Interactions between Smooth Muscle α-Actinin and Lipid Bilayers†

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ABSTRACT: α-Actinin has been proposed to be the actin−plasma membrane linker. This assumption is based on the discovery of direct interaction of α-actinin with two specific lipids, diacylglycerol and palmitic acid [Burn, P. (1988) *Trends Biochem. Sci. 13,* 79−83]. In our study, the binding of α-actinin with vesicles containing negatively charged phospholipids was measured by the method of 90° light-scattering. Our results show that α-actinin is able to bind membranes containing negatively charged phospholipids, but not to bind membranes composed of neutral lipids only. Diacylglycerol and palmitic acid, on the other hand, have little effect on the binding of α-actinin to lipid vesicles. Analysis of binding isotherms in terms of a membrane binding model gave apparent dissociation constants which varied between 0.2 and 3 μM over a range of 5−20 mol % negatively charged phospholipid. Comparing the kinetics of α-chymotrypsin digestion of α-actinin in solution to those of vesicle-bound α-actinin, it can be seen that the cleavage site at the junction between the C-terminal and the central rod domain of α-actinin and another cleavage site on the C-terminal domain can be most effectively protected by its membrane binding. Analysis of the amide I and II regions of Fourier-transform infrared spectra of α-actinin revealed that the association of α-actinin with negatively charged phospholipid vesicles resulted in some perturbation of the protein secondary structure. Monolayers containing negatively charged phospholipid were layered and incubated on the surface of a polymerization solution of actin and α-actinin, and observed with an electron microscope. The results show that the bundle structure of actin filaments can be formed if diacylglycerol and palmitic acid are present in lipid layers.

α-Actinin is a homodimer of two 100 kDa subunits (Amos & Amos, 1991). It is an actin cross-linking protein that can be found both in muscle and in nonmuscle cells (Baron et al., 1987). Under an electron microscope, α-actinins are rodlike molecules, 40−50 nm long and 4−5 nm in width (Podlubnaya et al., 1975; Suzuki et al., 1976; Meyer & Aebi, 1990). The actin binding of nonmuscle isoforms of α-actinin is inhibited by Ca^{2+} at micromolar concentrations, while the smooth muscle form studied in our experiments is not regulated by Ca^{2+} (Burredge & Feramisco, 1981). Proteolysis cleaves α-actinin down to a 27−36 kDa N-terminal region, i.e., the actin binding domain, and a 55 kDa fragment, which is responsible for the formation of homodimer (Mimura & Asano, 1986, 1987; Imamura et al., 1988; Kuroda et al., 1994). The C-terminal region of α-actinin contains two EF-hand, putative calcium binding motifs (Noegel et al., 1987).

α-Actinin is found in stress fibers and adhesion plaques in nonmuscle cells, as well as in Z disks and their homologues in muscle cells (Blanchard et al., 1989). A role for α-actinin in anchoring microfilament bundles to membranes (Loor, 1976) and for the movement of integral membrane proteins (Geiger & Singer, 1979) has been proposed according to its subcellular distribution. These point of views are supported in some extent by the discoveries that α-actinin is capable of interacting with some integral membrane proteins, such as integrins (Otey et al., 1990) and L-selectin (Pavalko et al., 1995), or even directly interacting with some membrane lipids. But there is still some confusion in the studies of α-actinin−lipid interactions so far.

It has been found that α-actinin is able to interact with several kinds of membrane lipids including diacylglycerol (DG)[1] and palmitic acid (PA) (Meyer et al., 1982; Burn et al., 1985), phosphatidylinositol 4,5-bisphosphate (PIP_2) (Fukami et al., 1992, 1996), and negatively charged phospholipids (Niggli & Gimona, 1993; Fritz et al., 1993). DG/PA were firstly found to be able to interact selectively with α-actinin (Meyer et al., 1982) and were assumed to be able to trigger the translocation of cytoplasmic α-actinin into the hydrophobic domain of membranes (Burn, 1988). In 1993, Niggli and Gimona reported that α-actinin could insert into the hydrophobic portion of liposomes if PS was present whereas Fritz et al. (1993) demonstrated that α-actinin was able to incorporate into the negatively charged phospholipid monolayers only if a high proportion of DG was present. From these rather contradictory results, it is obviously difficult to reach a clear conclusion about the lipid dependence in the α-actinin−lipid interactions.

In order to distinguish the different roles between DG/PA and negatively charged phospholipids in the membrane association of α-actinin, which is important in fully understanding the manner of α-actinin−membrane interactions, we investigated the binding properties of α-actinin in

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[1] Abbreviations: CD, circular dichroism; DG, diacylglycerol; DOPG, dioleoylphosphatidylglycerol; EDTA, ethylenediaminetetraacetic acid; FT-IR, Fourier-transform infrared spectroscopy; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; K_d, equilibrium dissociation constant; PA, palmitic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PMSF, phenylmethanesulfonyl fluoride; PS, phosphatidylserine; SDS, sodium dodecyl sulfate.

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association with model membranes by 90° light-scattering. The conformational changes of α-actinin induced by the binding to membranes were studied with proteolytic sensitivity and FT-IR measurements. The bundling of actin filaments by α-actinin on lipid monolayers composed of mixtures of phospholipids and DG/PA was also observed with an electron microscope.

