this protein very likely belongs to the class of reversibly membrane binding proteins (i.e., amphitropic proteins, *see* below), rather than to the integral membrane protein receptors.

In contrast, another microvillar protein of 140,000 Da is more likely integrally embedded in the membrane [48]. This protein partitions partially into the detergent-rich phase of Triton X-100 before, but not after, papain treatment. In the hydrophobic form it interacts in vitro with the 110,000-Da protein and could thus possibly function as its membrane receptor. An interesting aspect of the 140,000-Da protein is its immunological crossreactivity with the calcium ATPase of the sarcoplasmic reticulum. The former protein has no calcium ATPase activity, but both proteins may share a common structural domain involved in actin filament anchorage [149].

In isolated microvilli of rat mammary adenocarcinoma, a cell surface cytoskeleton-associated glycoprotein has been implicated to induce actin polymerization at the tip of the growing microvillus. The protein, a large disulfide-linked multimer with subunits of 75,000–80,000 Da, indeed interacts with actin filaments on sucrose density gradients, both in the presence and in the absence of phalloidin [91]. This multimer may be a receptor for a bundle of microfilaments.

### *Proteins of Cell-Cell Junctions (A-CAM, N-CAM)*

Not only regions of cell-substrate, but also sites of cell-cell contact interact on the cytoplasmic side with the cytoskeleton. Indeed, focal contacts between cultured cells and their substrate and adherens junctions between epithelial cells are comparable in many aspects. Both contain membrane-associated actin filaments and the cytoskeletal protein vinculin. Talin, in contrast, is absent from these junctions [183]. Comparable to focal contacts, specific transmembrane protein receptors may mediate

cell-cell and membrane cytoskeleton contacts simultaneously. Recently evidence has been provided for the involvement of a 135,000-Da protein in such interactions by B. Geiger's group [182–184]. This protein (A-Cam) is specifically associated with intercellular adherens-type junctions of many tissues, particularly in heart, lens and brain. A-Cam co-localizes with vinculin at the intercellular junctions, but is absent from focal contacts, indicating its specialized function. Some indirect evidence speaks for its linkage to the cytoskeleton. For example, Fab fragments of anti A-Cam antibodies not only inhibit reformation of cell-cell junctions in lens epithelial cells dissociated by EGTA, but they also induce a deterioration of stress fibers [184]. This effect may be specific since intact antibodies have no apparent effect on junction formation, possibly because they may bridge A-Cam molecules between neighboring cells, replacing normal receptor interactions. The intact A-Cam molecule appears to consist of a large extracellular domain with possibly a small transmembrane sequence [183]. It remains to be shown if the protein is indeed in direct contact with the microfilaments via a putative cytoplasmic domain.

The neural cell adhesion molecule N-Cam may also be associated with the cytoskeleton. The mobility of N-Cam appears to decrease significantly upon differentiation of a mouse neuroblastoma cell line, concomitant with a shift in the expression of the 140,000-Da form to the expression of the 180,000-Da form of N-Cam. [141]. These two forms differ in the length of their carboxy-terminal cytoplasmic regions. Possibly the 180,000-Da form contains a specific domain that links to the cytoskeleton, resulting in its immobilization. The preliminary finding that purified brain spectrin binds only to the 180,000-Da form of purified N-Cam immobilized on nitrocellulose, but not to the 140,000-Da form, supports this hypothesis.

## The Interaction of Cytoskeletal Proteins with Lipids and Membranes

As summarized in Table 1, a variety of cytosolic, cytoskeleton-associated proteins have been described that are located in sites of cytoskeleton membrane attachment and that may function as linkers. In very few cases a direct proof for such a function is available, except for the erythrocyte proteins [15]. Recent reports suggest that these cytosolic proteins may not only interact via specific protein receptors with the membrane but also via a direct insertion into bilayers. Moreover, specific lipids may modulate their function. Such examples will be discussed in the following sections. Most of the present evidence is based on in vitro studies, and this new concept will have to be confirmed in situ.

### *Microfilament-associated proteins*

*Actin.* Most of the present evidence, with a few exceptions, speaks against a direct insertion of actin itself into bilayers. For example, the viscosity of rabbit skeletal F-actin is not affected by liposomes consisting either of pure phosphatidylcholine or of *Dictyostelium* lipid extracts [106]. In addition, such liposomes do not significantly bind to F-actin affinity beads [107]. As discussed above, plasma membranes of *Dictyostelium,* in contrast, do interact with F-actin in these assays [106, 107]. Another approach involves the use of 5-iodo-naphtyl-1-azide (INA), a photoaffinity label that partitions into the hydrophobic phase of membranes. Endogenous actin which co-purifies with various membranes, for instance with chromaffin granule membranes, erythrocyte membranes, or brush border membranes, appears not to be significantly labeled by INA after equilibration of this label with the membranes [19, 117, 161]. The use of another hydrophobic photolabel, 3-trifluoromethyl-3-(*m*-iodophenyl)-diazirine (TID), equilibrated with brush border membranes, confirms these results [167].

One exception may be actin in platelets. Actin has been reported to be labeled by INA equilibrated with platelets [152]. Interestingly, actin retained in the Triton X-100-insoluble frameworks appears to be more strongly labeled than the cytosolic actin. Moreover, pure muscle actin in solution is not labeled by INA. Thus labeling of actin in platelets may be due either to an insertion of this protein into the lipid bilayer or to a conformational change of F-actin, leading to the formation of accessible hydrophobic domains. To conclude, the direct insertion of actin into bilayers appears not to be a mechanism of general importance, although it may occur, for instance in platelets. Moreover, none of the actin isoforms appear to be preferentially located at the plasma membrane [131]. Rather, specialized proteins very likely mediate actin-membrane linkage.

*α-Actinin.* As discussed in a previous section, it has been proposed that α-actinin is not involved in a direct actin-membrane linkage [36]. However, recent evidence now suggests that this protein, under specific conditions, can be induced to play such a role and may therefore exist both in a membrane-bound and in a cytoplasmic form (amphitropic).

