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Fragments from Actin Binding Protein (ABP-280; Filamin) Insert into Reconstituted Lipid Layers

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Previous computer analyses suggested two possible lipid binding sites, residues 49-71 and 131-155, of the primary amino acid sequence on ABP-280 (filamin), which could facilitate membrane attachment/insertion. We expressed these regions as fusion proteins with schistosomal GST and investigated their interaction with mixtures of zwitterionic (dimyristoyl-L-α-phosphatidylcholine, DMPC) and anionic (dimyristoyl-L-α-phosphatidylglycerol, DMPG) phospholipids in reconstituted lipid bilayers by differential scanning calorimetry (DSC). Using vesicles of mixed DMPC/DMPG with increasing fusion protein concentrations, we established in calorimetric assays a decrease of the main chain transition enthalpy, ΔH, and a shift in chain melting temperature. This is indicative of the insertion of these fragments into the hydrophobic region of lipid membranes. We confirmed these findings by the film balance technique using lipid monolayers (DMPG). The binding judged from both methods was of moderate affinity.

Many studies exist suggesting that the various interconnected assemblies in the extracellular matrix and the cytoskeleton play an important role in signal transduction events [1]. In signaling, where neighboring cells communicate via locally secreted chemical mediators either directly or through the extracellular matrix, the first step in the pathway is the binding to cell surface receptors i.e., integrins. Integrins are heterodimeric complexes in which both chains span the plasma membrane bilayer once, and the cytoplasmic domain of the β-chain is responsible for linkage to the actin cytoskeleton. Several cytoskeletal proteins have been reported to interact with integrin tails including α-actinin, talin, and filamin *in vitro* [2-6]. In addition to this pathway, it has been reported that these proteins as well as other membrane-associated proteins can bind directly with lipids [7-13]. Tempel *et al.* [14], for instance, determined three regions on the talin molecule and two regions the vinculin molecule that specifically associate with acidic phospholipids.

The actin binding protein (ABP-280; filamin) is a major constituent of the microfilament network determining the three-dimensional arrangement of actin filaments in smooth muscle and non-muscle cells. It is a homodimer with two polypeptide chains arranged in parallel, which only associate at the C-terminal ends, exposing actin binding regions at the N-terminal ends. This structural arrangement gives the molecule great flexibility and makes it a potent cross-linking and stabilizing protein for various actin filament aggregates [15]. It has been shown that the interaction of ABP-280 (filamin) with F-actin is biphasic [16] and of moderate affinity (μM) [17] but strongly influenced by cations [18]. The thermodynamic parameters and the viscoelastic moduli [19, 20] as well as the cross-linking/bundling [21] of F-actin in solution are also affected by the presence of filamin and gelsolin [22]. Furthermore, ABP-280 (filamin) is capable of interacting with phospholipid membranes directly. Tempel *et al.* [10], using lipid mono -and bilayer of various lipid compositions, and employing physical methods like calorimetry, film balance, and photolabeling established that filamin reconstitutes into the hydrophobic region of lipid layers. The incubation of filamin with lipid vesicles (DMPG/DMPC at 1:1 ratio) was described as 'condensed rounded-up' with a smooth surface when viewed by light microscopy [23]. More recently, structure predic-

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Abbreviations used: DMPC, dimyristoyl-L-α-phosphatidylcholine; DMPG, dimyristoyl-L-α-phosphatidylglycerol; DCS, differential scanning calorimetry; filamin; ABP-280, actin binding proteins; GST, glutathione *S*-transferase; DTT, dithiothreitol; PI, phosphoinositol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; DAG, diacylglycerol.

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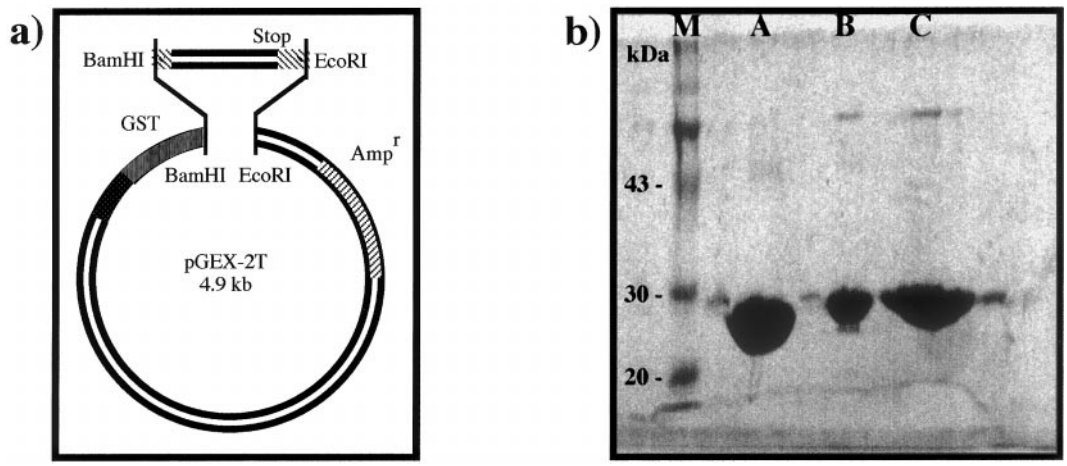