MATERIALS AND METHODS

Materials. Phosphatidylcholine (PC) from egg yolk, phosphatidylserine (PS) from bovine brain, phosphatidylinositol (PI) from soybean, dioleoylphosphatidylglycerol (DOPG), DG, PA, chicken muscle actin, and other chemicals used in the buffers were purchased from Sigma Chemical Co. Sepharose CL-6B and Sephadex G-50 were from Pharmacia Fine Chemicals. α-Actinin was purified from chicken gizzard by the method of Feramisco and Burridge (1980). D_2O (99.8% pure) was from Beijing Chemical Factory. Water used in all experiments was doubly distilled.

Preparation of Phospholipid Vesicles. Unilamellar vesicles were prepared with buffer A containing 30 mM NaCl, 10 mM Hepes, 0.2 mM EDTA, pH 7.4, or without EDTA (buffer B) in the Ca^{2+} effect experiments. Large unilamellar vesicles used in proteolysis experiments were prepared by the procedure of Mimms et al. (1981). These vesicles were about 200−400 nm in diameter. For the light-scattering measurements, small unilamellar vesicles about 30 nm in diameter were prepared by a cholate solubilization−gel filtration removal procedure (Brunner et al., 1976). The sizes of these lipid vesicles were sampled by use of negative staining electron microscopy. The vesicles were kept at 4 °C before use within 2 days. Triton X-100−lipid mixed micelles (1% aqueous solution of Triton X-100 corresponding to 14.7 mM) containing 20 mol % PS and 80 mol % Triton X-100 were prepared in buffer A according to the procedure of Orr and Newton (1992). Pure DOPG and DOPG/DG/PA (50%, 25%, 25%) vesicles for the FT-IR experiments were prepared in buffer C (50 mM NaCl, 20 mM Tris-HCl in D_2O, pD 7.4) by the standard sonication method. Phospholipid contents in all the samples were determined by phosphorus assay (Ames, 1966).

Determination of the Relative Molecular Weight of the Protein−Vesicle Complexes. 90° light-scattering measurements (Nelsestuen & Lim, 1977) were made at 25 °C with a HITACHI 850 fluorometer. For each isotherm, 1.5 mL of small vesicle was equilibrated at 25 °C in the cuvette at a concentration of 40 μg/mL. Particulate contaminants in the solutions were removed by centrifugation at 10000*g* for 15 min. Proteins were equilibrated in buffer A (or buffer B in the experiments involved Ca^{2+}) prior to their addition to the small vesicle suspension in a cuvette. Light-scattering intensities from the protein−vesicle complex were corrected for the scattering of unbound protein and otherwise analyzed by the method of Nelsestuen and Lim (1977). Data were plotted in the Hildebrand formats (Klotz, 1986) from which apparent dissociation constants were obtained. The outer leaflet stoichiometries of binding were calculated as described by Cutsforth et al. (1989). Protein concentration was measured according to Lowry et al. (1951).

α-Chymotrypsin Digestion. Unless otherwise stated, the lipid/protein complex at $R_i = 600$ (mol/mol) was digested up to 60 min with α-chymotrypsin at an enzyme to substrate ratio of 1:75 (w/w) at 37 °C in buffer A. The reaction was terminated by the addition of 1 volume of SDS solution containing 10% w/v SDS, 10% v/v glycerol, 5% v/v 2-mercaptoethanol, 1.0 mM PMSF, and 0.0625 M Tris-HCl, pH 6.8.

FT-IR Spectroscopy. Lyophilized α-actinin in 50 mM NaCl and 20 mM Tris-HCl (pH 7.4) was dissolved in D_2O at 15 mg/mL. To study infrared amide bands of α-actinin in the presence of different amounts of lipids, small sonicated vesicles were mixed at the desired proportions with α-actinin solution in buffer C to achieve the final protein concentration of 15 mg/mL. After incubation at room temperature for 30 min, the sample was assembled between two calcium fluoride windows separated by a 25-μm-thick Teflon spacer. Infrared spectra were recorded with a Bio-Rad FTS 165 FT-IR spectrometer equipped with a DTGS detector. Each spectrum was obtained by collecting 1024 interferograms with a nominal resolution of 4 cm^{-1}, and was then Fourier-transformed using a triangular apodization function. Spectra of the buffer were recorded under identical conditions. The criterion used for subtracting the background of buffers was the removal of the band near 2125 cm^{-1} and a flat base line between 1770 and 2100 cm^{-1}. Fourier self-deconvolution (Byler & Susi, 1986) of the subtracted spectra was carried out using a Bessel apodization function, a Lorentzian shape with a resolution enhancement parameter, *K*, of 2.4, and a full width at half-height of 13 cm^{-1}. No attempt was made to quantitate the percentages of different types of secondary structures from the amide I band contour.

Electron Microscopic Observation of the Actin Bundles Formed on Phospholipid Monolayers. A lipid monolayer was formed by spreading 0.6 μL of a 600 μM premixed lipid solution in 1:1 chloroform/hexane on the surface of 20 μL of the polymerization solution in a Teflon well (4 mm in diameter, 1 mm deep), which contained 4 μg of α-actinin and 10 μg of G(or F)-actin in buffer B. Incubation was allowed for 30 min at room temperature. The polymerization solution was then adjusted to 30 mM NaCl, 100 mM KCl, 10 mM $MgCl_2$, 0.2 mM ATP, and 10 mM Hepes (pH 7.4) by injection of concentrated salt solutions. After incubation for another 30 min at room temperature, the lipid film was picked up onto a carbon-coated grid after 5 min in contact with the surface of the polymerization solution. The grids were negatively stained by sequentially placing them on three drops of 0.75% uranyl acetate for ∼10 s each. Then, 5 μL of polymerization solution was carefully drawn out without disturbing the surface of solution, and negatively stained with the same method. All the samples were observed with a Philips EM 420 electron microscope.

