Interaction of Dystrophin Fragments with Model Membranes

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ABSTRACT The interaction with membrane lipids of recombinant fragments of human dystrophin, corresponding to a single structural repeating unit of the rod domain, was examined. Surface plasmon resonance, constant-pressure isotherms in a Langmuir surface film balance, and interfacial rheology were used to observe binding of the polypeptides and its effects on the properties of the lipid film. Modification of the monolayer properties was found to depend on the presence of phosphatidylserine in the lipid mixture and on the native tertiary fold of the polypeptide; thus a fragment with the minimum chain length required for folding (117 residues) or longer caused a contraction of the surface area at constant pressure, whereas fragments of 116 residues or less had no effect. The full extent of contraction was reached at a surface concentration of lipid corresponding to an average area of about 42 $Å^2$ per lipid molecule. A dystrophin fragment with the native, folded conformation induced a large increase in surface shear viscosity of the lipid film, whereas an unfolded fragment had no effect. Within a wide range of applied shear, the shear viscosity remained Newtonian. Binding of liposomes to immobilized dystrophin fragments could be observed by surface plasmon resonance and was again related to the conformational state of the polypeptide and the presence of phosphatidylserine in the liposomes. Our results render it likely that intact dystrophin interacts directly and strongly with the sarcolemmal lipid bilayer and grossly modifies its material properties.

INTRODUCTION

Dystrophin in striated muscle appears to be located mainly in the costameres, hoops in the sarcolemma surrounding the Z-discs of the myofibrillar lattice (Porter et al., 1992; Straub et al., 1992). Its function has not been precisely defined, but the likelihood is that it stabilizes the membrane against the continuous mechanical stresses to which it is subjected in the course of contractions and relaxations. It is clear at all events that when dystrophin is absent, or certain of its structural elements are genetically deleted, there is progressive and severe degeneration of the muscle, at least in humans (Ahn and Kunkel, 1993; Campbell, 1995). Muscles that lack dystrophin have a greatly reduced resistance to mechanical stress (Petrof et al., 1993) and an increased osmotic fragility (Hutter et al., 1991). Dystrophin-deficient myotubes, moreover, show a large reduction in membrane stiffness (Pasternak et al., 1995) and are again more susceptible to osmotic lysis (Menke and Jokusch, 1991).

Dystrophin is made up of four distinguishable domains (see Ervasti and Campbell, 1993; Ahn and Kunkel, 1993 for reviews). That at the N-terminus contains an attachment site for cytoskeletal actin, and two C-terminal domains are required for the attachment of other proteins, in particular the transmembrane glycoproteins that form a link to the extracellular matrix (Campbell, 1995; Ohlendieck, 1996). These regions are separated by a rod domain, comprising 24 degenerate repeats, homologous with those of the spectrin family of proteins (Koenig et al., 1988; Cross et al., 1990). It has been widely conjectured that the function of dystrophin in the sarcolemma is analogous to that of spectrin in the red blood cell, and indeed deficiencies or mutations of erythroid spectrin lead to pathological defects in the red cell membrane broadly similar to those engendered in dystrophic disorders in the sarcolemma.

If dystrophin exerts a major effect on the function of the sarcolemma, it must be presumed to modify the material characteristics of the membrane bilayer, as spectrin does in the red cell (see, e.g., Elgsaeter et al., 1986; Zeman et al., 1990). It is established through the work of several laboratories that spectrin interacts quite strongly with membrane phospholipids (see, e.g., Mombers et al., 1979; Maksymiw et al., 1987; McDonald, 1993; Diakowski and Sikorski, 1995). Here we show that an isolated repeat of the dystrophin rod domain binds strongly to phospholipid, and we examine the specificity of this interaction and its effect on the properties of a model membrane system.

MATERIALS AND METHODS

The expression in *Escherichia coli* and isolation of the dystrophin rod fragments, corresponding to the second repeat of the rod domain, has been described elsewhere (Kahana and Gratzer, 1995). The series of fragments used here have a common N-terminus, corresponding to Met^{439} in the dystrophin sequence, and lengths varying from 113 to 119 residues. Proteins were kept in 6 M guanidinium chloride at −20°C. Refolding occurs in seconds at room temperature, after dilution into a benign solvent (usually phosphate-buffered isotonic saline at pH 7.6).

