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α-Actinin interacts specifically with model membranes containing glycerides and fatty acids

(lipid–protein interactions/lipid specificity/lipid monolayer/liposomes/Z-line)

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ABSTRACT **A method was developed to identify specific protein–lipid interactions of complex lipid mixtures and to assess their effect upon the arrangement of such complexes in monolayers at an air–water interface. Its application to striated muscle α-actinin revealed that just two lipids selectively interact with α-actinin. One molecule of glyceride and one molecule of fatty acid were found to be associated in a constant stoichiometry with one molecule of the α-actinin dimer. In the presence of both glycerides and fatty acids unexpectedly rigid monolayer areas formed. This lipid specificity could be confirmed by brief protease digestion of α-actinin liposome mixtures followed by peptide analysis; the peptide patterns of α-actinin depended on the presence or absence of only these two lipids. Possible implications of these findings are discussed in the context of Z-line formation.**

α-Actinin is a constituent of the Z-line of muscle cells (1, 2). In nonmuscle cells it occurs in the general area of the contact zone between actin filaments and membranes (3, 4) and is also associated with microfilament bundles (5). A role for α-actinin in anchoring microfilament bundles to membranes (6) and for the movement of integral membrane proteins (7) has been proposed. In the case of muscle cells, the Z-line, which is the carrier of α-actinin, has contact with the membrane system of the sarcoplasmic reticulum (8). Very little is known about the exact α-actinin membrane contact, whether there is direct interaction with membrane lipids, whether it is mediated via other proteins, or whether both modes of interaction exist simultaneously. Some serious doubts have been raised as to a direct linker function of α-actinin between microfilaments and membranes in nonmuscle systems (9, 10).

In this communication interactions between isolated α-actinin and lipid systems are investigated. A method is described that allows an investigator to detect strong interactions between lipids and proteins entering lipid monolayers from solution. The application of this method to striated muscle α-actinin unambiguously revealed its strong and specific interaction with lipid layers. It took place only in the presence of certain lipids, not of the phospholipid class, that are rare constituents of natural membranes.

MATERIALS AND METHODS

Materials. Phospholipids and glycerides were purchased from Serdary Research Laboratories (London, ON), and synthetic lipids, trypsin (treated with diphenyl carbamyl chloride), and α-chymotrypsin (treated with tosyllysine chloromethyl ketone) were obtained from Sigma. Natural lipids were extracted from disrupted yeast cells as described by Bligh and Dyer (11). Precoated silica gel 60 high-performance TLC plates were from Merck and [^{3}H]palmitic acid was from the Radiochemical Centre (Amersham, England).

α-Actinin Isolation. α-Actinin was purified from pig skeletal muscle according to Suzuki *et al.* (12). Purity was tested by electrophoresis in NaDodSO$_4$/10% polyacrylamide slab gels. Only minor impurities were visible on overloaded gels. The protein was tested in the ultracentrifuge (Beckman model E). The $s_{20,w}$ value was 5.84 S, which corresponds to a molecular weight of 206,000 (α-actinin dimer); this is in fair agreement with Robson *et al.* (13). No aggregation of dimers was observed.

Monolayer "Sorting-Out" Studies. A Langmuir trough of 16 × 56 cm and 1 cm depth was used. It was equipped with a movable surface barrier. The protein chamber had the same width as the trough, i. e., a depth of 3 mm and a volume of 10 ml. At the beginning of sorting-out experiments the protein chamber was positioned at one end of the trough and filled with 12 ml of α-actinin solution (0.1–0.2 mg/ml). The interfaces to air of the protein solution and of the buffer in the trough were set at equal levels but both surfaces were not yet connected. (They were separated by the rim of the protein chamber, which was 0.5 mm below the interfaces.) The lipid monolayer was spread at the air–buffer interface of the trough between protein chamber and movable barrier by addition of aliquots of a lipid solution in *n*-hexane (2 mg/ml). The surface pressure (drop in surface tension due to the presence of a lipid monolayer) was measured by using a Wilhelmy plate.