FIG. 1. (a) The expression of GST-coupled proteins was accomplished by the annealing of corresponding f- and r-oligo-nucleotides. The double-stranded sequences were subsequently cloned into the vector pGEX-2T. (b) The purity of the recombinant proteins was checked by an analytical polyacrylamide gel (15%). Lane M: molecular weight marker; lane A: GST; lane B: GST/49-71; lane C: GST/131-155. The purity of the expressed proteins was higher than 95% judged by densitometry.

tions of the primary amino acid sequence of ABP-280 (filamin) by computer analyses showed that two segments could facilitate lipid membrane attachments or anchoring [24]: residues 49-71 (FTRWCNEHLKCVSKRIANLQTDL) of the amino-terminal may attach to phospholipid membranes, and residues 131-155 (DGNLKLILGLIWTLILHYSISMPMW) may anchor in the hydrophobic region of lipid membranes. In this study, we have expressed these regions as fusion proteins attached to schistosomal GST and used pure DMPG and lipid vesicles (DMPG/DMPC at 1:1 ratio of ~200 nm diameter), employing film balance technique and differential scanning calorimetry to probe their insertion into lipid mono -and bilayers. The findings in this study will be discussed in the context of recent developments in cell signaling.

MATERIALS AND METHODS

Generation of recombinant proteins. Two pairs of oligonucleotides (Genosys) coding for amino acids 49-71 and 131-155 of the filamin (ABP-280) sequence [24]—flanked upstream by a BamHI site and downstream by a stop-codon as well as an EcoRI site—were annealed:

(AA 49-71 f: 5′-GATCCTTCACGCGCTGGTGCAACGAGCACCTGAAGTGCGTGAGCAAGCGCATCGCCAACCTGCAGACGGACCTGTAAG-3′,
r: 5′-AATTCTTACAGGTCCGTCTGCAGGTTGGCGATGCGCTTGCTCACGCACTTCAGGTGCTCGTGCACCAGCGCGTGAAG-3′);
(AA 131-155 f: 5′-GATCCGACGGGAACCTGAAGCTGATCCTGGGCCTCATCTGGACCCTGATCCTGCACTACTCCATCTCCATGCCCATGTGGTAAG-3′,
r: 5′-AATTCTTACCACATGGGCATGGAGATGGAGTAGTGCAGGATCAGGGTCCAGATGAGGCCCAGGATCAGCTTCAGGTTCCCGTCG-3′).

The double-stranded oligonucleotides were cloned into pGEX-2T previously digested with BamHI/EcoRI (Fig. 1a). pGEX-2T is a bacterial expression vector that contains the GST gene. The plasmids were analyzed by restriction digests, and subsequently sequenced to confirm the presence of the inserts. The bacterial strain BL-21 was used for the expression of GST and the GST coupled filamin fragments GST/49-71 and GST/131-155, respectively. The proteins transformed from BL-21 were then purified according to the procedure by Smith and Johnson [25] (Fig. 1b).

Lipid monolayer and film balance technique. The anionic phospholipid dimyristoyl-L-α-phosphatidylglycerol (DMPG) from (Avanti Polar Lipids, Birmingham, AL) was dissolved in a chloroform/methanol solution of 9/1 (v/v). The lipid was then spread onto buffer (A) containing 20 mM HEPES/NaOH pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM DTT, 40 mM NaCl, 0.005% NaN_3, rendering a homogeneous lipid monolayer.

Monolayer experiments were performed in a film balance apparatus as described [26]. This unit consists of a light microscope which is placed above a 25 ml Langmuir trough to allow observation of the fluid surface. Peltier elements underneath the trough are used for temperature regulation (± 0.2°C). The trough is covered by a glass slide to protect the spread lipid monolayer. The surface pressure of the solution in the trough is measured by a Wilhelmy system. Time/pressure diagrams of the lipid monolayer are recorded at a lateral constant pressure of 19mN/m for DMPG.