SDS−Polyacrylamide Gel Electrophoresis. SDS−polyacrylamide gel electrophoresis was carried out according to Laemmli (1970) with a 3% stacking gel and a 10% separating gel. Gels were stained with 0.25% Coomassie brilliant blue G-250 in a 50% aqueous methanol solution containing 12.5% trichloroacetic acid and then destained with 7% acetic acid.

RESULTS

Membrane Binding Characteristics of α-Actinin Monitored by 90° Light-Scattering Intensity Measurements. α-Actinin has been found to interact specifically with negatively charged phospholipids and some other lipids. However, there have been few reports for a detailed characterization of the

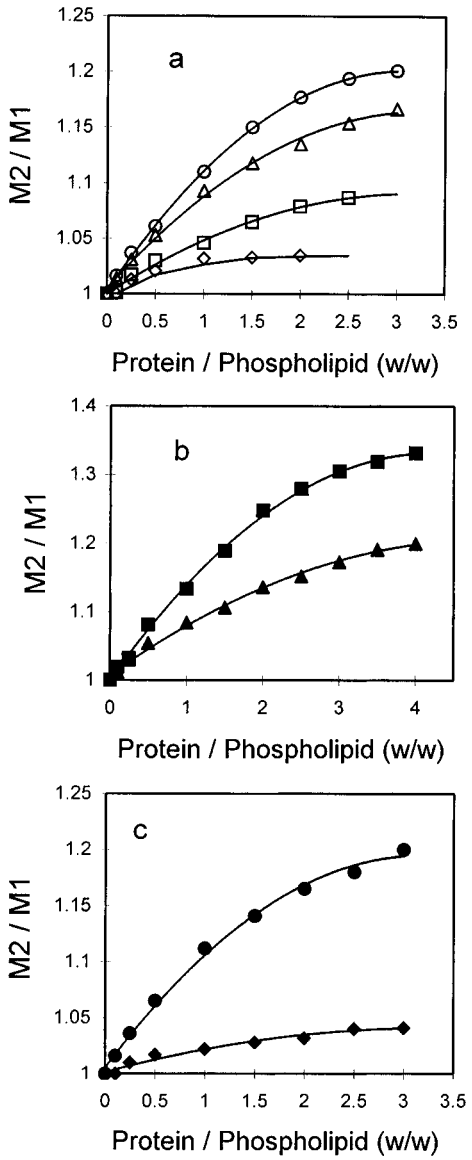

FIGURE 1: α-Actinin binding to small vesicles. The lipid content was (a) (○) 20% PS, (△) 10% PS, (□) 5% PS, (◇) 0% PS; (b) (■) 20% DOPG, (▲) 20% PI; (c) (●) 20% PS, 10% DG, 10% PA, or (◆) 10% DG, 10% PA. The remaining lipid was PC. Data were obtained from the 90° light-scattering measurements. M2/M1 stands for the molecular weight of the protein−vesicle complex relative to that of the vesicle alone.

binding of α-actinin with membranes containing those lipids. 90° light-scattering intensity measurement is most suitable to obtain the above information (Nelsestuen & Lim, 1977; Bazzi & Nelsestuen, 1987; Cutsforth et al., 1989)

Figure 1a presents the isotherms of the binding of α-actinin to lipid vesicles composed either of pure PC (PC vesicles) or with various amount of PS in addition to PC. Apparent dissociation constants and stoichiometries, i.e., the total number of lipids in the outer leaflet per bound protein, obtainable from these data (Cutsforth et al., 1989) are summarized in Table 1. Clearly, there was no significant binding of α-actinin to PC vesicles. In the presence of PS at an increasing concentration in the vesicles, a decrease in the outer leaflet lipid stoichiometry can be found. This indicates that PS is an essential component in the membrane for the binding of α-actinin, and the maximum binding is approximately proportional to the PS content. It implies that the factor limiting the maximum binding of α-actinin to membranes is the availability of negatively charged phospholipids. This point is further confirmed by the results shown in Figure 1b. Figure 1b presents the binding isotherms of α-actinin to vesicles containing either 20% DOPG (DOPG vesicles) or 20% PI (PI vesicles), respectively, to which the α-actinin molecules bind in a similar manner as that in the binding to vesicles containing 20 mol % PS (PS vesicles). Comparing the apparent dissociation constants and the stoichiometries (see Table 1) of α-actinin binding to PS, DOPG, and PI vesicles, respectively, it can be concluded that α-actinin interacts with lipid membranes with a selectivity on the order of DOPG > PS > PI.

Table 1: Experimental Parameters[a] for α-Actinin Binding to Lipid Vesicles

percentage (mol %)	lipid composition[b]	K_d[c] (μM)	stoichiometry[d]
5	PS	2.97 ± 0.34	1250 ± 153
10	PS	0.98 ± 0.51	765 ± 180
20	PS	0.50 ± 0.18	396 ± 69
20/10/10	PS/DG/PA	0.50 ± 0.03	446 ± 14
20	DOPG	0.22 ± 0.10	235 ± 88
20	PI	0.85 ± 0.20	579 ± 132

[a] Values and standard deviations are drawn from three to five independent determinations. [b] The remaining lipid was PC. [c] Hildebrand analysis of the binding data in Figure 1 was used to obtain K_d as the ratio of the slope to the intercept according to the standard Hildebrand form $1/\gamma = (1/P_f)(K_d/n) + 1/n$, where γ is molecules of protein bound per vesicle, n is the stoichiometry of binding in molecules of protein per vesicle, and P_f is the concentration of free protein. [d] Stoichiometry given as the ratio of the total outer leaflet lipid per bound protein; see Cutsforth et al. (1989).