After evaporation of the hexane the surface pressure was set to a particular value by moving the barrier and thus changing the monolayer area. The monolayer was then connected to the surface of the protein solution by sliding a glass rod along the separating Teflon rim of the protein chamber. The protein chamber was slowly and continuously (0.5–1 cm/min) shifted towards the barrier at constant surface pressure conditions. The chamber was stopped after the protein had access to 25–50% of the original monolayer area. This part of the surface was disconnected from the residual lipid monolayer by a surface barrier and removed from the surface by aspiration (400 cm^2 into 5–10 ml buffer). For all experiments described a 10 mM Tris·HCl/0.1 M NaCl, pH 7.3, buffer was used.

Digestion Experiments. Liposomes were prepared as described by Papahadjopoulos *et al.* (14), using a mixture of equal amounts (by weight) of phosphatidylinositol, phosphatidic acid, phosphatidylserine, phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylcholine; when indicated 5% (wt/wt) palmitoleic acid and 5% (wt/wt) 1,2-diolein (1,2-dioleoylglycerol) were added. The liposome concentration was adjusted to 4 mg/ml. α-Actinin (50 μg) was mixed with 20 μl of the liposome suspension. Buffer (10 mM Tris·HCl/0.1 M NaCl, pH 7.3) was added to 50 μl. After addition of 4 μg of trypsin (2 mg/ml) the mixture was incubated for up to 2 hr at 37°C. The reaction was stopped at the specified times by the addition of 20 μl of phenylmethylsulfonyl fluoride (0.1 M in isopropyl alcohol). The pep-

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tides were resolved on $NaDodSO_4$/polyacrylamide slab gels as described by Cleveland *et al.* (15).

RESULTS

Interactions between α-actinin and lipids in a surface adsorbed monolayer were investigated by using the assay shown in Fig. 1. A chamber containing the water-soluble dimeric form of α-actinin is slowly and continuously moved just below a monolayer of a native lipid mixture (with the aid of a motor). The monolayer area to which protein has access slowly increases. Protein insertion into the monolayer is monitored by the increase of monolayer surface area at constant surface pressure. Pronounced affinities between particular lipids and the protein should result in an enrichment of these lipids in the lipid–protein part of the monolayer. A general discussion of this assay beyond the present application will be published elsewhere.

In pilot experiments a total lipid extract of yeast cells was used to form a multicomponent lipid monolayer. Its composition was analyzed by TLC both for phospholipids (lane A in Fig. 2*A*) and components such as steroids, fatty acids, and glycerides (lane A* in Fig. 2*A*). The same analyses were carried out to determine the lipid composition in the lipid–protein part of the monolayer (lanes B and B* in Fig. 2*A*).

Only two lipids (nonphospholipids) were found in the lipid–protein monolayer (spots 5′ and 7′ in lane B* of Fig. 2*A*) repeatedly and reproducibly. The second unexpected observation was that these results were invariant over a wide range of surface pressure values held constant during the experiment (5–20 mN/m). They were also independent of the total amount of lipid available—i.e., independent of the fraction of the lipid monolayer to which protein had access if this fraction did not exceed 50% of the original lipid monolayer area. From experiments with different velocities of the sliding protein chamber and with different protein concentrations it became apparent that saturation of protein insertion is required for the high degree of lipid separation evident from Fig. 2*A*. Saturation was ensured by choosing sliding velocities less than 1 cm/min and protein concentrations exceeding 0.1 mg/ml at 5 mN/m monolayer pressure. Saturation was evidenced by the absence of a further surface area increase when the chamber shift was stopped. The assay in Fig. 1 under these experimental conditions will be referred to in the following as a "sorting-out experiment."