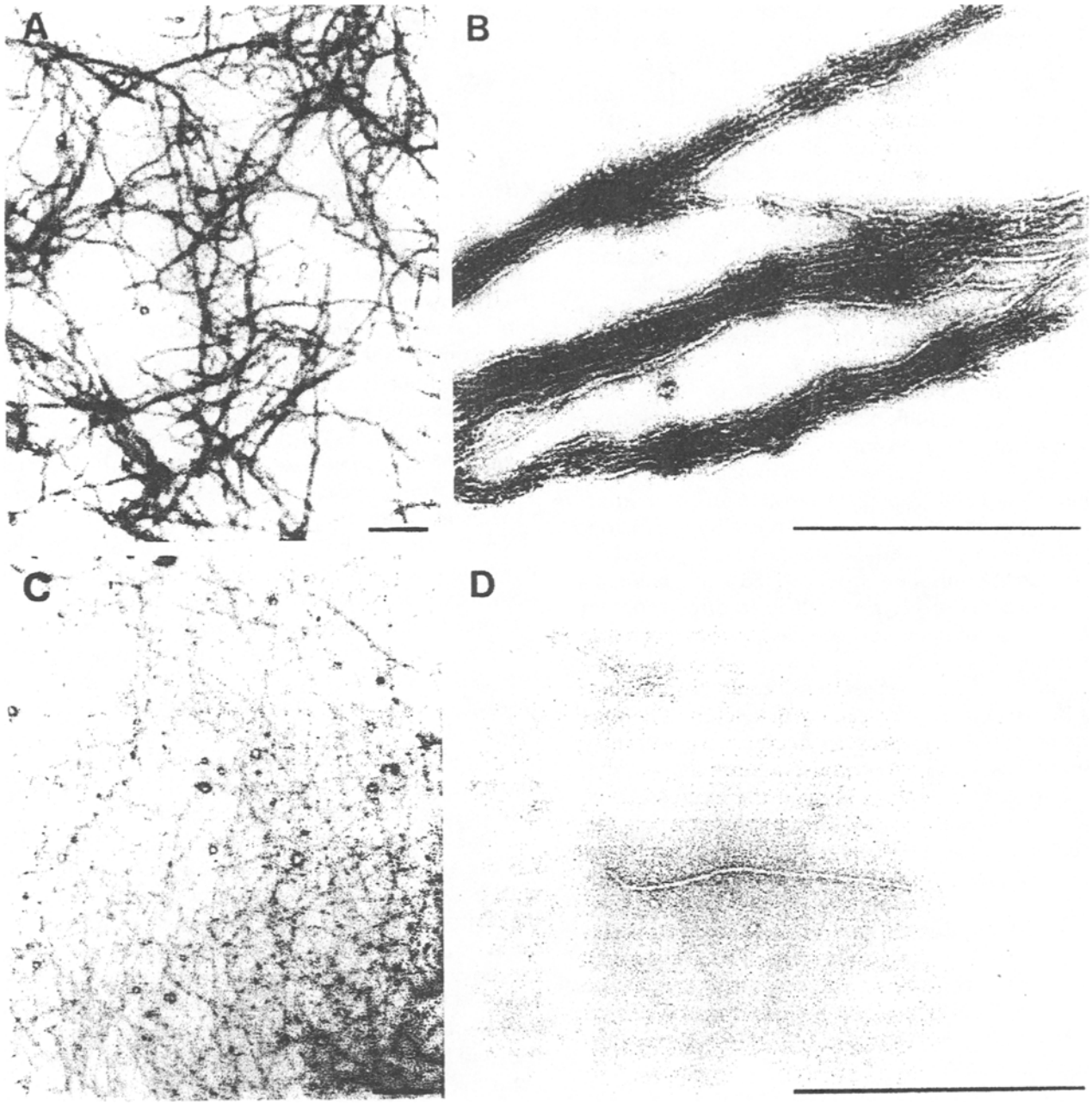


**Fig. 1.** The effect of specific lipids on the polymerization of actin in the presence of α-actinin. An actin-α-actinin mixture was polymerized in the presence (*A*, *B*) or in the absence (*C*, *D*) of diacylglycerol and palmitic acid. The whole mixture (*A*, *C*) or the 10,000 *g* pellets of the polymerized material (*B*, *D*) were then prepared for electron microscopy by negative staining (reprinted by permission from *Nature*, Vol. 314, No. 6010, pp. 470, copyright 1985, Magazines Ltd. [32]). Scale bars, 0.5 μM

Specific lipids have been shown to interact with α-actinin both in vitro and in situ and, in addition, appear to functionally regulate the protein, perhaps enabling it to link actin to the membrane. R.K. Meyer and co-workers [118] found that α-actinin, in two different model systems (monolayer and bilayer), specifically binds two lipids only, i.e., diacylglycerol and fatty acids (palmitic acid or oleic acid, but not arachidonic acid), out of a total yeast lipid extract. In monolayers, stoichiometric complexes of one protein molecule with one molecule of each diacylglycerol and of fatty acid are formed. These lipids not only interact strongly with α-actinin, but may also regulate its function [32]. When actin is allowed to polymerize in the presence of α-actinin or when α-actinin is added to F-actin, a network of single filaments results (Fig. 1*C* and *D*), which cannot be pelleted at 10,000 × *g*. Preincuba-

tion of α-actinin with diacylglycerol and palmitic acid leads to the formation of large actin filament bundles (10–40 filaments) pelletable at 10,000 × *g* (Fig. 1*A* and *B*). Arachidonic acid cannot replace palmitic acid. Thus binding of specific lipids to α-actinin enables this protein to bundle actin filaments, possibly at the membrane where these lipids are located.

