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Fragments from α-Actinin Insert into Reconstituted Lipid Bilayers

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Recent experiments have indicated that α-actinin interacts with phospholipid membranes. Using computer analysis methods we determined two possible lipid binding sites capable of membrane attachment/insertion, residues 281–300 and 720–739 of the primary amino acid sequence on smooth muscle α-actinin. Having expressed these regions as fusion proteins with schistosomal GST (glutathione *S*-transferase), we used differential scanning calorimetry (DSC) to investigate their interaction with mixtures of zwitterionic (dimyristoyl-L-α-phosphatidylcholine, DMPC) and anionic (dimyristoyl-L-α-phosphatidylglycerol, DMPG) phospholipids in reconstituted lipid bilayers. Calorimetric measurements showed that as fusion protein concentration increased, the main chain transition enthalpy decreased and chain melting temperatures shifted, which is indicative of partial protein insertion into the hydrophobic region of the lipid membranes. Centrifugation assay and subsequent SDS/Page chromatography confirmed this finding.

Proteins found between the cytoskeleton and the plasma membrane control cell shape and tension and stabilize attachments to other cells and to the substrate. Many of these proteins also regulate cell locomotion and transmit external stimuli. This diversity of cellular functions is matched by a large number of biochemical mechanisms that mediate the connections between membrane proteins and the underlying cytoskeleton (1).

α-Actinin is a prominent component determining the three-dimensional arrangement of actin filaments in smooth muscle and non-muscle cells (2). It is an antiparallel homodimeric rod-shaped protein of about 30–40 nm length with extensive homologies to spectrin and dystrophin (3, 4). It consists of two identical polypeptide chains, divided into three functional domains: an actin-binding region at the N-terminus, a central α-helical region, and a C-terminus containing two EF-hands. This structural arrangement allows the molecule to act as "cross-linker" and "spacer" for various actin filament aggregates (2, 5). It has been shown that the interaction of α-actinin with F-actin is biphasic (6) and of moderate affinity (μM) (7) but influenced by cations and ionic strength (8, 9). The thermodynamic parameters and the viscoelastic moduli (10, 11) as well as the cross-linking and/or bundling (12, 13) of F-actin in solution are also affected by the presence of α-actinin. α-Actinin also interacts with other proteins in the vicinity of the plasma membrane including ICAM-1, ICAM-2, β1-integrin, β2-integrin, L-selectin, vinculin, and zyxin (14–20). Furthermore, there is evidence that α-actinin is capable of interacting with phospholipid membranes directly (21–24). Han *et al.* (25) recently established, using various mono- and bilayer of charged lipid compositions and static light-scattering techniques that α-actinin reconstitutes into the hydrophobic region of lipid bilayers.

In this study, we used computer analyses to predict the structure of the primary amino acid sequence of α-actinin from smooth and non-muscle cells. Two segments were shown to facilitate lipid membrane attachments and anchoring: residues 281–300 (EKLAS-DLLEWIRRTIP-WLEN) and residues 720–739 (QLLTTIARTINEVEN-QILTR) of the amino-terminal may attach to phospholipid membranes and anchor in the hydrophobic region of

Abbreviations used: DMPC, dimyristoyl-L-α-phosphatidylcholine; DMPG, dimyristoyl-L-α-phosphatidylglycerol; DSC, differential scanning calorimetry; α-actinin, actin-binding protein; GST, glutathione *S*-transferase; DTT, dithiothreitol.

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lipid membranes. To probe their insertion into lipid layers, we expressed these regions as fusion proteins attached to schistosomal GST (glutathione S-transferase), using lipid vesicles consisting of DMPG, dimyristoyl-L-α-phosphatidylglycerol and DMPC, dimyristoyl-L-α-phosphatidylcholine at a molar ratio of 1:1 and of ~200 nm diameter as well as centrifugation assay and SDS/Page chromatography.