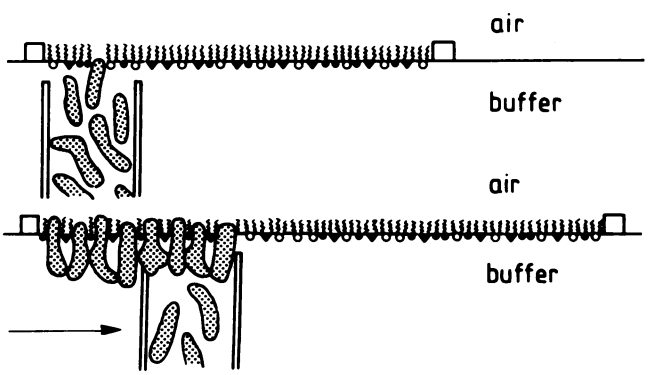

FIG. 1. Schematic illustration of the sorting-out technique. The figure shows a lipid monolayer at the surface of a Langmuir trough and an immersed chamber containing protein molecules. The lipid monolayer is confined to part of the surface by means of surface barriers (square symbol). At the beginning of the experiment the chamber containing protein is positioned close to one barrier (*Upper*). The chamber is then continuously moved towards the other barrier (*Lower*). During chamber shift the increase of surface area due to protein insertion is compensated by an increase of total surface area (constant surface pressure conditions). In this way the area of the lipid monolayer to which protein has access increases in a defined way. Proteins are not drawn to scale with respect to lipids; ⥣, phospholipids; ⥥, fatty acids; ⥣, glycerides.

The next obvious step in this study was to characterize the lipids in the positions 5′ and 7′, which appear to preferentially interact with α-actinin. They could be identified by elementary chemical analysis and mass spectrometry as a mixture of simple glycerides (spot 5′) and as a mixture of fatty acids (spot 7′), predominantly palmitic acid and palmitoleic acid.

Both component 5 and component 7 in the yeast lipid mixture were necessary for sorting out. When only component 5 or component 7 was removed with a preparative TLC step and the sorting-out experiment was carried out, the other component was not enriched in the protein monolayer. When both component 5 and component 7 were removed none of the other lipids were enriched by α-actinin. To put the conclusion that both fatty acids and glycerides are required on a more quantitative basis, sorting-out experiments were carried out with mixtures of synthetic glycerides and fatty acids. In a first control experiment the components 5 and 7 in the yeast lipids were replaced by corresponding amounts of 1,2-diolein and palmitoleic acid. Sorting out yielded the same results as shown in Fig. 2*A*. This was followed by sorting-out experiments using only 1,2-diolein and palmitoleic acid in different stoichiometries. In Fig. 2*B* these stoichiometries in the original monolayer (abscissa) are plotted against the stoichiometries found in the protein-containing sorted-out part of the monolayer (ordinate). The six experimental points unambiguously indicate a 1:1 molar ratio of the two lipids in the lipid–protein association independent of their stoichiometry in the monolayer before protein intrusion. This particular choice of diolein as the glyceride and palmitoleic