DG and PA have been found to play important roles in the process of actin bundling by α-actinin and in the association of α-actinin to the membrane. But it has remained dubious whether these lipids are directly involved in the membrane binding process of α-actinin. Figure 1c presents the isotherms of α-actinin binding respectively to DG/PA (10%, 10%) vesicles or PS/DG/PA (20%, 10%, 10%) vesicles. It is interesting to note that the presence of DG and PA exerted only a negligible influence on the binding of α-actinin to the PC or PS vesicles; this conclusion can be drawn either from the binding isotherms or from the apparent dissociation constant and stoichiometry shown in Table 1.

Kinetics of α-Actinin Digestion by α-Chymotrypsin in Soluble and Membrane-Bound Form. In Figure 2, the kinetics and the extent of α-chymotrypsin digestion of α-actinin in solution can be compared to those of α-actinin bound to vesicles containing 20% negatively charged phospholipids. Figure 2a shows the time course of digestion of pure α-actinin with α-chymotrypsin. After 1 min, six fragments, i.e., the 105, 100, 90, 70, 65, and 32 kDa fragments, were the major products. A 55 kDa fragment appeared, and the 105, 100, 90, and 70 kDa fragments disappeared within 5 min. The 65 kDa fragment totally disappeared after 30 min. Two peptides, the 55 and 32 kDa fragments, survived till the end of digestion. The digestion time course of the PS vesicle-bound α-actinin (Figure 2b) turned out to be greatly different from that in Figure 2a. After 1 min, only two bands, 105 and 70 kDa, showed up, and the 32 kDa fragment appeared after 5 min. The appearance of the 55 kDa fragment and the disappearance of the 105, 100, 90, 70, and 65 kDa fragments were all delayed obviously.