MATERIALS AND METHODS

Detection methods of secondary, hydrophobic, and amphipathic structures. The primary amino acid sequence of smooth muscle (PID g112956) and non-muscle cell α-actinin (PID g2493432) were used for structure predictions (cf. http://www.ncbi.nlm.nih.gov). The secondary structure predictions were made by PHD: Profile-fed neural network systems from Heidelberg (26–28) (cf. http://www.dodo.cpmc.columbia.edu/ProteinPredict). Briefly, PHD searches for local sequence homologies, using an internal data base of non-homologous polypeptides chains to predicts α-helices, β-sheets, and random coils. To search for highly hydrophobic or amphipathic segments within the α-actinin amino acid sequence, we constructed plots for the average hydrophobicity and the average hydrophobic moment (29). The normalized 'consensus' scale of Eisenberg *et al.* (30) was taken as the hydrophobicity scale for the amino acids. An amino acid window size of 11 was used throughout the analyses.

Generation of recombinant proteins. Two pairs of oligonucleotides (Genosys) coding for amino acids 281–300 and 720–739 of the smooth muscle (chicken) α-actinin sequence were annealed by the method previously described (31). The bacterial strain BL-21 was used for the expression of GST and the GST-coupled α-actinin fragments GST/281–300 and GST/720–739, respectively. The proteins transformed from BL-21 were then purified according to the procedure by Smith and Johnson (32). The purity was ≥95% judged by densitometry.

Lipid bilayer, DSC, and centrifugation assay. Lipid stock solutions were prepared by dissolving pure lyophilized phospholipids (zwitterionic DMPC and anionic DMPG) from Avanti Polar Lipids, Birmingham, AL; in chloroform/methanol 2/1 (v/v). From aliquots of these solutions, a dry lipid film was formed on the walls of an extensively rinsed glass beaker by evaporating the solvent with a stream of nitrogen followed by vacuum desiccation for at least 2h. The lipid film was dissolved in 20 mM Hepes/NaOH pH 7.4, 0.3 mM EGTA, 0.3 mM EDTA, 0.2 mM DTT, 5 mM NaCl, 0.005% NaN_3 for the preparation of unilamellar vesicles. The lipid dispersion was then subjected to five freeze/thaw cycles and pressed 10 times through 200-nm filters. Samples containing unilamellar vesicles with ~200 nm diameter were equilibrated at 4°C for 30 min. DSC samples, containing 1 mg/ml of lipids were injected into the sample cell, and scans were performed at a rate of 30°C/h. Data were collected at 0.05°C intervals and stored on a computer. At the end of the heating/cooling cycles at P/L molar of 1:100, the proteo-liposomes were centrifuged at 20,000*g* for 20 min at 4°C to remove unbound protein, and followed by SDS/Page analysis.

RESULTS

Computational predictions. Smooth muscle (continuous line) and non-muscle (dotted line) α-actinin show a high amino acid sequence homology—results from computer analyses are summarized in Fig. 1. Two regions of significant hydrophobic character can be seen within the examined amino acid range (Fig. 1A, continuous line). Around segments number 281–300 and 720–739, amino acid configurations of an obviously surface-seeking character are predicted (Fig. 1B, continuous line), suggesting lipid interactions via amphipathic helices. The primary amino acid limits of these amphipathic regions are selected according to predicted α-helices (Fig. 1), i.e., the secondary structure prediction method classified both stretches as α-helical. The average charge of these stretches is positive (Fig. 1D). The amino acid composition and arrangement of residues 281–300 and 720–739 are most notable with regards to the amphipathic helix motif (Fig. 1C), i.e., the consensus score values are fairly high. The probability that the given amphipathic arrangement of residues occurs by chance is very small (Fig. 1C, vertical bars). All in all, the strong amphipathic structure of residues 281–300 and 720–739 seem good candidates for membrane interactions.