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Phospholipids were bought from Lipid Products (UK). Liposomes were prepared by sonication: lipid solutions in chloroform/methanol were deposited on the wall of a flask under a stream of nitrogen and freed of solvent by rotary evaporation. They were suspended in 0.15 M sodium chloride, 20 mM Tris, 0.1 mM EDTA, 0.2 mM β-mercaptoethanol (pH 7.4) and sonicated for 15 min in bursts of 3 min with cooling in ice. After 2 h at room temperature, the liposomes were centrifuged at 70,000 × *g*. Phospholipid concentrations were determined by phosphorus analysis (Kates, 1974).

For surface plasmon resonance measurements, the proteins were dialyzed against 5 mM sodium phosphate (pH 7.4) and then mixed with 5 volumes of 40 mM sodium formate (pH 3.8) and centrifuged for 15 min at 70,000 × *g*. The protein was coupled to the BIAcore sensor chip (Pharmacia) by injection into the flow cell with an equimolar mixture of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide, following the manufacturer's instructions. To follow binding, the sensor chip was first equilibrated with the liposome suspension buffer, and 30 μl of the liposome suspension was then introduced. To observe dissociation, the liposome buffer was again injected. Before the next binding cycle the sensor chip was washed with 0.1% Tween 20 in the same buffer. Binding studies were conducted with liposomes of the following compositions: phosphatidylcholine:phosphatidylethanolamine, 2:3; phosphatidylcholine:phosphatidylserine, 2:3; phosphatidylethanolamine:phosphatidylserine, 2:3.

For monolayer experiments, a computer-controlled automated Langmuir surface film balance with a capacity of 60 ml and a surface area of 58.5 cm^2 (DeWolf, 1996) was used to obtain pressure-area and constant-pressure (area-time) isotherms. The surface pressure was measured with a Wilhelmy plate of filter paper, using a Cahn D-202 digital recording balance. The apparatus was enclosed and operated under a positive pressure of filtered, humidified air. All observations were made at room temperature, which was constant to within 1°C. Lipid mixtures were spread by deposition from a chloroform solution. The standard lipid mixture, corresponding to the approximate composition of a typical (e.g., red blood cell) plasma membrane cytoplasmic leaflet, comprised phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingomyelin, phosphatidylinositol, and cholesterol in the weight ratios 1:2:3:0.7:0.4:2.75. When phosphatidylserine was excluded, the ratios of the other components were kept the same. To ensure that the protein could not reach a water-air interface, a condensed monolayer lacking phosphatidylserine was spread on the surface between the outer edges of the barriers and the ends of the trough. The protein stock solution was injected into the aqueous subphase, followed by gentle mixing with a steel rod lying on the bottom of the trough. The final guanidinium chloride concentration in the trough was about 3 mM; this additive has no measurable effect on the lipid film properties.

Surface viscosity was measured with a biconical-bob, interfacial torsion rheometer (Mohammed et al., 1993), operated in the free oscillation mode, in which the applied shear stress ranged from 0.1 to 0.39 mN m^{-1}. Surface monolayers were generated as above, and protein was injected into the subphase.

Protein concentrations were measured spectrophotometrically throughout, using calculated specific absorptivities (Perkins, 1986). Circular dichroism spectra were measured in a Jobin-Yvon CD6 instrument in a path length of 0.5 mm. Absorption by the liposome preparations confined measurements to the wavelength range above ~208 nm.

RESULTS

It was shown earlier (Kahana and Gratzer, 1995) that a critical polypeptide chain length is required for the dystrophin fragments to enter the native triple-helical fold. Thus of the available fragments, those of 117 and 119 residues (here designated F117 and F119) are folded, whereas those from 113 to 116 residues in length (F113 to F116) have no stable tertiary structure (but a considerable, temperature-dependent content of α-helix). These fragments allowed us to assess the conformational specificity of the interaction with lipid.