Lipid bilayer and differential scanning calorimetry (DSC). Lipid stock solutions were prepared by dissolving pure lyophilized phospholipids (zwitterionic (dimyristoyl-L-α-phosphatidylcholine, DMPC and anionic dimyristoyl-L-α-phosphatidylglycerol, 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.

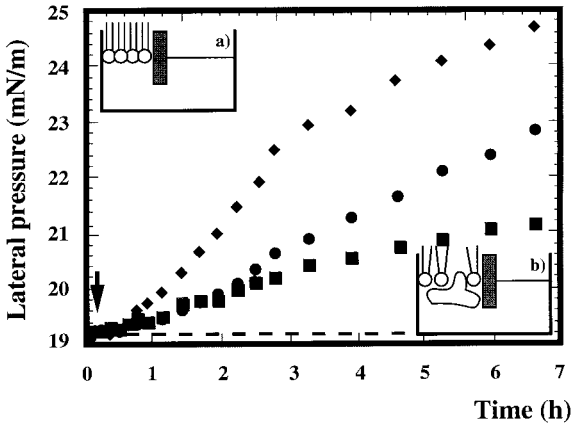

FIG. 2. A time/pressure diagram as measured by the film balance technique. Pure DMPG phospholipid monolayers were spread on buffer and 25 μg/ml GST (■), GST/49-71 (●), or GST/131-155 (◆) was injected into the subphase. The arrow marks the time of injection. The pressure of 19 mN/m was constant for pure DMPG (— —) over 10 h. (Note: the equilibrium pressure reached after 15 h was 28.5, 25, and 21.5 mN/m for GST/131-155, GST/49-71, and GST, respectively). Measuring temperature 20°C. Buffer (A): 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. (*Inset*: schematic representation of the film balance technique. Changes in pressure with time (a+b) are due to the insertion of the protein into the lipid layer).

RESULTS

Insertion of filamin fragments into lipid monolayer measured by film balance technique. Prior to monitoring the insertion of filamin fragments 49-71 and 131-155 coupled to glutathione S-transferase (GST), we determined the surface tension of (a) the anionic DMPG monolayer and (b) protein solutions at the liquid-air interface in the absence of DMPG. Using buffer (A) containing 20 mM HEPES/NaOH pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM DTT, 40 mM NaCl, 0.005% NaN_3 in the presence of DMPG, we measured an equilibrium lateral pressure of 19 ($\pm$1) mN/m over 10 hours. GST, GST/49-71, and GST/131-155 in the absence of DMPG showed a lateral pressure of less than 1 mN/m with time, indicating no interference of these fragments with the air-water interphase. All subsequent experiments were therefore carried out at a lateral pressure of 19 mN/m. Figure 2 shows a time/pressure diagram recorded by the film balance apparatus. In these experiments, we used pure DMPG monolayers (— —), which were spread on buffer (A), and 25 μg/ml GST (■), GST/49-71 (●), or GST/131-155 (◆), which were injected into the subphase. The arrow marks the time of injection of each protein. Recording pressure changes with time (Fig. 2) and equilibrium pressures after 15 hours of 28.5, 25, and 21.5 mN/m for GST/131-155, GST/49-71, and GST (data not shown), we observed an increase in the order of GST/131-155 > GST/49-71 > GST. These increases are indicative of protein incorporation into the lipid monolayer. The cartoons a+b (inset to Fig. 2) describe these events.

Insertion of filamin fragments into lipid bilayer measured by differential scanning calorimetry (DSC). The change of specific heat with rising temperature for vesicles consisting of DMPG/DMPC (1 mg/ml at 1:1 ratio and 200nm diameter) in the presence of increasing fragment (GST/49-71 and GST/131-155) concentration is shown in Fig. 3A+B. The effect of filamin fragments on the thermotropic properties of the lipid vesicles shows a shift of the chain melting temperature. The change in heating profile $T_s^* \rightarrow T_s$ and $T_l^* \rightarrow$