Diacylglycerol is a specially interesting component, a recently identified second messenger resulting from increased phosphatidylinositol breakdown induced by various stimuli [21, 109, 119]. Thrombin, for instance, stimulates its production in platelets [21], concomitant with secretion, aggregation and a marked increase in polymerized actin [38]. According to the results discussed above, this component may be directly involved in the thrombin-induced reorganization of the cytoskeleton and its membrane linkage rather than acting indirectly via its well-known stimulatory effect on protein kinase C [128]. Indeed, in support of this hypothesis it was found that thrombin markedly increases the incorporation of lipid components into platelet frameworks [32]. The retained lipids correspond mainly to palmitic acid, diacylglycerol and some phospholipids [32]. One main lipid receptor in the frameworks appears to be α-actinin, as diacylglycerol and palmitic acid co-purify with this protein during immunoprecipitation from the SDS-solubilized cytoskeleton. The binding appears to be very strong, as some of the immunoprecipitated α-actinin is radioactively labeled even after SDS polyacrylamide gel electrophoresis. However, the strongly bound component has not yet been identified, and it cannot be excluded that α-actinin contains covalently linked fatty acids. Neither the significance nor the molecular mechanism of lipid binding to α-actinin are yet understood. Figure 2 illustrates a possible model: In unstimulated platelets α-actinin is partly cytosolic or in peripheral association with the membrane, and actin is mainly depolymerized (Fig. 2*A*). Thrombin initiates an increased turnover of phosphoinositol lipids, resulting in the generation of diacylglycerol and inositoltrisphosphate, followed by a further breakdown of diacylglycerol to either phosphatidic acid or to fatty acids and glycerol [109]. The necessary lipid components are thus temporarily available and could either induce the association of α-actinin with the plasma membrane or could interact with this protein already peripherally attached to the membrane (Fig. 2*B*). The α-actinin lipid complexes could now serve as membrane anchors for actin filaments. Association of these complexes (shown to occur in monolayer models [118]) could lead to actin filament bundling at the membrane (Fig. 2*C*). Some preliminary evidence exists that α-actinin indeed inserts into the lipid bilayer in platelets. When platelets are incubated with INA, labeling of a 95,000-Da component associated with the cytoskeleton, which has a mobility comparable to α-actinin in two-dimensional gels, has been observed [152]. However, these results do not yet conclusively demonstrate that labeling of this protein occurs indeed in the bilayer. Further studies are necessary to clarify this point.

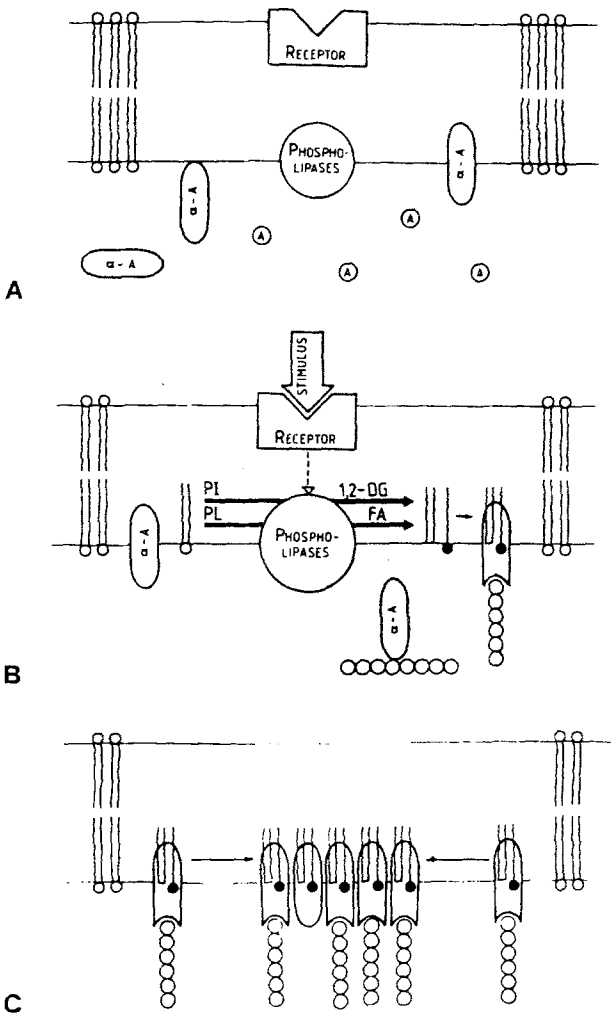


**Fig. 2.** A model for the involvement of an amphitropic protein in actin filament-membrane attachment in thrombin-stimulated platelets. (*A*) In unstimulated platelets the amphitropic protein α-actinin (*α-A*) is located mainly in the cytosol, and actin Ⓐ is in the monomeric form. (*B*) Thrombin induces the generation of diacylglycerol (*1,2-DG*) and fatty acids (*FA*) by activation of phospholipases, thereby promoting the insertion of the amphitropic α-actinin into the membrane, which can then function as an anchor for actin filaments (*PL* = phospholipids). (*C*) α-Actinin clusters may bundle actin filaments at the membrane (kindly provided by Dr. P. Burn)

*Profilin, Gelsolin.* Profilin, a cytosolic protein of 15,000 Da, forms a stable complex with monomeric actin (=profilactin), preventing it from poly-

merization. The protein is widely distributed in eukaryotic cells [25] and, complexed to actin, functions as a microfilament precursor [115]. It is not clear how dissociation of the profilactin complex is induced, for instance in platelets, during thrombin stimulation of actin polymerization. Recently evidence has been presented that specific lipids may regulate profilactin dissociation [101]. Profilactin, incubated under conditions where monomeric actin polymerizes, does not dissociate. However, the addition of specific phospholipids leads to dissociation of the complex, allowing actin filament formation. Phosphatidylinositol 4,5-bisphosphate ($PIP_2$) and phosphatidylinositol 4-monophosphate (PIP) are most effective, whereas phosphatidylinositol (PI) or phosphatidylserine (PS) have very little effect. These lipids do not affect actin polymerization in the absence of profilin. Column chromatography experiments indicate that profilin dissociates from actin and instead strongly binds to the liposomes. G-actin does not appear to interact with these lipids. The authors propose that the induction of actin polymerization in platelets could occur at the plasma membrane where profilactin complexes would dissociate in the presence of increasing amounts of $PIP_2$ produced in the initial phase of thrombin stimulation. Interestingly, both profilin and profilactin appear to have hydrophobic domains, based on their interaction with detergents as shown by charge-shift electrophoresis [111].

According to a recent report [87], not only profilin, but also gelsolin, a protein which in a calcium-dependent manner severs actin filaments, binds to their ends and promotes the polymerization of monomeric actin, is functionally regulated by $PIP_2$. This lipid specifically inhibits the actin filament-severing properties of gelsolin in the presence of $\mu M$ $Ca^{2+}$, but has much less inhibitory effect on its nucleating properties. Moreover, the lipid induces removal of one of the actin monomers from 2 : 1 actin-gelsolin complexes formed in the presence of $\mu M$ $Ca^{2+}$, which lack the ability to sever actin filaments. The resulting 1 : 1 actin-gelsolin complexes have regained their severing function, as demonstrated by the authors after subsequent addition of Triton in order to reverse the inhibitory effect of the lipid on this process.

If one considers also the data on $\alpha$-actinin, the assembly, bundling and membrane linkage of microfilaments may thus be directly regulated by components of the phosphatidylinositol cycle.