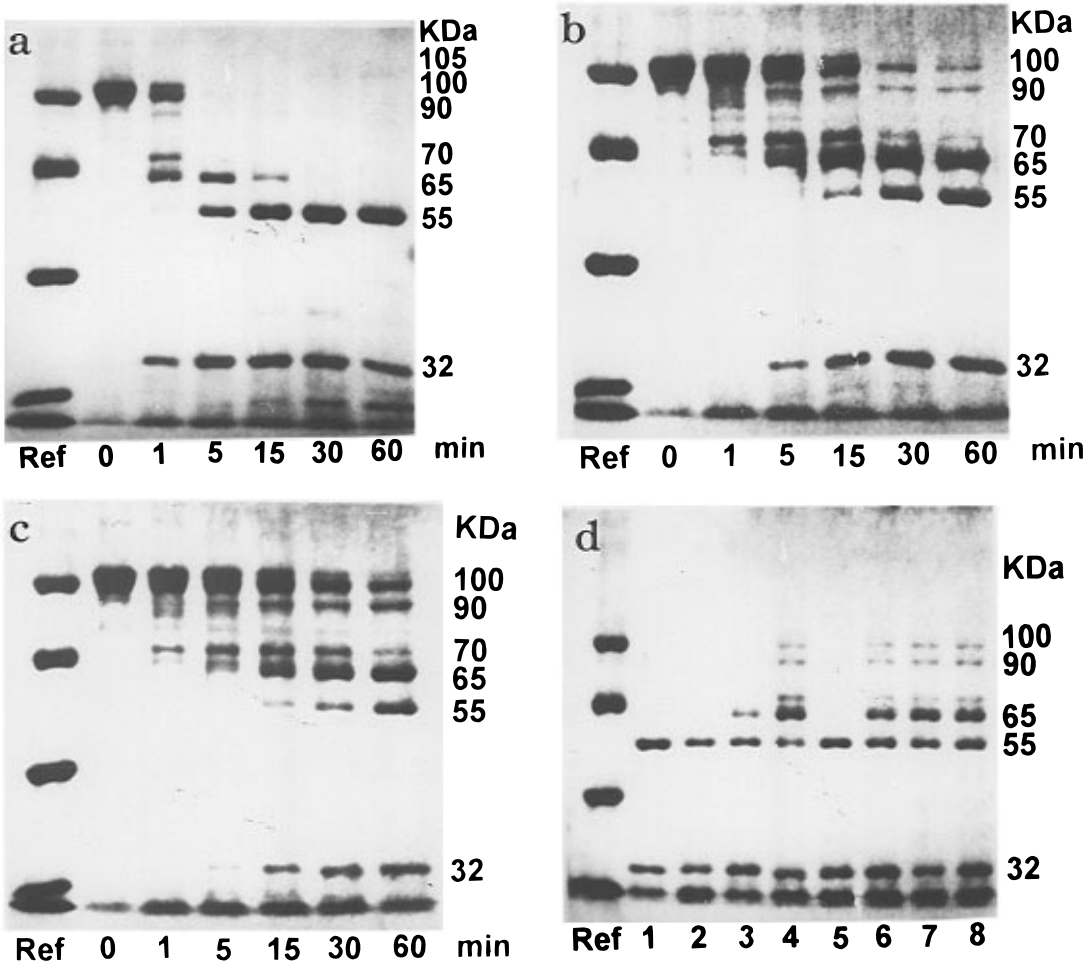

FIGURE 2: Time course of α-actinin digestion by α-chymotrypsin from 0 to 60 min at 37 °C. (a) Digestion of pure α-actinin; (b) digestion of α-actinin in the presence of 20% PS vesicles; (c) digestion in the presence of PS/DG/PA (20%, 10%, 10%) vesicles; (d) digestion of pure α-actinin for 60 min at 37 °C (lane 1), or in the presence of PC vesicles (lane 2), PS (20%)−Trition X-100 mixed micelles (lane 3), PS/DG/PA (20%, 10%, 10%) vesicles (lane 4), DG/PA (10%, 10%) vesicles (lane 5), 20% PS vesicles (lane 6), 20% DOPG vesicles (lane 7), or 20% PI vesicles (lane 8). Ref., molecular mass standards: carbonic anhydrase (M_r = 29 kDa), actin (45 kDa), bovine serum albumin (66 kDa), and phosphorylase *b* (97.4 kDa).

The data in Figure 2c do not suggest any further change in the α-chymotryptic sensitivity of α-actinin with the presence of DG and PA in the 20% PS bilayer. It has been reported that the addition of α-actinin to liposomes containing DG and PA resulted in some protection of at least one cleavage site of α-actinin against trypsin digestion (Meyer et al., 1982). This discrepancy is possibly due to the differences in the site of cleavage by trypsin and that by α-chymotrypsin.

It can be seen from Figure 2d that, first, the presence of PC or DG/PA vesicles without the inclusion of any negatively charged phospholipids did not have any significant influence on the time course of α-actinin digestion (lanes 2 and 5 vs lanes 1 and 4). Second, the presence of vesicles containing other negatively charged phospholipids, DOPG or PI vesicles, had the same protective effects as that from the PS vesicles (lanes 7 and 8 vs 6). Furthermore, the 65 kDa fragment of α-actinin was obviously protected when the α-actinin was mixed with PS−Triton X-100 micelles (lane 3). All these results indicate that a negatively charged phospholipid is essential for the interaction of α-actinin with membranes.

Conformational Changes of α-Actinin Induced by the Association with Lipid Vesicles. Figure 3 shows the 1800−1500 cm^{-1} infrared spectra of α-actinin, either in the presence or in the absence of lipid vesicles. The amide I band of α-actinin in D_2O buffer (trace 1 in Figure 3a) is characterized by a maximum absorption at 1649 cm^{-1}, and can be confirmed by Fourier self-deconvolution results (see trace 1 in Figure 3b). It indicates a high α-helical content of α-actinin in solution, which is in agreement with the results of some CD measurements (Suzuki et al., 1973, 1976; Malhotra et al., 1986; Wenegieme et al., 1994). There is also absorption of α-actinin at 1558 and 1564 cm^{-1} in the amide II region, which can be detected by Fourier self-deconvolution of the original spectrum (see trace 1 in Figure 3b).

In contrast, incubation of α-actinin with pure DOPG vesicles (lipid:protein ratio = 600:1) resulted in an increased absorption at 1634 cm^{-1} in the amide I region, indicating an increase in the amount of β-sheet structure of α-actinin. Besides, the absorption at 1564 cm^{-1} of α-actinin in the amide II region was almost diminished (see trace 2 in Figure 3). The addition of DG and PA in the pure DOPG bilayer resulted in only slight, if any, further perturbation of the protein secondary structure (see trace 3 in Figure 3). We also studied the extents of structural perturbation on α-actinin caused by interaction of the protein with the lipid vesicles

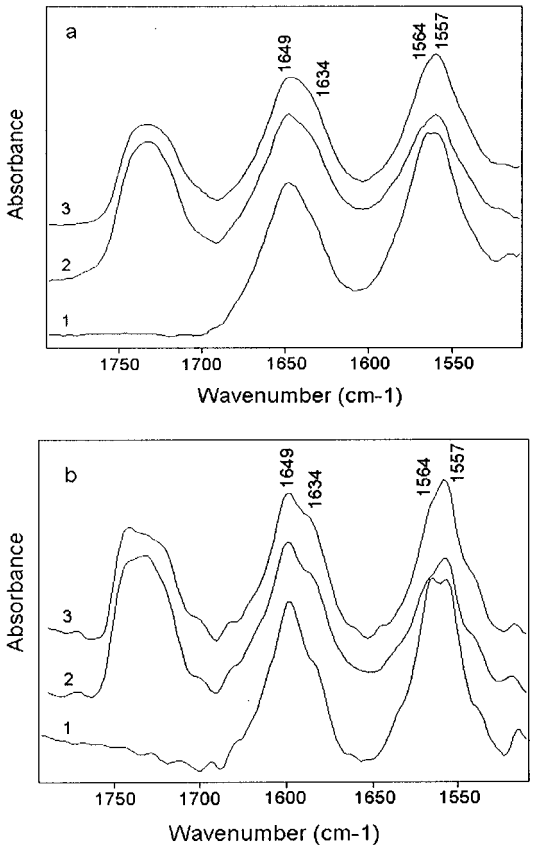

FIGURE 3: Infrared spectra in the amide I and II regions of α-actinin in solution (1) and in the presence of pure DOPG vesicles at a lipid to protein ratio of 600:1 (2) and in the presence of 2:1:1 DOPG/DG/PA vesicles at a total lipid to protein ratio of 600:1 (3). (a) Original spectra; (b) spectra after band-narrowing by the Fourier self-deconvolution method with a resolution enhancement parameter of 2.4 and a full width at half-height of 13 cm^{-1}. The protein concentration in all samples was approximately 15 mg/mL.

composed either of pure DOPG at lipid to protein ratios of 100:1 and 25:1 or of 20% DOPG and 80% PC at a lipid to protein ratio of 600:1. In FT-IR spectra of all these samples, the absorption at 1634 cm^{-1} of the protein increased, and the absorption at 1564 cm^{-1} of the protein decreased upon membrane binding of the protein. These spectral changes were most profound in the measurement made with the sample that had a lipid to protein molar ratio of 600:1 and the vesicles made up with pure DOPG (trace 2 in Figure 3).