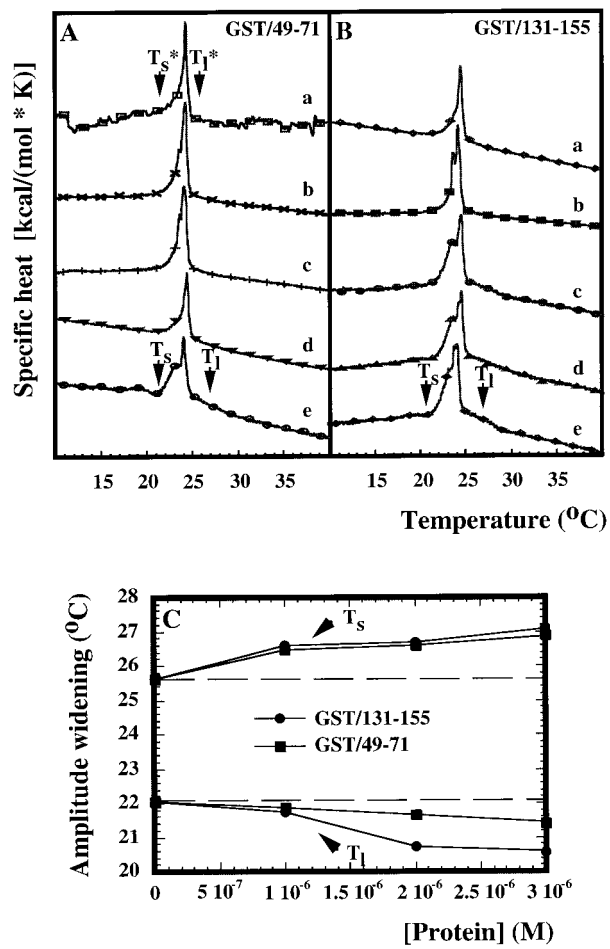

FIG. 3. DSC endotherms in panel A and B show the variation of the main phase transition of lipid vesicles (DMPC/DMPG at 1 mg/ml and 1:1 ratio) in the presence of GST/49-71 (A), GST/131-155 (B), or GST (B; a). T_s depicts the solidus and T_l the liquidus line. T_s^* and T_l^* show DMPC/DMPG vesicles in the absence of GST/49-71, GST/131-155, or GST, and T_s and T_l in the presence of these proteins at various protein concentrations. (A; a) pure lipids; (B; a) pure GST (P/L = 1/10); (A+B; b) P/L = 1/800; (A+B; c) P/L = 1/400; (A+B; d) P/L = 1/200; and (A+B; 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) T_s (solidus line) and T_l (liquidus line) are plotted as a function of various protein concentrations (—●— GST/131-155, and —■— GST/49-71).

T_l are signs for the proteins to interact with the hydrophobic region of the liposomes. The incubation of DMPC/DMPG vesicles with filamin fragments significantly shift the onset of the chain melting temperature, T_s (solidus line) to lower temperatures and the completion of the chain melting temperature T_l (liquidus line) to higher temperatures. Figure 3C shows the onset (T_s) and completion (T_l) of the main phase transition for DMPC/DMPG vesicles with temperature and increasing fragment (GST/49-71 and GST/131-155) concentration. Filamin fragment GST/131-155 inserts with higher efficiency into the hydrophobic region compared to GST/49-71. This effect is probably due to higher electrostatic attraction of this fragment for the inner leaflet of the lipid bilayer.

DISCUSSION

Elucidating the possible interaction of actin-associated proteins with lipids is important for the understanding of their function at the cellular level. Protein-lipid interaction may not only play a central role in (i) mediating the anchorage of the cytoskeleton to the lipid membrane, but also (ii) in defining a specific membrane topology. The release of actin-binding proteins or certain lipids e.g., inositolphosphates or diacylglycerol from a protein-lipid complex may represent a more direct way for a cell to trigger intracellular events than through transmembrane signaling [27].

Our results obtained by differential scanning calorimetry show that the proposed regions on ABP-280 (filamin)—amino acid residues 49-71 and 131-155 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 same type of interaction is demonstrated by film balance analyses. Recording the thermodynamic behavior of a system at constant pressure, we observe an increase in lateral pressure (mN/m) with time when the fusion proteins are injected into the subphase. The small increase is due to the insertion of the proteins. GST/131-155 shows a higher lateral pressure compared with GST/49-71 which confirms predictions by Tempel *et al.* [24] of the suitability of GST/131-155 to insert more efficiently into the hydrophobic region of the lipid layer. Computer alignment analyses of fragment 131-155 (residues: DGNLKLILGLIWTLILHYSISMPMW) with other membrane associated proteins from humans gave the following consensus score and value: 57, $3e^{-9}$ (ABP-280); 35, 0.019 (α-actinin); 34, 0.044 (dystrophin, spectrin). Interestingly, all proteins consisted of the sequence: DGN. . . .LG.IW. .IL, thus the transmembrane protein, e.g. glycophorin A did not show this sequence.