*Protein 4.1* Protein 4.1 is a monomeric soluble protein present in erythrocytes, platelets, granulocytes, human fibroblasts and brain. In erythrocytes this protein not only mediates the interaction between spectrin and actin, but it is also involved in anchorage of the cytoskeletal network to the membrane, predominantly via binding to the cytosolic domain of the transmembrane protein glycophorin. In addition, protein 4.1 binds with lower affinity to band 3 [105]. Recently evidence has been put forward that protein 4.1 interacts directly with lipids and that specific phospholipids regulate its association with glycophorin. According to Ref. 155, protein 4.1 binds to about the same extent to erythrocyte inside-out vesicles and to PS vesicles. In contrast, very little binding occurs to vesicles of neutral lipids such as phosphatidylcholine (PC) or sphingomyelin. The affinity and saturation of binding were not determined. Protein 4.1 bound to lipid vesicles may still interact with the actin-spectrin network, as protein 4.1-lipid complexes increase the viscosity of actin-spectrin mixtures. Protease treatment of the erythrocyte vesicles does not markedly decrease binding of protein 4.1, indicating that lipids may be the main binding sites [155]. This finding is somewhat in contrast to data shown in Ref. 3, where it is shown that specific antibodies to glycophorin inhibit binding of protein 4.1 to inside-out vesicles by at least 60%. On the other hand, it has been confirmed that, indeed, protein 4.1 binds to some extent to phospholipid vesicles, especially if they contain $PIP_2$, although with low affinity [4]. When these vesicles also contain glycophorin, the binding affinity is markedly increased. The affinity of the interaction of protein 4.1 with glycophorin in the presence of $PIP_2$ appears to be comparable to that of the interaction of protein 4.1 with erythrocyte vesicles, as glycophorin, reconstituted in $PIP_2$-containing liposomes, inhibits the latter interaction in a competitive manner. Low, physiological amounts (0.9 mol %) of the specific lipid in the liposomes are sufficient to induce this effect. Other lipids, such as PIP or PS, cannot substitute for $PIP_2$ [4]. The mechanism of the modulatory control exerted by $PIP_2$ has not yet been clarified. The lipid may affect the conformation of glycophorin, inducing a high-affinity binding site for protein 4.1, but it could also change the conformation of protein 4.1 itself, enabling it to bind with higher affinity to glycophorin. It remains to be seen how the lipid modulates the interaction of protein 4.1 with spectrin and actin.

*Spectrin.* Structure and function of spectrin, the major protein of the erythrocyte cytoskeleton, has been studied in great detail. Spectrin tetramers associate with actin oligomers via protein 4.1. This network is linked to the membrane mainly via ankyrin and protein 4.1. Evidence also exists for a direct, weak interaction of spectrin with lipid bilay-

ers. As discussed in Ref. 83, spectrin is not a very hydrophobic molecule, but it may contain some hydrophobic domains as shown by its behavior in charge-shift electrophoresis and by proton magnetic resonance studies. Moreover, 2-bromo-stearate has been found to quench the intrinsic fluorescence of spectrin at low concentrations, indicating a large number of hydrophobic binding sites on the molecule [83]. The presence of hydrophobic domains is also supported by the labeling of spectrin, associated with erythrocyte membranes, by hydrophobic reagents such as phenylisothiocyanate, INA, etc. [24, 162], and by the finding that anilinonaphtyl-groups covalently linked to spectrin exhibit a fluorescence emission spectrum characteristic of a highly hydrophobic environment [26]. Both fatty acids and PS (but not PC) quench markedly the fluorescence of the anilinonaphtyl group, indicating specific binding of negatively charged hydrophobic molecules to spectrin. Evidence for a direct interaction of spectrin with especially negatively charged phospholipids has been obtained both with monolayer and bilayer techniques. Monolayer studies show that acidic phospholipids allow high rates of penetration of spectrin into the monolayer at a high initial surface pressure, whereas only very little penetration was observed in the presence of PC [120]. Electron microscopy of spectrin samples incubated with PS vesicles shows indeed a direct binding of up to seven molecules of spectrin per vesicle [44]. Crosslinking of vesicles by spectrin also occurs, indicating multiple binding sites on the protein. Some of the binding sites appear to be located near the tail end of spectrin. To exclude artifacts, the interaction of actin, ankyrin, albumin and protein 4.1 with PS liposomes was also studied. Except for protein 4.1 (in agreement with Ref. 155) none of these proteins appears to associate with liposomes [44].

From these data it cannot be concluded whether spectrin indeed inserts into the hydrophobic part of the bilayer. The interaction with acidic phospholipids could be mainly of electrostatic nature. Labeling by phenylisothiocyanate could also occur in accessible hydrophobic pockets of spectrin outside of the membrane. It is also not clear how important such interactions are in situ. As summarized in Ref. 44, some indirect evidence exists that spectrin indeed affects lipid organization in intact erythrocytes. For instance, in diseases associated with erythrocyte spectrin deficiency, PS and phosphatidylethanolamine, normally located mainly in the inner half of the bilayer, are found in the outer half, suggesting that spectrin, through a direct interaction with specific lipids, maintains the phospholipid asymmetry of the membrane.