Surface plasmon resonance measurements were carried out on two coupled fragments, one folded (F117), the other (F113) not. Both gave evidence of some binding of liposomes, but there was a marked difference between them (Table 1). Because a liposome must be assumed to have many potential binding sites on its surface for such a small protein, nearly all of which must remain sterically inaccessible when only one has attached to an immobilized protein molecule, meaningful quantitative analysis is essentially precluded. Indeed, apparent binding constants, determined from the measured association and dissociation rate constants, proved to be concentration-dependent, and no further attempts to extract thermodynamic data were made. It is possible, however, to determine apparent plateau levels of binding of the liposomes; these values are shown in Table 1. They reveal that the folded fragment binds much more lipid than the unfolded fragment, and that there is only vestigial binding of phosphatidylcholine (PC) liposomes, more binding of mixed phosphatidylcholine-phosphatidylethanolamine (PC/PE) liposomes, and still more binding of phosphatidylcholine-phosphatidylserine (PC/PS) liposomes. That phosphatidylserine is the principal binding component is confirmed by the high binding titer for phosphatidylethanolamine-phosphatidylserine (PE/PS) liposomes. It does not appear that binding of the liposomes to F113 induces a shift in the conformation toward that of the native fold: circular dichroism revealed no significant change in secondary structure of the polypeptide on the addition of a 15-fold (w/w) excess of PC/PS liposomes.

We examined the ionic strength dependence of liposome binding (Fig. 1). Binding is suppressed at low ionic strength, and its increase with increasing concentration of univalent counterions follows to a good approximation the shielding of the membrane surface charge predicted by the Gouy-Chapman theory (see, e.g., Barber, 1980).

To approach more closely the situation in a planar membrane, we examined the interaction of the series of fragments with a lipid monolayer, spread at the air-water interface, using a Langmuir surface film balance. In these experiments the composition of the monolayer was made

TABLE 1 Relative lipid-binding capacities of unfolded (F113) and folded (F117) dystrophin fragments, estimated by surface plasmon resonance

Liposome composition*	F113	F117
PC	160 ± 30#	520 ± 105
PC/PE	280 ± 80	1000 ± 60
PC/PS	1030 ± 130	1680 ± 200
PE/PS	1190 ± 90	1570 ± 130

*Molar ratios of lipids in the mixed liposomes were 2:3 phosphatidylcholine (PC):phosphatidylethanolamine (PE) or phosphatidylcholine:phosphatidylserine (PS) and phosphatidylethanolamine:phosphatidylserine.

#Values are surface plasmon resonance units corresponding to apparent saturation of the immobilized polypeptides with liposomes.

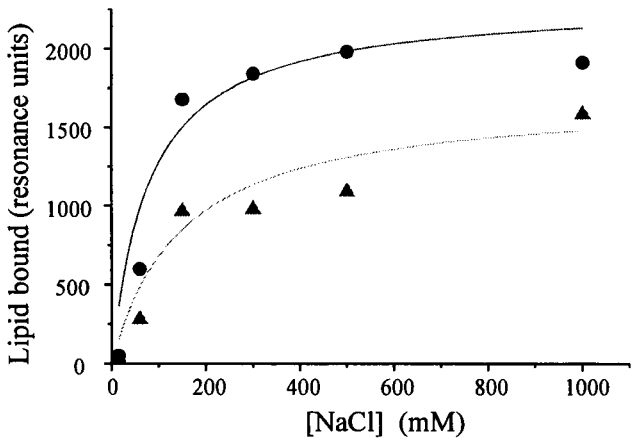

FIGURE 1 Ionic strength dependence of binding of phosphatidylcholine-phosphatidylserine liposomes to immobilized dystrophin fragments, followed by surface plasmon resonance. Fragment F117 (●) possesses the native conformation, whereas fragment F113 (▲) is not correctly folded. Curves represent the calculated fit for a single class of binding sites.

comparable to that of a typical cytoplasmic membrane leaflet (Devaux, 1988). The interaction with the lipid of the polypeptides, injected into the subphase, depended on the surface pressure of the film. Thus, at low surface pressures (below ~32 mN m^{-1}), expansion of the film area was observed (Fig. 2). We take this to be a consequence of penetration of the protein and its probable denaturation at the air-water interface, as could be observed in the absence of the lipid film. All measurements were therefore conducted at surface pressures of not less than ~36 mN m^{-1}. In these conditions interaction of protein with the film could be observed, as reflected by an area contraction. Equilibrium was attained very slowly if the subphase was not stirred, but in about 30 min after gentle mixing; this is demonstrated in Fig. 3, which shows the time course of equilibration for protein at an air-buffer interface (with no lipid film). The lag therefore reflected the rate of diffusion of the protein to the interface, and, because continuous stirring was precluded by the exacting stability requirements, it was rather variable.