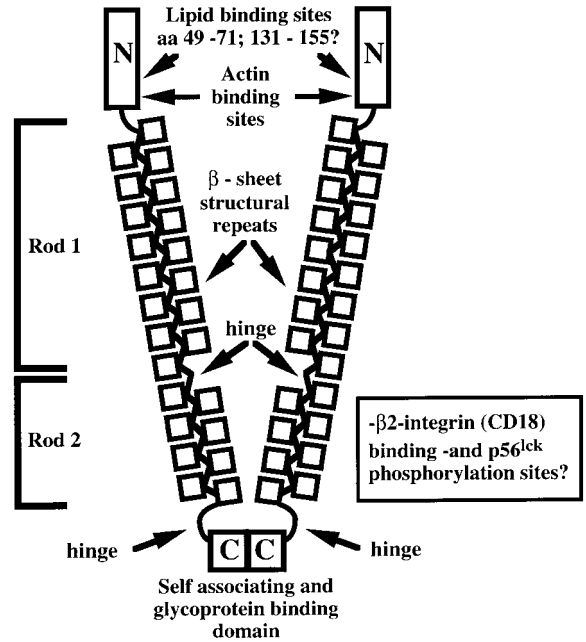

FIG. 4. Model of ABP-280 (non-muscle filamin) interactions with β2-integrin, F-actin, phospholipid membranes, and $p56^{lck}$. We present a detailed schematic view which is based on recent experimental and theoretical data. According to Tempel *et al.* [24], two regions in the ABP-280 (filamin) molecule (amino acids 49-71 and 131-155) are good candidates for lipid interactions. Sharma *et al.* [31] showed that *scr* kinase $p56^{lck}$ interacts with tyrosine-phosphorylates ABP-280 (filamin) probably in the rod 2 domain.

Recent studies have focused on elucidating interactions of the cytoplasmic tails of integrins with the cytoskeleton at focal contacts in cells [28-30]. Sharma *et al.* [5] identified the actin-binding protein (ABP-280, filamin) as a major cytoskeletal protein that binds directly and specifically to the cytoplasmic tail of the β2-integrin subunit CD18 using co-immunoprecipitation and co-immunolocalization assays. The ABP-280 (filamin) binding site in CD18 was localized on the N-terminus (amino acids 724 to 747). More recently, Sharma *et al.* [31] showed that $p56^{lck}$—a lymphoid-specific *src* tyrosine kinase, which is critical for T-cell development and activiation—and (ABP-280, filamin) interact. In this *in vitro* study these workers investigated the role of $p56^{lck}$ phosphorylating ABP-280 (filamin). They found that purified $p56^{lck}$ catalyzed the phosphorylation at an approximate stoichiometry of 1:1 and that tyrosine phosphorylation was restricted to two peptides of labeled (ABP-280, filamin). Using isolated epithelial cell membranes the addition of unphosphorylated, but not $p56^{lck}$-phosphorylated ABP-280 (filamin), suggested that tyrosine phosphorylation may interfere with ABP-280 (filamin) binding to actin and subsequently with the link between the actin cytoskeleton, integrin, and lipid membrane. A recently pub-

lished study has shown that actin is linked to integrin in quiescent cells and that the disruption of the interaction activates integrins [32]. We are currently testing the hypothesis of whether dissociation of ABP-280 (filamin)-bound integrin [5] from the cytoskeleton or ABP-280 (filamin)-bound lipid [10] is only required for early cell signaling.

In Fig. 4 we show a composite map of the structural and functional domains of the non-muscle ABP-280 (filamin) molecule, indicating the location of binding sites for actin, and phospholipid membranes at the amino terminus. β2-integrin (CD18) binding and $p56^{lck}$ tyrosine phosphorylation sites are assumed in the rod 2 region at the carboxy-terminal end, thus further research is required for more complete elucidation.