*Vinculin.* The cytoskeletal protein vinculin (130,000 Da) is widely distributed in vertebrate muscle and nonmuscle tissues. It is a monomeric, soluble protein, partly located in the cytosol, partly at very specific sites of actin membrane attachment, such as focal contacts, or adherens junctions of epithelial cells [65]. Purified in a soluble form from chicken gizzard, followed by attachment of a fluorescent label and microinjection into chicken embryo fibroblasts, vinculin inserts specifically into focal contacts [63]. Thus chicken gizzard vinculin appears to be able to bind, as endogenous fibroblastic vinculin, to specialized plasma membrane areas. Fluorescence photobleaching recovery experiments [97] suggest that cytoplasmic vinculin and vinculin in focal contacts are in a steady-state equilibrium. It is not clear whether a specific protein receptor in focal contacts is responsible for this very specific localization, whether vinculin self-associations are also important, or whether lipids are also involved. B. Geiger [63] has postulated a model for events occurring in focal contacts during cell attachment. Putative transmembrane receptors are induced to cluster in sites of cell-cell and cell-substrate contact. Vinculin may then bind to the clustered receptors and initiate the formation of microfilament bundles. However, the true function of vinculin has not yet been demonstrated convincingly. On one hand, microinjection of specific anti-vinculin antibodies into living fibroblasts has been reported to induce disruption of stress fibers and disappearance of focal contacts [60]. On the other hand, the actin bundling activities of vinculin are now attributed not to vinculin itself, but to co-purifying impurities [56]. Vinculin in focal contacts is very likely not retained there via binding to the end of actin bundles, as removal of actin from isolated focal contacts does not result in removal of vinculin [10]. Other factors must be responsible for its specific localization. We have studied the interaction of vinculin with bilayers and plasma membranes in more detail. Plasma membranes purified from chicken embryo fibroblasts reproducibly contain small amounts of vinculin, about 0.7 $\mu$g vinculin per 100 $\mu$g plasma membrane protein, as determined by an antivinculin antibody (V. Niggli and M.M. Burger, *unpublished results*). Vinculin appears to be very tightly bound to the plasma membrane, as not more than 20% can be removed by washing with buffers containing 600 mM NaCl, or 2 mM Tris, pH 7.4. In agreement with B. Geiger and co-workers [10], we found that removal of most of the actin from the plasma membranes using DNase I under depolymerizing conditions results in the removal of less than 10% of the endogenous vinculin (V. Niggli, J. Muser and M.M. Burger, *unpublished observations*). Plasma mem-

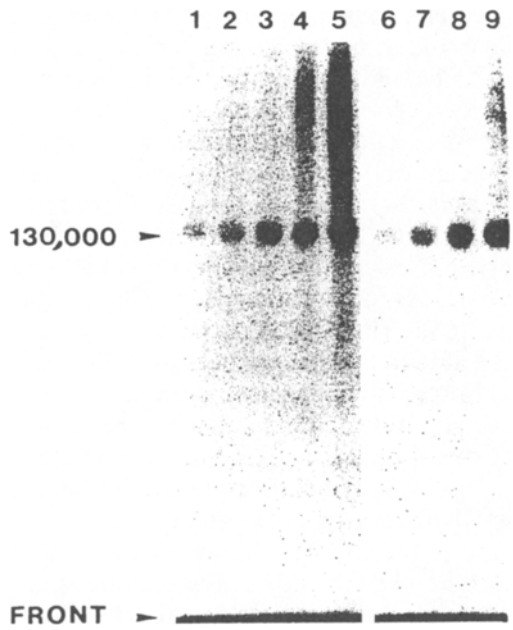


**Fig. 3.** Vinculin-bilayer interactions in the presence of low concentrations of acidic phospholipids. Vinculin, purified from chicken gizzard, was incubated and photolyzed with liposomes containing 1% (wt/wt) [$^3$H]PTPC, and either 99% (wt/wt) phosphatidylcholine (lane 1), or increasing concentrations of acidic phospholipids (lanes 2 and 6: 0.5%; lanes 3 and 7: 1%; lanes 4 and 8: 5%; lanes 5 and 9: 10%; wt/wt). In lanes 2–5 $PIP_2$, and in lanes 6–9 PI was used (reprinted from Ref. 127, with permission of the publisher). The figure shows an autoradiogram of the dried gel

branes of cells detached by EGTA reproducibly contain 40% less vinculin, in $\mu$g per $\mu$g of total membrane protein, than membranes of attached cells (V. Niggli and M.M. Burger, *unpublished results*). This result suggests that vinculin dissociates partly from the membrane during the process of cell detachment and stress fiber disruption induced by EGTA. Such a process has also been observed to occur in EGTA-dissociated epithelial cells [181]. How is the linkage of vinculin to the membrane mediated? Possibly talin is involved via attachment to the fibronectin receptor [80]. According to a recent report [13], a protein different from talin may also be important. Vinculin, fluorescently labeled, binds to focal contacts of Triton X 100-permeabilized myoblasts. Binding is optimal in low ionic strength buffers. Cells washed with high ionic strength buffers do not bind vinculin anymore, possibly due to the removal of a peripheral protein. Mild proteolysis of the cells also abolishes vinculin binding. Neither talin nor actin can reconstitute vinculin binding in washed cells. However, a heat labile, nondialyzable, water soluble factor from a chicken gizzard fraction again appears to promote vinculin binding [13].

Evidence for a direct insertion of vinculin into bilayers also exists. S. Ito and co-workers [85] show that vinculin interacts with liposomes consisting of acidic phospholipids, but not with neutral phospholipids. This interaction is markedly inhibited by an increase in the ionic strength. However, it is not clear if the protein inserts directly into the hydrophobic part of the bilayer. Moreover, it cannot be excluded that acidic lipids induce vinculin aggregation, resulting in co-elution of vinculin and the liposomes in the void volume of Bio-Gel A-5m columns. We have now confirmed and extended these findings by using a photoactivatable phospholipid [127]. This phosphatidylcholine analogue ([$^3$H]PTPC) has a photoactivatable diazirine group on its apolar portion, which generates a highly reactive carbene group upon photolysis. Significant and reproducible labeling of vinculin could be detected upon incubation and photolysis in the presence of liposomes containing low amounts of the photoactivatable phospholipid. Labeling was specifically dependent on the presence of acidic phospholipids. Labeling is maximal at low ionic strength and in the presence of >50% of acidic phospholipids, in agreement with Ref. 85. Due to the sensitivity of the method, small but reproducible increases in the labeling of vinculin can also be observed at physiological salt concentrations and acidic phospholipid content of the membrane (*see* Fig. 3). All acidic phospholipids tested so far induce the insertion of vinculin into the bilayer, PS and phosphatidic acid being the most efficient. As vinculin, separated from the liposomes by a dialysis membrane, is not measurably labeled and as lipids other than the photolabel influence labeling, it is very likely that vinculin labeling occurs in the bilayer.

We propose a two-step model. First, vinculin interacts with liposomes via polar interactions, these interactions inducing a conformational change of the protein and allowing in a second step its insertion into the bilayer with a hydrophobic domain. Indeed, evidence has been presented for such a conformational change to occur [85]. The presence of hydrophobic domains is supported by the finding that soluble vinculin can be labeled by the hydrophobic molecule TID [127]. Moreover, vinculin surfaces with high velocity in monolayer assays [59], again indicating its amphiphilicity.