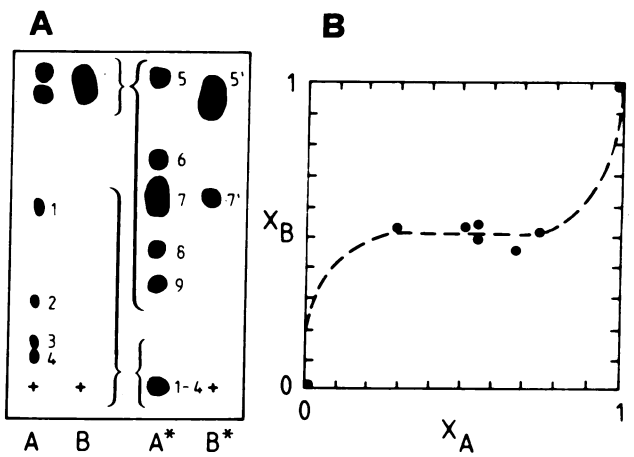

FIG. 2. (*A*) Qualitative lipid analysis. A TLC plate is shown with total yeast cell lipids (lanes A and A*) and the lipid components remaining in the protein–lipid part of the monolayer (lanes B and B*), analyzed after removal of the proteins with chloroform/methanol (11). The plate with lanes A and B was developed in chloroform/methanol/water, 65:25:4 (vol/vol), separating primarily phospholipids. The plate with lanes A* and B* was developed with *n*-hexane/chloroform/methanol/diethyl ether, 18:13:5:2 (vol/vol), separating steroids primarily. 1, Phosphatidylethanolamine; 2, phosphatidylcholine; 3, phosphatidylinositol; 4, lysophosphatides; 5, glycerides; 6, not identified; 7, cholesterol, palmitoleic acid, and palmitic acid; 8 and 9, not identified; +, start position. Brackets indicate corresponding substances resolved differently in the two different solvent systems. (*B*) Lipid stoichiometry in the α-actinin-enriched monolayer after sorting out. α-Actinin was introduced under a monolayer consisting of synthetic lipids, palmitoleic acid, and 1,2-diolein, in different molar ratios (abscissa, X_A, mole fraction of palmitoleic acid). The ratio of these two lipids in the protein–lipid part of the monolayer (see Fig. 1) was analyzed, after chloroform/methanol extraction, by quantitative gas chromatography (ordinate, X_B, mole fraction of palmitoleic acid).

acid as the fatty acid was arbitrary: saturated and mono-unsaturated fatty acids from C_{14} to C_{18} gave the same qualitative results together with saturated and mono-unsaturated mono- and diglycerides.

The protein-to-lipid stoichiometry was also assessed. It was suspected to be rather high because the increase in monolayer area during protein insertion (at constant surface pressure) approached the area exposed to protein. In four independent experiments α-actinin was inserted into monolayers formed from [^{3}H]palmitic acid and equimolar amounts of 1,2-diolein. Care was taken that insertion reached saturation. The resulting lipid–protein surface layer was washed by moving it at a constant surface pressure of 5 mN/m over the surface of a shallow buffer tank (0.5 mm deep and 3 m long) before removing it and analyzing it for protein according to Lowry *et al.* (16) and for lipids by tritium determination. A value of 1.3 ± 0.4 palmitic acid molecules per 1 α-actinin dimer was found. This may be a low estimate because the presence of α-actinin that may have remained unspecifically associated to the α-actinin–lipid monolayer during the extensive washing along the buffer tank could not be ruled out and quantitatively assessed.

Concomitant with effective sorting out of lipids in the presence of α-actinin there was a dramatic increase of surface viscosity of the α-actinin–lipid layer. This was assayed in the trough illustrated in Fig. 3*A*. A U-type channel of 4 mm width was introduced between two sides of a coherent monolayer between a surface balance and a movable surface barrier. Monolayers from yeast lipids exhibited normal reversible surface pressure–area curves as shown by the broken line in Fig. 3*B*. However, when α-actinin (0.1 mg) was injected into the channel area, changes on the right side did not translate into surface pressure changes on the left side of the channel. The surface pressure remained constant at the value (such as 13 mN/m in Fig. 3*B*) to which it had been set before protein injection. A rigid lipid–protein surface layer had formed in the channel. This rigidity was dependent on exactly the same lipid specificity as the sorting-out effect: First, it was observed only when glycerides and fatty acids were both present in sufficient amounts, regardless of whether they were applied as components of natural yeast lipids, as components of artificial lipid mixtures, or the two lipids alone. Second, rigidity could not be observed for any lipid mixture that lacked any one of the two specific lipids. Third, α-actinin alone, in the absence of any lipid, reduced surface tension by up to 15 mN/m (0.1 mg/ml). When such a surface layer was formed at the surface of the channel and connected to lipid layers on either side the protein layer did not impede lipid flow through the channel in the presence of surface pressure gradients between both sides.