With further increase in initial surface pressure, the lipid packing reached a point at which the protein could induce no area contraction. This occurred above ~40 mN m^{-1}.

There was an abrupt discontinuity in the behavior of the monolayer as the chain length passed from 116 to 117 residues, that is at the critical length for folding (Fig. 4). Thus we were able to detect no interaction between the protein and the membrane when the protein was not folded, and an apparently strong interaction (considering the very low concentration of protein in the subphase) with folded species. The diminutions in surface area seen in Fig. 4 are not the result of collapse of the monolayer, which indeed sometimes occurs, even slightly below the critical collapse pressure (see, e.g., Gershfeld, 1974, for a review), because of overshoot in the feedback system. Collapse reveals itself in an instantaneous, catastrophic contraction to a new plateau level at about half the previous surface area. The steplike changes in area seen in Figs. 2 and 4 probably reflect the limited sensitivity of the feedback system, but at this stage we cannot exclude the possible cooperativity of binding or of the response of the monolayer to bound protein.

Increasing the protein concentration above a saturating level did not result in any greater reduction in area. Thus at

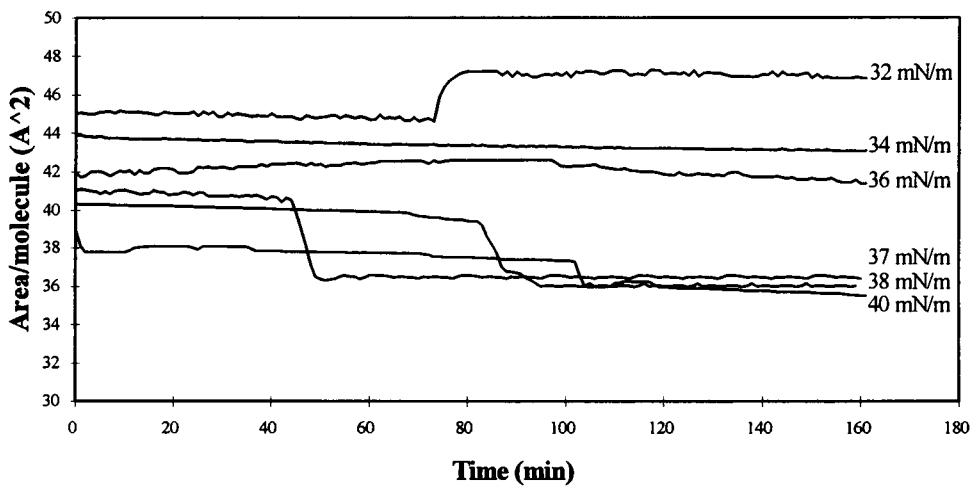

FIGURE 2 Constant-pressure isotherms for a lipid monolayer, with the composition of a typical (red blood cell) plasma membrane cytoplasmic leaflet, spread on isotonic aqueous buffer at the indicated surface pressures. Dystrophin fragment F119 was added to the subphase at zero time. Expansion of the surface layer, implying penetration of protein to the air-water interface, is reflected by an increase in the area per molecule; this is seen at low surface pressure (32 mN m^{-1}). At higher surface pressures (37 and 38 mN m^{-1}), at which the protein cannot penetrate the lipid film, interaction with the film causes an area contraction. This effect diminishes at higher pressures (40 mN m^{-1}) and vanishes when the pressure is so high that further contraction is prohibited by the lipid packing in the film. The times at which the changes occur are diffusion dependent and variable because continuous mixing is not possible, and the relatively abrupt nature of the changes probably reflects the limited sensitivity of the feedback system (see text).