It remains to be shown whether vinculin inserts also in situ, for instance in focal contacts, into the bilayer, and how important such an interaction is, compared to protein-protein linkage. Preliminary results indicate that vinculin is indeed labeled when chicken embryo fibroblasts are incubated with the photoactivatable free fatty acid, followed by photolysis of the cells and immunoprecipitation of vincu-

lin. Moreover, only the membrane-associated, but not the cytosolic, vinculin appears to carry the photolabel (V. Niggli, L. Sommer, J. Brunner and M.M. Burger, *in preparation*). Possibly the recently detected covalent modification of vinculin by fatty acids [31] may play a role in its membrane attachment (*see* below). It would also be of interest to study the in vitro interaction of vinculin with other proteins, such as talin or actin, in the presence of acidic phospholipids, which could possibly modulate these interactions via induction of a conformational change in vinculin.

*Metavinculin.* Metavinculin is a protein of 152,000 Da, which shows immunological crossreactivity with vinculin [58]. Metavinculin is very likely not just a precursor of vinculin [58, 164]. Rather, these two proteins could be encoded by two different genes or result from a single gene by differential splicing. In contrast to vinculin, this protein occurs exclusively in muscular tissues [154], and it appears to be located in the same sites as vinculin, as shown by microinjection of the fluorescently labeled proteins into the cytoplasm of cultured myotubes [154]. Metavinculin and vinculin appear to be retained in the cells by somewhat different mechanisms, as high ionic strength buffers appear to be necessary to extract metavinculin quantitatively from gizzard smooth muscle, whereas a sizable amount of vinculin can already be extracted with low ionic strength buffers at alkaline pH [70, 164]. However, vinculin extraction is also increased at high ionic strength. These findings very likely are not just due to insolubility of metavinculin at low ionic strength, as both proteins are soluble under a wide variety of conditions [70], and as a 152,000-Da protein related to vinculin has been isolated by low ionic strength extraction from gizzard muscle [58]. Rather, metavinculin may interact differently with membranes and/or other proteins, compared to vinculin, necessitating a different extraction procedure. Certainly metavinculin is not an integral membrane protein, as postulated previously [163], as more recent results show that are based on its solubility properties and on its lack of interaction with Triton X-114 [70, 164]. The function of both proteins has not yet been clarified. Metavinculin has been shown to lower the viscosity of F-actin in vitro (58), suggesting that it may bundle actin filaments. However, these results have to be interpreted with caution in view of the finding that extensively purified vinculin loses the ability to decrease F-actin viscosity [56] due to the removal of contaminating proteins. Such contaminants could also co-purify with metavinculin. According to M. Gimona and co-workers [70], the reduction of F-actin viscosity by both vinculin and metavinculin from pig stomach is very variable for different preparations, and no direct binding of these proteins to actin could be detected.

*Microtubules, microtubule-associated proteins*

As discussed in a previous section, some evidence exists for the existence of tubulin-like molecules which are very tightly bound to plasma membranes. In addition, soluble tubulin can interact with lipid bilayers under specific conditions in vitro. As discussed in Ref. 6, $\alpha$- and $\beta$-tubulin do not contain any strongly hydrophobic domains. However, large amounts of octyl glucoside (60 mol/mol protein) and deoxycholate bind to purified cytoplasmic calf brain tubulin [5]. Detergent binding induces a partial transition from $\alpha$-helix to disordered structure in the protein and inhibits its polymerization. Tubulin also becomes more accessible to controlled proteolysis in the presence of detergents [6]. These data point to the presence of amphiphilic regions in tubulin, which could possibly be involved in membrane binding. Indeed, tubulin could be shown to interact in vitro with phospholipid vesicles, although some controversy exists on the specific lipid requirement of this process. According to Ref. 95, tubulin interacts with dipalmitoylphosphatidylcholine, but only at the lipid phase transition temperature. Tubulin liposome complexes are proteolyzed much slower than soluble tubulin, and the $\alpha$-helical content of the protein is increased upon binding of lipids [98], in contrast to the effect of detergents, which appear to unfold and denature the protein [6]. As the interaction with lipids is insensitive to an increase in the ionic strength of the medium, hydrophobic interactions may play a role. According to another report [89], pig brain tubulin does not interact with dimyristoylphosphatidylcholine at the phase transition temperature, as measured by free flow electrophoresis. However, tubulin appears to interact with vesicles containing anionic lipids such as phosphatidylglycerol. The authors attribute this interaction mainly to the presence of copurifying microtubule-associated proteins (MAPs), as extensively purified tubulin interacts only very weakly with acidic phospholipids. In contrast, purified MAPs interact with high affinity, probably due to their positive charge. They may also insert into the hydrophobic part of the bilayer, as high ionic strength does not inhibit this interaction and as MAPs appear to affect membrane fluidity [90]. Moreover, acidic phospholipids, especially PI, appear to inhibit microtubule assembly, possibly via binding to MAP-2 [191]. The basis of the discrepancy between the different reports is not clear.

The phosphorylation state of tubulin may play a role, as evidence has been recently provided for an increased interaction of phosphorylated tubulin with PC liposomes in vitro [77]. When tubulin, purified from pig brain, is phosphorylated in vitro by the calcium and calmodulin dependent protein kinase, only the phosphorylated tubulin forms a stable complex with liposomes, separatable from free, not phosphorylated, tubulin on a Sepharose CL-4B column. This interaction, is reversible, as treatment of vesicle-bound tubulin with alkaline phosphatase releases about 40% of the covalently bound phosphate and about the same amount of tubulin from the liposomes. The phosphorylated domain of tubulin appears not to insert directly into the bilayer, as it is accessible to proteolysis in the membrane-bound form. Very likely phosphorylation stabilizes a more hydrophobic conformation of tubulin, as confirmed by charge-shift electrophoresis of the two forms [77]. Phosphorylated tubulin may correspond to the membrane-associated form of tubulin, detected in various purified membrane preparations as discussed above. Interestingly, phosphorylation of tubulin also inhibits its ability to self-assemble into microtubules. Thus phosphorylated, membrane-bound tubulin very likely cannot serve as a nucleating site for tubulin assembly. Polymerized tubulin may also be able to interact with lipid bilayers, as microtubules, formed between two lipid bilayers (but not monomeric tubulin or bovine serum albumin), have been reported to allow electrical coupling between these two membranes [180]. In conclusion, both a subpopulation of tubulin itself, possibly phosphorylated, and MAPs are able to interact with bilayers in vitro. It remains to be seen how important these observations are for the in situ linkage.