From these results it appeared that the specific interactions between glyceride, fatty acid, and α-actinin result in unusually stable associations over visible dimensions (mm). Formation of such associations was spontaneous although facilitated by surface pressures exceeding 5 mN/m during protein insertion. Once a "rigid" layer had formed it stayed coherent irrespective of the surface pressure difference between the adjacent monolayers, from 0 mN/m to the collapse pressure and for at least 1 hr.

For electron microscopy α-actinin–lipid surface layers were transferred to carbon grids (for details see legend to Fig. 4), resulting in two distinctly differing observations. Electron microscopy of α-actinin molecules adsorbed to carbon grids from solution has shown structures 30 nm in length and 4 × 2 nm in width (17). Such structures are also seen on micrographs from lipid–α-actinin monolayers in the absence of the specific lipids (see Fig. 4 *Inset*). The same preparation displays, however, additional structures, which are knob-like with a diameter of approximately 8 nm. No elongated structures but only knob-like structures of the same diameter were observed in micrographs of rigid surface layers obtained from lipid–α-actinin monolayers in the presence of the specific lipids (Fig. 4). From this comparison it may be inferred that the α-actinin molecules in association with the two specific lipids may be oriented with their long axis perpendicular to the plane of aggregation. So far no definite order could be detected within the plane. This may be

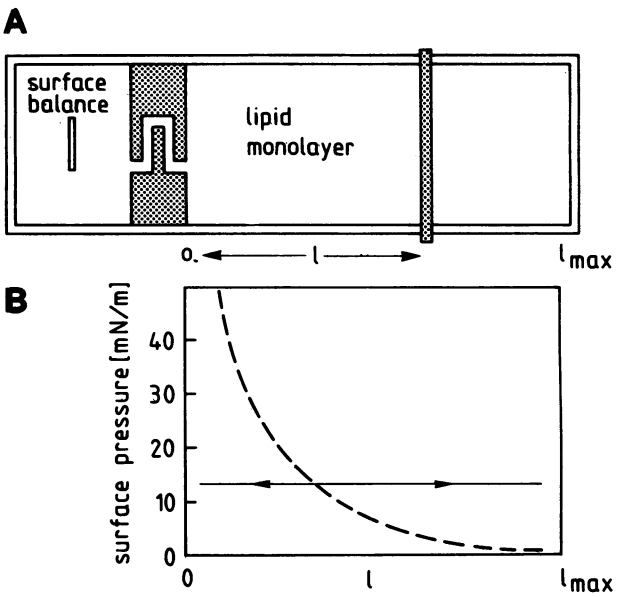

FIG. 3. (*A*) Schematic view of a Langmuir trough, used to test the viscosity of an α-actinin–lipid layer. A U-shaped channel is introduced between the surface pressure balance and a movable barrier. Then a lipid monolayer is spread at the surface. Drop in surface tension is measured in relationship to the movement of the barrier before and after injection of α-actinin into the channel. (*B*) Dependence of the surface pressure on the movement of the barrier, measured in the device shown in *A*. The usual reversible surface pressure–area curves of the lipid monolayer were obtained before α-actinin was injected into the channel (broken line) but, when tested 15 min after injection, the channel was blocked by α-actinin; that is, the surface pressure remained at the value set during protein injection (13 mN/m, continuous line). Such a block was not observed when one or both of the specific components were absent.

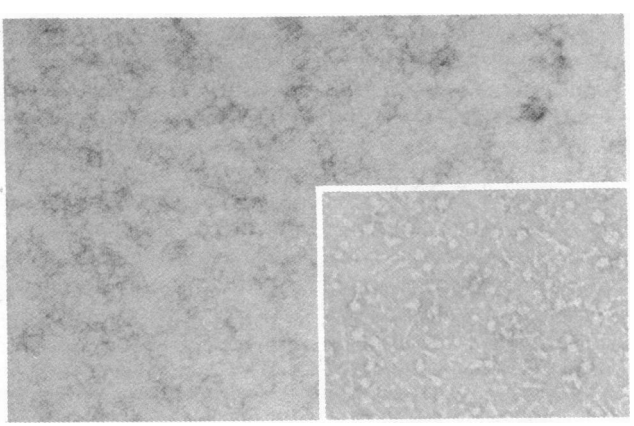

FIG. 4. Electron microscopic picture of a rigid α-actinin–lipid layer. An α-actinin–lipid layer was formed and adsorbed onto a carbon grid. It was then stained by several drops of 1% uranyl acetate and observed in a Zeiss EM 10 electron microscope. (*Inset*) Control in which α-actinin was adsorbed from the air–water interface in the absence of the specific lipid components. (×126,000.)