Electron Microscopic Observation of the Bundling of Actin Filaments by α-Actinin on Phospholipid Monolayers. As shown in Figure 4a, the actin filament and the single α-actinin molecule can be resolved. The α-actinin molecules in contact with the lipid monolayer composed of PC and 20% PS appeared as rodlike structures, very similar to those observed by Meyer and co-workers (1982). When 10% DG and 10% PA were included in addition to the 20% PS in the lipid components of the monolayer, actin bundles on monolayers could be observed as shown in Figure 4b.

Examination of the polymerization solution, which contained 11 μM actin and 1.0 μM α-actinin covered with a 20% PS monolayer, shows that there are many large complexes of F-actin and α-actinin (Figure 5a). However, if 10% DG and 10% PA were present in the 20% PS monolayer, few such kinds of protein assemblies could be found in the polymerization solution (Figure 5b).

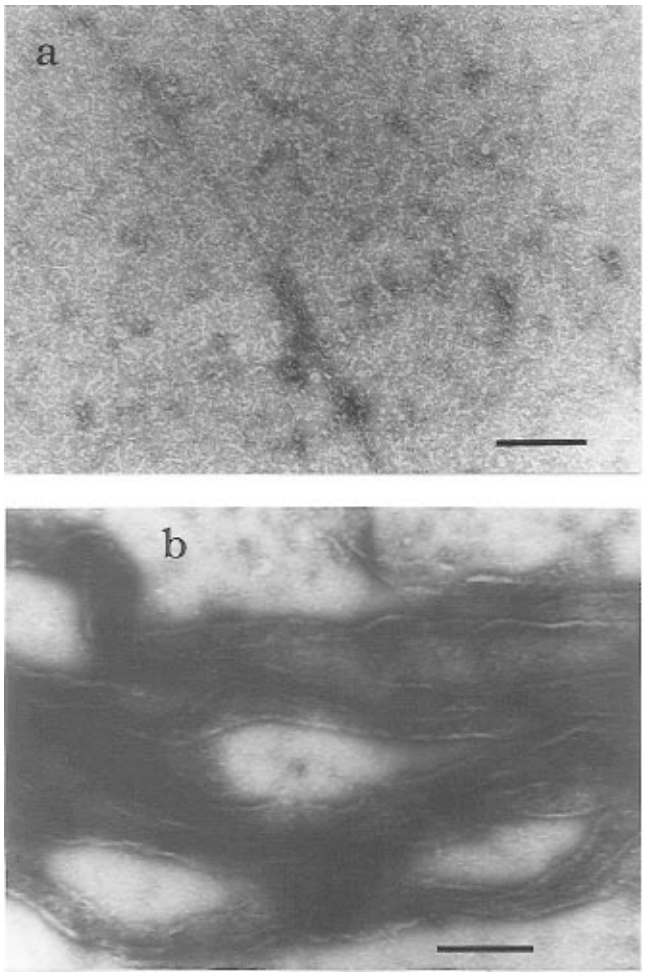

FIGURE 4: Negatively stained electron micrographs of lipid monolayers layered and incubated on the surface of the polymerization solution containing the mixture of α-actinin and G(or F)-actin. The lipid compositions of the monolayers were (a) 20% PS or (b) PS/DG/PA (20%, 10%, 10%). The remaining lipid component in the monolayers was PC. Bar = 0.2 μm.

DISCUSSION

In brief, the membrane binding properties of α-actinin were characterized in our study. It can be concluded that a negatively charged phospholipid is an essential component in the membrane for the binding of α-actinin. However, there is no absolute requirement for any particular negatively charged phospholipid, though there is a preference in the order of DOPG > PS > PI. Analysis of binding isotherms in terms of a membrane binding model gives apparent dissociation constants varying from 0.2 to 3 μM over a range of 5−20 mol % negatively charged phospholipid (see Table 1). We also find that 10% DG and 10% PA do not affect the binding behavior of α-actinin to vesicles. Since both of these two lipids are required simultaneously in the actin bundling by α-actinin (Burn et al., 1985), and DG and PA contents in the membranes at physiological conditions are usually very low, effects on the binding of α-actinin to membranes of DG or PA added separately, or those of the two lipids added together at higher concentrations, were not examined in the present study.