### *Intermediate Filaments*

Starting from the observation that vimentin purified from Triton-insoluble frameworks by affinity chromatography in the presence of urea still contains substantial amounts of lipids [176], the interaction of the purified protein with lipids has been studied. Indeed, vimentin, delipidated with organic solvents, could be shown to interact with liposomes consisting of Ehrlich-Ascites tumor cell lipids, by sucrose and KBr density gradient centrifugation [177]. The protein binds with highest affinity to acidic phospholipids. Different types of intermediate filament proteins (desmin, neurofilaments) share this ability. The study of the lipid-binding properties of different vimentin fragments suggests that the arginine-rich N-terminus is involved in this interaction. Vimentin filaments can be labeled by the hydrophobic photolabel 1-azidopyrene only in the presence of acidic lipids, but not in their absence [138, 139], indicating not only polar but also hydrophobic interactions. The filaments appear to interact with liposomes in a lateral manner, as shown by electron microscopy [138, 139]. This finding is very probably not due to a fixation artifact, as this interaction is specifically dependent on the presence of acidic phospholipids. Moreover, lipids inhibit vimentin assembly, $PIP_2$ being the most efficient. Very likely this effect occurs via binding of the lipids to the N-terminus, which is essential for filament assembly. The question if intermediate filament proteins indeed insert in situ directly into the bilayer of cell organelles and the plasma membrane, or if peripheral proteins such as ankyrin or spectrin are also involved, has yet to be solved. A primary interaction with these putative linker proteins, followed by a secondary stabilizing insertion into the bilayer, may also occur. As discussed in Ref. 177, interaction of intermediate filaments with membranes may also be a signal for their depolymerization, followed by their migration into the nucleus, to exert functions related to gene expression.

## The Covalent Interaction of Cytoskeletal Proteins with Fatty Acids

Although covalent linkage of fatty acids to eukaryotic proteins is a well-established post- or cotranslational modification [108], the actual function of this process is not yet well understood. According to Ref. 130, proteins containing palmitate linked via hydroxylamine labile ester bonds are mainly located in membranes, whereas proteins containing myristate linked via amide bonds are found both in cytosolic and in membrane fractions. Acylation appears to be common to a variety of different proteins (soluble proteins, N-glycosylated and nonglycosylated integral membrane proteins, peripheral membrane proteins). Acylation may influence protein folding, affecting both protein-protein and protein-lipid interactions. Indeed, recent evidence suggests that acylation allows membrane insertion of otherwise soluble proteins [99, 137]. It is, however, not clear if the acylated site itself inserts directly into the bilayer or if binding occurs due to a change in protein folding. Acylation could be one possible mechanism for enabling otherwise soluble cytoskeletal proteins such as vinculin and $\alpha$-actinin to interact with bilayers. Acylation of several cytoskeletal proteins has recently been reported. Such proteins may therefore be amphitropic, i.e., directed towards both the membrane under some conditions and the cytoplasm under others.

Actin of *Dictyostelium discoideum* appears to contain covalently bound palmitate, in an ester linkage [168]. The fatty acid-to-protein ratio was not determined. The authors find no evidence for the preferential interaction of acylated actin with membranes. Acylated actin is located mainly in the 100,000 × *g* supernatant of cell homogenates, comparable to total actin. The covalently linked fatty acids may therefore influence actin polymerization or its interaction with other proteins, rather than its membrane linkage. The fatty acid content of vertebrate actin has not been extensively studied. According to Ref. 92, purified rabbit skeletal muscle actin does not contain significant amounts of covalently bound fatty acid. This actin modification thus appears not to be universal, and its significance is unclear.

The erythrocyte cytoskeletal component ankyrin contains a high-affinity binding site for both spectrin and the cytoplasmic domain of bound 3 [105], therefore constituting an important link of the cytoskeletal network to the erythrocyte membrane. Ankyrin appears to be one of the major labeled proteins upon incubation of chicken erythrocytes with [$^3$H]palmitate, as identified with a specific antibody [169]. Most of the label can be released by hydroxylamine, but not by organic solvents, Triton or SDS. The fatty acid is therefore very likely linked via ester or thio-ester bonds to the protein. The actual identity of the fatty acid bound to ankyrin has not yet been determined. Acylation appears not to be obligatorily coupled to protein synthesis, as inhibitors of this process do not affect ankyrin acylation markedly. Moreover, acylation occurs throughout erythroid differentiation and also in adult chicken and rabbit erythrocytes. Ankyrin thus appears to be acylated at the plasma membrane, after its assembly into the cytoskeleton, with some turnover. Presently the extent and the function of this modification are unknown. Acylation may lead to a secondary insertion of ankyrin into the bilayer, after its primary linkage to band 3. Ankyrin on its own does not appear to interact directly with bilayers in vitro, at least not with PS liposomes [44]. Of course, these findings do not exclude that ankyrin may insert into the bilayer under some specific conditions. Alternatively, acylation could modulate its interaction with other cytoskeletal proteins or with band 3.

As discussed above, erythrocyte protein 4.1 appears to interact with liposomes of specific lipid composition. Interestingly, this protein may contain substantial amounts of possibly covalently bound fatty acids. As analyzed by gas chromatography, alkali treatment of protein 4.1, eluted from polyacrylamide gel slices, releases about 2 mol palmitic acid and 1 mol stearic acid per mol protein. These fatty acids are not removed by extraction with chloroform/methanol [92]. This modification may allow the interaction of protein 4.1 with bilayers observed in vitro discussed above. However, the possibility cannot be excluded that not protein 4.1 itself but a comigrating impurity has contributed to these fatty acids.

As discussed above, vinculin interacts with membranes and inserts into bilayers. It appears to contain covalently attached fatty acids, palmitic acid [31] as well as myristic acid [93]. In both cases vinculin has been immunoprecipitated from chicken embryo fibroblasts, which were preincubated for 2–5 hr with labeled myristic or palmitic acid. Myristic acid, identified by thin-layer chromatography of HCl-hydrolysates of the myristate-labeled vinculin band, may be amide linked to the protein, as the band is resistant to hydroxylamine. Palmitate, on the other hand, identified by HPLC analysis of methanolic HCl hydrolysates of immunoprecipitated vinculin is possibly linked via ester bonds. It is tempting to conclude that the covalent attachment of fatty acid to vinculin is involved in its association with liposomes, membranes and focal contacts. However, a conclusive proof for this hypothesis is yet lacking. For example, the actual stoichiometry of fatty acid bound per vinculin has not yet been determined. A value of about 4% of the total vinculin carrying covalently bound myristate has been estimated [93], but this value is very likely not correct, as the actual specific activity of the bound myristate is not known. Moreover, no difference could be detected in the fatty acid content of cytosolic and membrane-bound vinculin upon labeling with myristic acid [93].