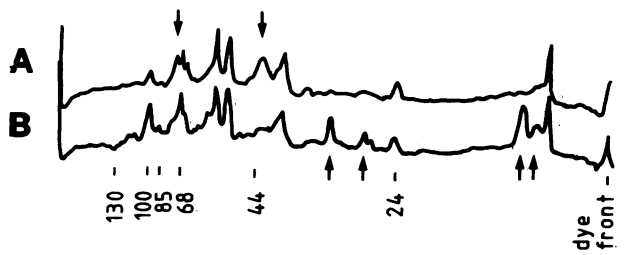

FIG. 5. Densitometer tracings of a $NaDodSO_4$/10% polyacrylamide slab gel. The peptides of α-actinin obtained after a 15-min trypsin digestion in the presence of phospholipid liposomes containing diolein (5% wt/wt) and palmitoleic acid (5% wt/wt) (curve A) and in the presence of phospholipid liposomes without these two lipids (curve B) are shown. The position of molecular weight standards ($\times 10^{-3}$) are indicated: β-galactosidase (130,000), α-actinin (100,000), human transferrin (85,000), bovine serum albumin (68,000), ovalbumin (44,000), and trypsin (24,000). Two new bands appear in curve A (molecular weights 75,000 and 43,000, arrows) and four bands seen in curve B disappeared in curve A (molecular weights 31,000, 27,000, 14,000, and 13,000, arrows).

due to the apparent partial collapse of α-actinin molecules during preparation, which may be overcome by more advanced preparations of such rigid surface layers for electron microscopy.

Do the above findings of specific lipid–α-actinin interactions in monolayers also apply to the interactions of α-actinin and artificial bilayer membranes? This question was addressed by adding α-actinin to liposomes formed from mixtures of phospholipids with or without 1,2-diolein and palmitoleic acid (5%, wt/wt each). α-Actinin–liposome interactions were assayed by brief (15-min) trypsin digestion and analysis of proteolytic products by $NaDodSO_4$ gel electrophoresis. In Fig. 5 a densitometer tracing of α-actinin peptides obtained in the presence of liposomes with palmitoleic acid and diolein (curve A) is compared to the pattern obtained from liposomes without these two lipids (curve B). The presence of both lipids led to the protection of at least two cleavage sites, as could be judged from the appearance of two peptides of high molecular weight and the concomitant disappearance of four peptides of lower molecular weight (arrows). The ratio of unprotected to protected α-actinin was dependent on the α-actinin and liposome concentrations and on the experimental time. The data in Fig. 5, curve A, correspond to conditions in which about 50% of the α-actinin molecules were protected. The digestion pattern of α-actinin obtained in the absence of any liposomes is almost indistinguishable from the pattern obtained in the presence of pure phospholipid liposomes. It should be added that very long exposures to 10% (wt/wt) trypsin, even in the presence of the two specific lipids, led finally to complete digestion of α-actinin. Similar results were obtained with α-chymotrypsin.