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REFERENCES

1. Yamada, K. M. (1997) *Matrix Biol.* **16,** 137–141.
2. Horwitz, A., Duggan, K., Buck, C., Beckerle, M. C., and Burridge, K. (1986) *Nature* **320,** 531–533.
3. Otey, C. A., Pavalko, F. M., and Burridge, K. (1990) *J. Cell Biol.* **111,** 721–729.
4. Schaller, M. D., Otey, C. A., Hilderbrand, J. D., and Parsons, J. T. (1995) *J. Cell Biol.* **130,** 1181–1187.
5. Sharma, C. P., Ezzell, R. M., and Arnaout, M. A. (1995) *J. Immunol.* **154,** 3461–3470.
6. Hemler, M. E. (1998) *Curr. Biol.* **10,** 578–585.
7. Goldmann, W. H., Niggli, V., Kaufmann, S., and Isenberg, G. (1992) *Biochemistry* **31,** 7665–7671.
8. Fritz, M., Zimmermann, R., Baermann, M., and Gaub, H. E. (1993) *Biophys. J.* **65,** 1878–1885.
9. Niggli, V., Kaufmann, S., Goldmann, W. H., Weber, T., and Isenberg, G. (1994) *Eur. J. Biochem.* **224,** 951–957.
10. Tempel, M., Goldmann, W. H., Dietrich, C., Niggli, V., Weber, T., Sackmann, E., and Isenberg, G. (1994) *Biochemistry* **33,** 12565–12572.
11. Isenberg, G. (1991) *J. Muscle Res. Cell Mot.* **12,** 136–144.
12. Isenberg, G., and Goldmann, W. H. (1995) *in* The Cytoskeleton (Hesketh and Pryme, Eds.), Vol. 1, pp. 169–204, JAI Press Inc., Greenwich, CT.
13. Isenberg, G., and Niggli, V. (1998) *Int. Rev. Cytology* **178,** 73–125.
14. Tempel, M., Goldmann, W. H., Isenberg, G., and Sackmann, E. (1995) *Biophys. J.* **69,** 228–241.
15. Hartwig, J. H., and Kwiatkowski, D. J. (1991) *Curr. Opinion Cell Biol.* **3,** 87–97.
16. Goldmann, W. H., Guttenberg, Z., Ezzell, R. M., and Isenberg, G. (1998) *in* Modern Optics, Electronics and High Precision Techniques in Cell Biology (G. Isenberg, Ed.), pp. 159–171, Springer–Verlag, Heidelberg.
17. Goldmann, W. H., and Isenberg, G. (1993) *FEBS Lett.* **336,** 408–410.
18. Senger, R., Goldmann, W. H., and Isenberg, G. (1995) *Biochem. Soc. Trans.* **23,** 57S.
19. Goldmann, W. H. (1992) *Biochem. Soc. Trans.* **20,** 89S.
20. Ruddies, R., Goldmann, W. H., Isenberg, G., and Sackmann, E. (1993) *Biochem. Soc. Trans.* **21,** 37S.
21. Goldmann, W. H., Senger, R., and Isenberg, G. (1994) *Biochem. Biophys. Res. Commun.* **203,** 338–343.
22. Goldmann, W. H., Tempel, M., Sprenger, I., Isenberg, G., and Ezzell, R. M. (1997) *Eur. J. Biochem.* **246,** 373–379.
23. Goldmann, W. H., Kaes, J. Isenberg, G., and Sackmann, E. (1993) *Biochem. Soc. Trans.* **21,** 133S.
24. Tempel, M. Goldmann, W. H., and Isenberg, G. (1994) *FEBS Lett.* **350,** 169–172.
25. Smith, D. B., and Johnson, K. S. (1988) *Gene* **67,** 31–40.
26. Dietrich, C., Boscheinen, O., Scharf, K. K., Schmitt, L., and Tampe, R. (1996) *Biochemistry* **35,** 1100–1105.
27. Isenberg, G. (1996) *Sem. Cell Dev. Biol.* **7,** 707–715.
28. Pfaff, M., Liu, S., Erle, D. J., and Ginsberg, M. H. (1998) *J. Biol. Chem.* **273,** 6104–6109.
29. Loo, D. T., Kanner, S. B., and Aruffo, A. (1998) *J. Biol. Chem.* **273,** 23304–23312.
30. Glogauer, M., Arora, P., Chou, D., Janmey, P. A., Downey, G. P., and McCulloch, C. A. (1998) *J. Biol. Chem.* **273,** 1689–1699.
31. Sharma, C. P., Goldmann, W. H., and Arnaout, M. A. (1998) *Mol Biol. Cell* **9**(Suppl.), 17a.
32. Jones, S. L., Knaus, U. G., Bokoch, G. M., and Brown, E. J. (1998) *J. Biol. Chem.* **273,** 10556–10566.