One of the earliest events in transformation of chicken embryo fibroblasts with RSV is a disruption of microfilament bundles and a reduction in the number of focal adhesion plaques, concomitant with a striking change in vinculin organization [50]. How this change is induced is not known, but a post-translational modification of vinculin may be involved. According to P. Burn and M.M. Burger [31], vinculin, immunoprecipitated from chicken embryo fibroblasts transformed by a temperature-sensitive transformation mutant of RSV at the nonpermissive temperature, indeed contains about threefold more [$^3$H]palmitate (related to [$^{35}$S]methionine) than the protein isolated from fully transformed cells grown at the permissive temperature. However, vinculin isolated from cells transformed by wild-type RSV shows no significant change in its myristate content, compared to normal cells [93]. Furthermore, the exact degree of metabolic amino acid labeling has to be assessed in all of these experiments, even though some label can be ascribed to tightly attached fatty acids. Thus, the

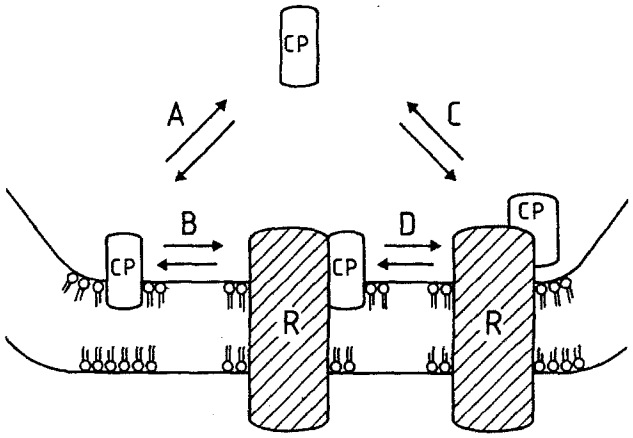


**Fig. 4.** Possible modes of interaction of amphitropic cytoskeleton-associated proteins with the bilayer and with putative transmembrane receptor proteins, starting from their soluble, cytosolic form. Cytosolic amphitrophic cytoskeletal proteins (*CP*) may insert in a first step into the bilayer, possibly dependent on specific lipids (*A*), followed by lateral diffusion of the inserted protein in the plane of the bilayer and by a secondary stabilizing interaction (*B*) with putative receptor proteins (*R*). Alternatively, a primary interaction with a receptor protein may occur (*C*), which is then stabilized by a secondary insertion of the linker into the bilayer (*D*)

functional role and relevance of vinculin acylation remains unclear.

## Conclusions

Several mechanisms appear to be operative in cytoskeleton-membrane attachment: integral membrane proteins may link cytoskeletal filaments to the membrane, either directly (laminin receptor, 5′-nucleotidase) or indirectly (fibronectin receptor, band 3), via peripheral cytoskeletal proteins (ankyrin, protein 4.1, talin). In addition, a growing number of reports supports a new concept whereby soluble cytoskeleton-associated proteins ($\alpha$-actinin, vinculin, protein 4.1, profilin, gelsolin, spectrin) or filament proteins themselves (tubulin, vimentin) interact directly with bilayers, at least under certain conditions. In most cases, a preference for specific lipids has been observed. Vinculin, spectrin and vimentin require the presence of acidic phospholipids. The lipids of the receptor-regulated phosphatidylinositol cycle play a special role. $PIP_2$ appears to interact with and functionally regulate protein 4.1, gelsolin and profilin, whereas diacylglycerol and fatty acids increase the actin-bundling capacity of $\alpha$-actinin. Receptor-stimulated changes in the phosphatidylinositol lipids, which occur in a wide variety of cells, may thus directly initiate the reorganization of the actin cytoskeleton. Many questions remain open. For instance, it is not clear in most cases whether the association of these proteins with lipids is just peripheral, via ionic interactions, or if these proteins also insert into the hydrophobic part of the bilayer. Only vimentin and vinculin have been shown to be labeled by hydrophobic photolabels, dependent on the presence of specific lipids. Tubulin, spectrin, profilin and gelsolin seem to contain hydrophobic domains, potentially able to insert into bilayers. In no case does convincing evidence exist for such interactions to occur also in situ, except for $\alpha$-actinin. Furthermore, it is not clear whether only a covalently modified subpopulation of these soluble proteins interacts with bilayers, what the dynamics of these interactions are, and whether they are reversible. A detergent treatment appears, for example, to be necessary to dissociate gelsolin from $PIP_2$, thereby restoring its severing action. Tubulin, upon dephosphorylation, dissociates from lipid vesicles. Such proteins may belong to a new class of membrane proteins, defined as amphitropic proteins, which are neither integral nor peripheral, which can exist in a soluble form in the cytosol, and which can alternatively insert directly into lipid bilayers with a hydrophobic domain. However, the existence of such a special class of proteins is yet hypothetical and has to be demonstrated by future experiments. In any case, the ability of cytosolic proteins to interact, at least in vitro, with lipids appears to be more common than previously thought. Criteria for the relevance of such interactions are, for instance, a preference for specific lipids, the identification of lipid binding domains and the functional regulation of such proteins by lipids. However, such interactions have to be demonstrated to occur also in intact cells, which in very few cases has been done so far. A possible sequence of events depicting the reversible membrane association of, e.g. vinculin, is shown in Fig. 4. Vinculin may either first insert into lipid bilayers of appropriate composition (local increases in specific lipids may induce a local insertion), followed by lateral diffusion in the plane of the bilayer and complex formation with integral membrane proteins (possibly via talin). Alternatively, the protein may first bind via electrostatic interactions to an integral membrane protein and then, in a second step, insert into the membrane.

To conclude, not only protein-protein interactions but also specific lipids appear to be involved in membrane cytoskeleton linkage, leading to an even more complex system of multiple interactions. These findings suggest new possibilities of information transfer via the plasma membrane, occuring during dynamic events such as shape change and

cell motility. Particularly the receptor-regulated phosphatidylinositol cycle may be of importance.

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