The association of a water-soluble protein with a synthetic lipid bilayer is initiated by their nonspecific (Hartmann & Galla, 1978) or specific (Nelsestuen & Lim, 1977; Cutsforth et al., 1989) binding through electrostatic interactions. If

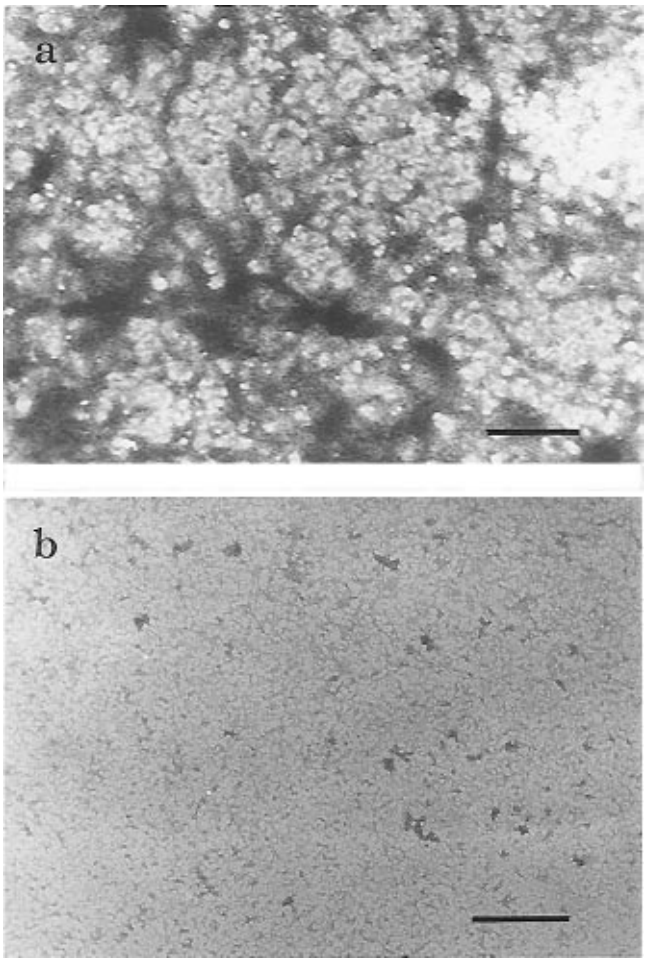

FIGURE 5: Negatively stained electron micrographs of the subphase under lipid monolayers. The lipid compositions of the monolayers were (a) 20% PS and (b) PS/DG/PA (20%, 10%, 10%). The remaining lipid component in the monolayers was PC. Bar = 1.0 μm.

the membrane-bound protein contains a region of hydrophobic amino acids, it may insert into the bilayer interior [Beschiaschvili & Seelig, 1990; Berliner & Koga, 1987; for reviews, see Selinsky (1992) and Sankaram and Marsh (1993)]. In solution, α-actinin can bind to membranes only if the negatively charged phospholipid was present according to our studies. In Meyer et al. (1982)'s work, DG/PA were emphasised as the essential components when α-actinin inserted into the lipid monolayers. Since the lipid monolayers they used were composed of a total lipid extract of yeast cells which obviously included many kinds of negatively charged phospholipids, the role of the negatively charged phospholipids in the binding of α-actinin to monolayers was neglected. The important role of the negatively charged phospholipid in the association of α-actinin with membranes was also noticed by Fritz et al. (1993), who demonstrated that, after the injection of α-actinin into the subphase, the surface pressure of the lipid monolayer increased significantly only if the negatively charged phospholipid and a high proportion of DG were present simultaneously. As stated in Fritz et al. (1993), the increased surface pressure was attributed to the insertion of α-actinin into the monolayers. Therefore, they cannot clarify if DG is still needed besides the negatively charged phospholipid when α-actinin only binds to the lipid headgroups through electrostatic interaction but does not insert into the membranes. Our experimental results showed that it is the negatively charged phospholipid, but not DG/PA, responsible for the binding of α-actinin to membranes (Figure 1a,b,c). This means that the electrostatic interaction is a prerequisite for the binding of α-actinin to membranes. Considering the insertion experiment results, it seems that DG (Fritz et al., 1993) or DG/PA (Meyer et al., 1982) would play some special roles in the insertion of α-actinin into membranes containing negatively charged phospholipids. But it is still immature to draw such a definite conclusion, since there were some other evidences indicating that α-actinin was able to insert into vesicles containing PS only (Niggli & Gimona, 1993).

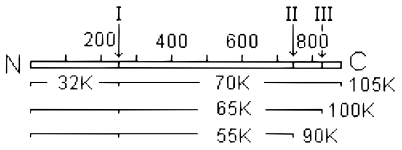

FIGURE 6: Cleavage and domain map of α-actinin, based on the results of Imamura et al. (1988), Gilmore et al. (1994), and Mcgregor et al. (1994).

α-Actinin is an acidic protein with a pI of 6.0 (Pollard et al., 1986). Ca^{2+} is usually an obligatory cofactor for many other acidic membrane binding proteins, such as protein kinase C (Newton, 1993) and blood coagulation proteins (Nelsestuen & Lim, 1977). Muscle α-actinin does possess cation binding ability (Wenegieme et al., 1994), although it cross-links actin filaments in a Ca^{2+}-insensitive manner (Duhaiman & Bamburg, 1984; Bennett et al., 1984; Landon & Olomuchi, 1983). This motivated us to examine if Ca^{2+} would play an important role in the process of α-actinin binding to membranes. We measured the binding isotherms of α-actinin binding to vesicles containing negatively charged phospholipid in the presence of Ca^{2+} at concentrations as high as several millimolar, but lower than the Ca^{2+} concentration needed to trigger the aggregation of the 4:1 PC/PS lipid vesicles, which should be avoided in the 90° light-scattering measurements (Nelsestuen & Lim, 1977). It was found that Ca^{2+} is unable to influence the membrane binding behavior of α-actinin (data not shown). The results of Ca^{2+} titration experiments also supported this argument (data not shown).