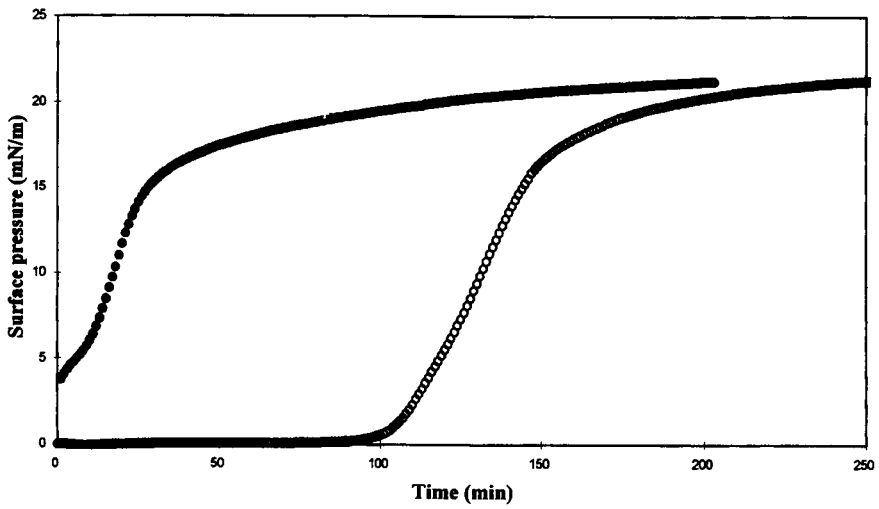

FIGURE 3 Adsorption isotherm for dystrophin fragment F119 to a buffer-air interface. Protein was added to the subphase at zero time with (●) and without (○) subsequent stirring.

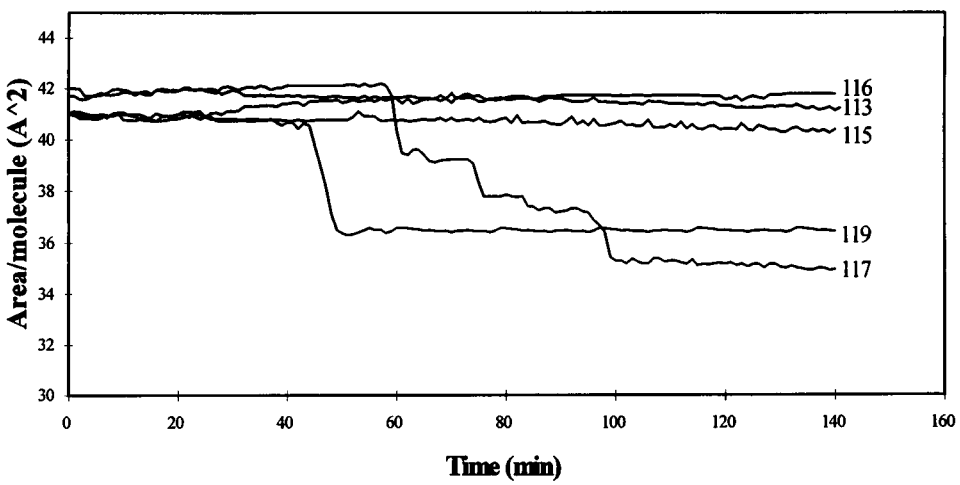

FIGURE 4 The effect of dystrophin fragment length on lipid monolayers, with the composition of a plasma membrane cytoplasmic leaflet, held at a constant pressure of 37 mN m^{-1} (precision of pressure control ± 1 mN m^{-1}). The area contraction is expressed in terms of the average area per lipid molecule. The lengths of the polypeptide chains of the fragments are indicated. The minimum chain length for formation of the native tertiary fold is 117 residues.

concentrations of F119 from 50 to 150 ng ml^{-1} the area contraction at constant pressure was the same within experimental error at 3.3 ± 0.5 $Å^2$ per molecule of lipid. This shows that the area contraction was not caused by lipid loss into the protein-containing subphase.

The interaction of the dystrophin fragments with the lipid film proved to be highly specific in its requirement for the anionic component of the membrane, phosphatidylserine, which is generally, perhaps indeed always, largely confined to the inner plasma membrane leaflet of eukaryotic cells (Zachowski, 1993; Williamson and Schlegel, 1994). Thus omission of PS from the lipid mixture essentially eliminated interaction of the monolayer with the protein, as also previously observed for spectrin (Maksymiw et al., 1987; DeWolf et al., 1996).