DISCUSSION

The aim of this study was to analyze interactions between α-actinin and lipids in order to contribute to the unresolved question of whether α-actinin could be anchored to membranes. First, interactions between α-actinin and lipid monolayers were analyzed. This has the disadvantage that a monolayer is far from being a biological membrane, but it has the advantage that interactions can be identified and quantitated. The search for α-actinin-specific lipids out of a given mixture of different naturally occurring lipids required a technique that had to be developed specifically for our purpose. With such a sorting-out technique only two lipid components out of an entire lipid extract of yeast cells were found to interact with α-actinin specifically. They are any fatty acid as one of the components and a glyceride as the other. The affinity of these two lipids for α-actinin is extremely high because not even trace amounts of other lipids were found to be present in the vicinity of α-actinin.† These two lipids, when present in different stoichiometries in the lipid monolayer, were always found in a 1:1 ratio in the protein part of the monolayer. Furthermore, interactions between α-actinin and these two lipids resulted in an extremely viscous or rigid monolayer compared with monolayers that lacked at least one of the two lipids.

These three observations, taken together, provide conclusive evidence for specific interactions between α-actinin and two types of lipids in equimolar amounts. For various pairs of differently structured synthetic fatty acids and glycerides the same qualitative result was obtained, although their relative affinity for α-actinin varied on a quantitative scale. We know of no other report on a highly specific interaction between a protein and simple glycerides. Association of free fatty acids with certain membrane-related proteins of cultured human cells (19, 20) and with certain virus-specific proteins (21) has been shown with about the same molar ratio of 1–2 fatty acids per 1 protein as estimated here for α-actinin.

These studies on monolayers were (22) corroborated by investigations of interactions between α-actinin and bilayer membranes of liposomes (22). Addition of α-actinin to suspensions of liposomes containing the two specific lipids resulted in the protection of at least one cleavage site of α-actinin against trypsin or α-chymotrypsin digestion. No such protection was observed under any condition in the absence of the two specific lipids. This may be explained either in terms of a lipid-dependent penetration of the membrane or by lipid-dependent protection within the membrane as discussed below.

Monolayer experiments at lipid densities and pressures as high as that expected for natural membranes showed a slow but still significant penetration of α-actinin into the monolayer. The penetration rate was, within experimental error, the same in the presence and in the absence of the two specific lipids. This argues against lipid-dependent penetration of the bilayer as an explanation for the protection against cleavage. We are left with the second possibility—i.e., lipid-dependent protection. The effect may be due to (*i*) irreversible penetration of α-actinin in the presence of the specific lipids and reversible penetration in their absence, or (*ii*) the formation of stable and close contacts between the specific lipids and α-actinin not occurring with other lipids. In view of the rigidity—i.e., unusually high viscosity—and the fixed stoichiometry of specific lipid to α-actinin monolayers, the second explanation seems more likely, although the first may apply in addition.

The α-actinin used in these experiments is of muscle cell origin. It is well known that Z-lines, in which α-actinin occurs, have a direct relationship with sarcoplasmic reticulum membranes (8). Muscle cell α-actinin may therefore have access to lipids, as non-muscle cell α-actinin possibly does. Provided the two specific kinds of lipid specified here occur in these membranes and interact with α-actinin, it remains difficult to assess whether this interaction itself is sufficient to explain a possible anchoring of α-actinin to membranes *in vivo*. Such lipid–α-actinin interactions may, however, be instrumental in stabilizing contacts between adjacent α-actinin molecules. This is consistent with the presence of only a few lipids per α-actinin in stable

† Sorting-out experiments with smooth muscle α-actinin from chicken gizzard instead of striated muscle α-actinin did not reveal an absolute and exclusive occurrence of the two lipids with the protein, but they were again seen to be highly enriched. This may be related to fewer hydrophobic regions in α-actinin from smooth muscle compared to that from striated muscle (18).

association and supports the assumption that protein–protein interactions contribute to the primary cohesion forces. They may occur within the monolayer but also between the approximately 80% of the α-actinin molecule protruding into the water phase. Such interactions, both in and below the monolayer, would also explain the high lateral viscosity of these protein–lipid associations.

The biological significance of this model of α-actinin anchoring to membranes remains uncertain. The observation has, however, a predictive value that is open to experimental tests. Both fatty acids and glycerides occur in native membranes as well as in the Z-line, to which α-actinin may be directly associated, and these lipids may contribute to α-actinin associations during Z-line formation (23).

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