Addition of α-actinin to suspensions of micelles or vesicles containing negatively charged phospholipids resulted in the protection of some fragments of α-actinin against α-chymotrypsin digestion. On the basis of the cleavage map of smooth muscle α-actinin by α-chymotrypsin [see Figure 6 and cf. Imamura et al. (1988)], the time course of digestion showed in Figure 2b can be interpreted as that the cleavage site I is not protected because the 70 and 32 kDa bands appear as early as 1 and 5 min, respectively; this is not significantly different from the situation when PS is not present (Figure 2a). Since the appearance times of the 100, 90, 65, and 55 kDa fragments are delayed and the 105, 100, 70, and 65 kDa fragments are very effectively protected, it is clear that cleavage sites II and III are protected. This phenomenon may be attributed either to the conformational changes of α-actinin induced by the negatively charged phospholipids or to the insertion of the cleavage sites of α-actinin into the phospholipid environments. It is interesting to note that the above protection effect is rather insensitive to structural changes of the lipid assembly as

drastic as the change from large unilamellar vesicles to micelles (see lanes 3 and 6 in Figure 2d).

FT-IR spectroscopy is a superb method in the study of conformational changes of proteins (Jackson & Mantsch, 1995; Muga et al., 1991a, 1991b), despite that quantitative estimates of protein secondary structure by FT-IR spectroscopy suffer from some problems (Jackson & Mantsch, 1995; Surewicz et al., 1993). Conformational changes of α-actinin induced by salt and cations have been reported recently (Wenegieme et al., 1994; Kuroda et al., 1994). In our study, we found that the conformation of α-actinin could also be affected by its membrane binding. The FT-IR results show that both the amide I and amide II regions in the spectra of membrane-bound α-actinin are significantly different from that of α-actinin in solution. From the analysis of the amide I absorption of α-actinin shown in Figure 3, it can be concluded that the proportion of β-sheet structure in α-actinin increased when bound to DOPG membranes, as compared to that in solution. In the amide II region of the membrane-bound α-actinin, the disappearance of the absorption at 1564 cm^{-1} also indicates its conformational changes. Because there is no definite assignment of amide II frequencies to the secondary structure of proteins yet, it is difficult to explain this phenomenon precisely so far.

α-Actinin-induced actin filament bundling in solution has been reported by several authors (Burn et al., 1985; Meyer & Aebi, 1990; Podlubnaya et al., 1975; Condeelis & Vahey, 1982; Endo & Masaki, 1982; Grazi et al., 1994; Wachsstock et al., 1993). Among the many conditions explored, low temperature (Endo & Masaki, 1982; Grazi et al., 1994), addition of DG and PA (Burn et al., 1985), the critical molar ratio of α-actinin/actin [∼1:20 for chicken gizzard α-actinin at 22 °C in Meyer and Aebi (1990)], and the threshold concentrations of α-actinin and actin filaments (Wachsstock et al., 1993) have been reported to be essential for actin bundle formation in solution. Both α-actinin and F(or G)-actin (Weisenhorn et al., 1990; St-Onge & Gicquaud, 1990) are able to bind to membranes containing negatively charged phospholipids. This motivated us to see if the actin bundles could also be formed by α-actinin on the lipid monolayers containing negatively charged phospholipids. Extensive search of the specimens with an electron microscope failed to find any of the large complexes either in the network form or in the bundle form on the monolayer containing 20% PS, though actin filaments and a single α-actinin molecule were resolved (Figure 4a). Because DG and PA are able to induce actin bundle formation (Burn et al., 1985), 10% DG and 10% PA were added into the compositions of the lipid monolayer. The lipid monolayer was observed by using an electron microscope after incubation on the surface of the polymerization solution containing 11 μM actin and 1.0 μM α-actinin. The actin bundles on such a monolayer were found repeatedly and reproducibly (see Figure 4b). We also found that the presence of DG and PA in the monolayer containing negatively charged phospholipids greatly affected the distribution of the protein assemblies between the monolayer surface and the polymerization solution, as qualitatively demonstrated in Figure 5a,b. It can be deduced from these two figures that most of the protein assemblies seem to transferred from the solution to the monolayer surface if DG and PA were present. This may also be counted as evidence that DG and PA may play some special roles in the F-actin/α-actinin/membrane interactions.

So far, most of the attention in the field of cytoskeleton−plasma membrane interaction has been paid to the interaction between cytoskeleton proteins and membrane receptors [for a review, see Luna and Hitt (1992)]. However, recently many cytoskeleton proteins, such as actin (St-Onge & Gicquaud, 1990), spectrin (Cohen et al., 1986), vinculin (Niggli et al., 1986), myosin I (Hayden et al., 1990), band 4.1 (Sato & Ohnishi, 1983), synapsin I (Benfenati et al., 1989), and α-actinin, etc., have been found to be able to interact directly with negatively charged phospholipids. These proteins have been categorized as the amphitropic proteins by Burn (1988). It is also particularly noteworthy that the functions of some cytoskeleton proteins, such as α-actinin (Burn et al., 1985; Fukami et al., 1992, 1996), profilin (Lassing & Lindberg, 1985), cofilin (Yonazawa et al., 1991), gelsolin (Janmey & Stossel, 1987), and gCap (Yu et al., 1990) etc., can be mediated by phosphoinositides or their metabolites. Consequently, studies on the interactions between cytoskeleton proteins and membrane lipids will become more and more important in achieving a full understanding of cytoskeleton−plasma membrane interaction.

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