As a first step toward determining whether adsorption of the dystrophin modifies the material characteristics of the membrane, we measured the surface viscosity with the biconical-bob surface rheometer. The lipid monolayer alone gave a surface shear viscosity of 3.34 ± 0.35 $\mu N\ s\ m^{-1}$, which was independent of applied shear stress within experimental error over the range 0.1–0.39 mN m^{-1}. When the unfolded dystrophin fragment, F113, was introduced into the subphase, there was no significant change in shear viscosity, which was found to be 3.57 ± 0.38 $\mu N\ s\ m^{-1}$. By striking contrast, the folded fragment, F117, at the same concentration increased the shear viscosity of the film by a factor of about 7 to a value of 21.6 ± 0.50 $\mu N\ s\ m^{-1}$. The lipid concentration in these experiments corresponded to an area density of 42 $Å^2$ per molecule, assuming all of the protein (amounting to about one molecule per surface area of 1500 $Å^2$) to be adsorbed. The shear viscosity remained Newtonian over the range of shear stresses explored.

DISCUSSION

Our results show that a structural repeat from the dystrophin rod domain has a high-affinity binding site for membrane phosphatidylserine. This site is specific, in the sense that it is formed only by the native tertiary fold. In this, dystrophin resembles spectrin, although single repeats of the latter have not been studied with respect to lipid-binding propensity. The specificity of binding for PS and its depen-

dence on the native fold were less stringent, although still marked, in the liposome binding experiments: it is possible that the surface plasmon resonance technique detects weaker binding events, but, as indicated, the system does not in this case lend itself to quantitative analysis.

The effect on the lipid monolayer of the adsorption of dystrophin repeats is qualitatively rather similar to that of intact spectrin (Maksymiw et al., 1987), in that it is accompanied by an area contraction. Because the magnitude of this effect reaches a plateau with increasing polypeptide concentration, it is unlikely that the area contraction arises from loss of lipid from the monolayer through binding to protein in the subphase; nor could such a mechanism explain the increase in surface shear viscosity, engendered by the folded dystrophin fragment.

Our results imply that, unless the dystrophin repeat that we have studied is grossly atypical, the affinity of the whole dystrophin rod of 24 repeats for the membrane could be extremely high. This inference is consistent with the observations that genetic variants of dystrophin lacking the C-terminal, dystroglycan-binding elements nevertheless locate on the sarcolemma (Helliwell et al., 1992; Recan et al., 1992; Matsumura et al., 1993) and that dystrophin, expressed in COS cells, which are devoid of dystroglycans, also migrates to the plasma membrane (Lee et al., 1991).

With regard to the character of the interaction of the dystrophin fragments with the model membrane system, it should be kept in mind that the polypeptide chain (like the entire rod domain, and indeed those of the other known spectrin-like proteins) is highly anionic. Phosphatidylserine thus probably binds at a positively charged site on the protein, and a hydrophobic contribution to the association is also possible. At all events, the suppression of unfavorable global electrostatic effects on the apposition of the reactants by counterion shielding (Fig. 1) is not surprising. Binding between phosphatidylserine and intact spectrin, bearing a similarly high negative charge, has also been demonstrated (Maksymiw et al., 1987). We cannot exclude a possible effect of ionic strength on the structure of the liposomes, which might perturb the protein-lipid interactions.

The adsorption of the dystrophin fragment to the lipid film causes a striking increase in shear viscosity, which may be important in protecting the membrane against persistent stresses. If, as has been widely surmised, dystrophin exerts a function in the sarcolemma similar to that of spectrin in the red blood cell, elastic and rheoviscous properties should be generated in the intact membrane skeletal network. Little is known about this structure in the sarcolemma, in which it is evidently confined mainly to specialized regions (probably the costameres). In the red cell membrane at least some of the characteristic material properties are lost if the continuity of the lattice is interrupted (Mohandas and Evans, 1994). Thus the appearance of such properties in a model system of the kind studied here may be contingent on the presence of dystrophin-binding proteins that will allow formation of a continuous network. The deletion of the β-dystroglycan-binding site from the cysteine-rich domain has been shown to annihilate the functional efficacy of dystrophin (Rafael et al., 1996).

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