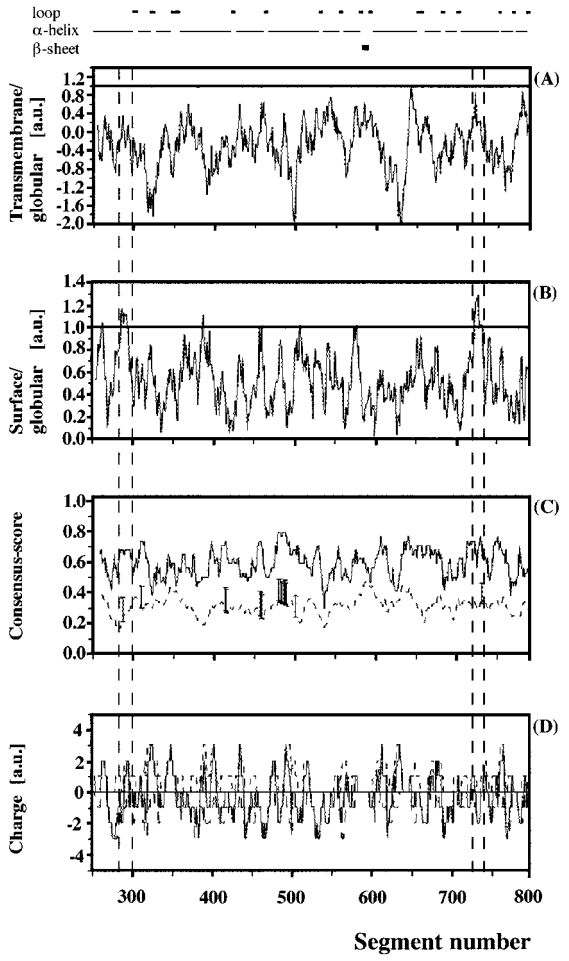
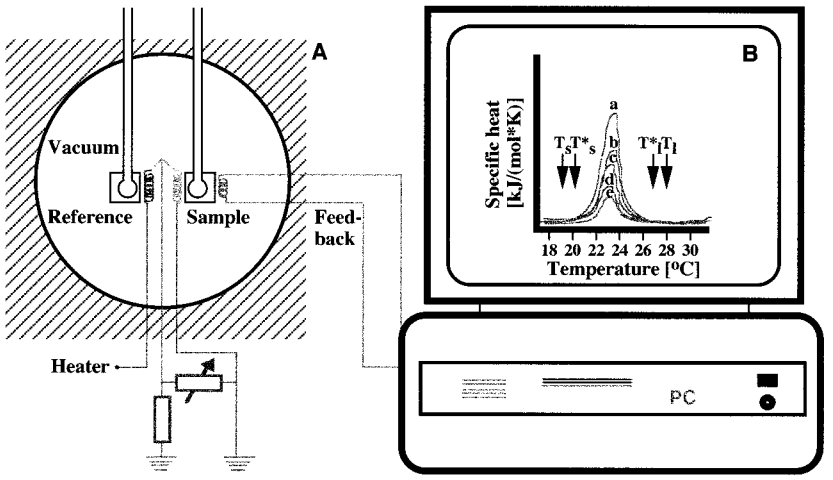


FIG. 1. Structure prediction plots: (A) hydrophobicity, (B) hydrophobic moment, and (C) probability for residues 250–800 of α-actinin according to the methods in (29, 30, 34). Secondary structures (D), i.e., α-helical, β-strand, and random coil were predicted by the methods described in (25–27). The amino acid window was 11, and the results were plotted above the middle residue of the window.



C	Lipid melting temperature (°C)			
Lipid melting transition points and shift	GST/281-300	GST/720-739	GST	DMPG/DMPC (control)
Pre-transition point (T_S or T^*_S)	19.5	19.8	20.1	20.3
Post-transition point (T_l or T^*_l)	28.0	27.7	27.0	27.1
(T_l - T_S) or (T^*_l - T^*_S)	8.5	7.9	6.9	6.8
Shift [(T_l - T_S) or (T^*_l - T^*_S)]	1.7	1.1	0.1	-

FIG. 2. (A) Schematic representation of the differential scanning calorimeter. (B) Variation of the main phase transition of lipid vesicles (DMPC/DMPG at 1 mg/ml and 1:1 ratio) in the presence of GST/281–300. T_s depicts the solidus (pretransition) and T_l the liquidus (posttransition) line of thermograms, T^*_s and T^*_l show DMPC/DMPG vesicles in the absence of GST/281–300, and T_s and T_l in the presence of the protein at various protein concentrations. (a) pure lipids; (b) P/L = 1/800; (c) P/L = 1/400; (d) P/L = 1/200; and (e) P/L = 1/100. Buffer: 20 mM Hepes/NaOH, 1 mM EGTA, 1 mM EDTA, 0.2 mM DTT, 40 mM NaCl, and 0.005% NaN_3, pH 7.4. (C) Lipid melting temperatures in the presence of GST/281–300, GST/720–739, and GST at protein/lipid molar ratios of 1:100 as determined from baseline-corrected DSC measurements.

Insertion of α-actinin fragments into lipid bilayer measured by DSC. Figures 2A and 2B show the principle of the calorimeter and the recorded changes of specific heat with rising temperature for vesicles consisting of DMPG/DMPC (1 mg/ml at 1:1 ratio and 200 nm diameter) in the presence of increasing fragment GST/281–300 concentration. The effect of GST/281–300 on the thermotropic properties of the lipid vesicles is to shift the chain-melting temperature. The change in heating profile $T^*_s \rightarrow T_s$ (pretransition point) and $T^*_l \rightarrow T_l$ (posttransition point) are signs for the proteins to interact with the hydrophobic region of the liposomes. At the highest protein/lipid (P/L) molar ratio of 1:100, the difference between the pre-transition points in absence and presence of GST/281–300 (T^*_s and T_s, respectively) was 0.8°C (Fig. 2B, trace a versus e). The respective T^*_s and T_s value for GST/720–739 of 0.5°C reflects weaker interaction with the liposomes. A comparison of the total main chain-melting transition T^*_s or T_s and T^*_l or T_l, respectively, in protein-free lipid vesicles (Fig. 2B, trace a) with that of lipid vesicles in the presence of GST/281–300 (P/L molar ratio of 1:100; Fig. 2B, trace e) shows a shift from 6.8 to 8.5°C (a shift of 1.7°C). The values for GST/720–739 and GST under identical conditions showed a shift of 1.1 and 0.1°C, respectively, compared with protein-free vesicles (Fig. 2C). These results indicate stronger hydrophobic interactions with lipid vesicles for GST/281–300 than for GST/720–739.

To investigate whether GST/281–300, GST/720–739, and GST were reconstituted into the lipid vesicles, the protein/lipid solutions (at molar ratio of 1:100) were used at the end of DSC scans and centrifuged at 20,000*g* for 20 min. Unbound and stably incorporated proteins were separated into supernatant (S) and vesicular pellet fractions (P) and analyzed by SDS/Page (Figs. 3 and 4). The results clearly indicate protein/lipid incorporation of the order GST/281–300 > GST/720–739 > GST, which is consistent with findings using DSC. We further used the helical wheel projection method (33) to determine whether α-helicity corresponds with the ability of the peptides to form amphipathic structures. The α-helix constructed from peptide 281–300 is stronger amphipathic than peptide 720–739 (data not shown).

DISCUSSION

Many cytoskeletal proteins close to the plasma membrane exist in soluble form in the cytoplasm, which could allow them to associate transiently with the lipid boundary. In this aggregate form, proteins are likely to interact in two steps: an initial electrostatic attraction is followed by lipid insertion after refolding of the protein. This event occurs only when proteins find compatible lipid membrane configurations, i.e., α-helices or β-strands. Surface binding to polar lipid head groups is usually achieved by exposing amphipathic α-helices; thus insertion into one-half of the hydrophobic bilayer requires a β-barrel or hydrophobic α-helix formation. Normally, when a primary amino acid sequence is known, the method described above under Materials and Methods section affords highly accurate predictions. The hydrophobicity index for each amino acid and the probability, if a protein is membrane spanning, is derived from hydrophobic plots (29, 30). We used a purpose-written matrix program to discriminate between surface-seeking and transmembrane configurations of α-actinin (34). By applying this method, we have been able to predict potential lipid-binding motifs for several proteins with high accuracy: vinculin, talin, and filamin (34, 35). The proposed lipid-binding sites have been confirmed *in vitro* by other techniques, including hydrophobic labeling, differential scanning calorimetry, film balance technique, CD spectroscopy, and isothermal titration calorimetry (31, 36–38).

Examining the sequence of α-actinin for transmembrane stretches revealed that this protein does not contain a 20-residue-long region with a mean hydrophobicity greater than the minimal required of 0.42 (30). Thus the hydrophobicity of α-actinin with acidic lipids probably results from the incorporation of short hydrophobic stretches and/or attachment of amphipathic α-helical stretches. The secondary structure motif (amphipathic α-helix) also appears to bind to charged lipids; presumably, when the helical axis is parallel to the membrane surface, the polar side of the amphipathic helix binds electrostatically to the charged lipid head groups whereas the non-polar helix side interacts hydrophobically with the lipid chains.

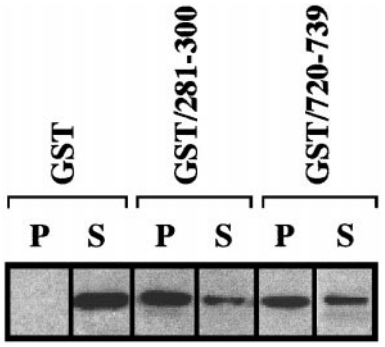


FIG. 3. Reconstitution of GST/281–300, GST/720–739, and GST into mixed DMPG/DMPC (1:1; 1 mg/ml) vesicles. Unbound protein (supernatant, S) is separated from stable vesicle fractions (pellets, P) at a protein/lipid molar of 1:100. Conditions: 10% SDS/Page; 50 μl per lane; Coomassie blue stain.

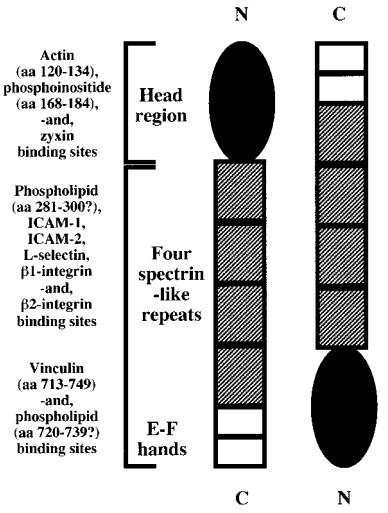


FIG. 4. Schematic view of the domain structure of α-actinin. The molecule consists of a globular head region, four spectrin-like repeats, and two E-F hands. The molecule is an antiparallel homodimer of approximately 30–40 nm length with binding sites for actin (2), ICAM-1 (14), ICAM-2, L-selectin, β2-integrin (15, 16), β1-integrin (17), vinculin (19), and zyxin (20). The E-F hands were described by Noegel *et al.* (18).

Our results obtained with DSC show that the proposed regions on α-actinin amino acid residues 281–300 and 720–739 attached to schistosomal GST interact with phospholipids. Using this method, we found that the fusion proteins suppress the enthalpy, ΔH and broaden the main phase transition. This effect—although small—is indicative of hydrophobic insertion. It can be explained by the penetration of the fusion proteins into the lipid bilayer core which expands, destabilizes, and rearranges the phospholipid structure. The centrifugation assay and subsequent SDS/Page chromatography confirmed the incorporation of these fragments into the hydrophobic region of the liposomes.

In conclusion, the notion that many cytoskeleton-associated proteins may be regulated by lipids in a selective and specific manner particularly stresses the impact that protein-lipid interactions may have on structure/function relations, and thus on signaling pathways that originate at membrane interfaces.

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