Volume 301, number 3, 243–245 FEBS 10956 April 1992

Expression of the N-terminal domain of dystrophin in *E. coli* and demonstration of binding to F-actin

M. Way*, B. Pope, R.A. Cross**, J. Kendrick-Jones and A.G. Weeds

MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

Received 20 February 1992

The N-terminal head domain of human dystrophin has been expressed in soluble form and high yield in *E. coli*, allowing us to test the previously unconfirmed assumption that dystrophin binds actin. DMD246, the first 246 amino acid residues of dystrophin, binds F-actin in a strongly co-operative manner with a Hill constant of 3.5, but does not bind G-actin. Dystrophin heads are thus functionally competent actin-binding proteins. This result opens the way to identifying critical residues in the actin-binding site and encourages us that the other domains of dystrophin might also be treated as functionally autonomous modules, accessible to a similar approach.

Dystrophin; Actin-binding; Muscular dystrophy; Alpha-actinin

1. INTRODUCTION

Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are X-linked human degenerative muscle disorders [1]. Both arise from alterations in the dystrophin gene: DMD from a total absence of dystrophin and BMD from the expression of truncated forms [2]. The dystrophin molecule can be divided into four domains (I–IV) based on its amino acid sequence. The N-terminal domain (I) of the human isoform, containing ~240 amino acids, is 44% identical to the F-actin binding domain of chick smooth muscle α-actinin and related cross-linking proteins [3–6]. The major domain (II) comprises 25 repeating elements similar to those in spectrin [6,7]. Deletions of up to 50% in domain II are responsible for the Becker phenotypes [8], suggesting that dystrophin can tolerate limited changes in structure and still retain partial function. Domain III is a cysteine rich region of ~150 amino acids that contains two 'EF-hand' calcium-binding motifs, though the match to the consensus calcium-binding sites is poor [6]. Domain IV is a unique C-terminal domain of ~420 amino acids [6] that associates with membrane glycoproteins and may provide membrane anchorage [9,10].

Progress has been made in analyzing these dystrophies at the cytogenetic level [11,12], and antibodies have localized the protein in the sarcolemma [13] as a component of the junctional folds [14]. However, very little is known about the biochemical properties, interactions and cellular functions of dystrophin. This is because it has not been possible to obtain adequate amounts of protein from muscle tissue for biochemical studies [9,14,15]. However, very small amounts of pure dystrophin have been prepared for electron microscopy [15]: these confirmed the potential for self-association predicted from sequence studies [6]. In particular, actin-binding has yet to be unequivocally demonstrated, although NMR measurements using short synthetic N-terminal peptides of dystrophin are suggestive [16]. Here we have expressed domain I of human dystrophin in *E. coli* and shown that it binds F-actin co-operatively with 50% binding occurring at 44 μM.

2. MATERIALS AND METHODS

In the absence of a crystal structure it is not possible to define the C-terminal end of domain I of dystrophin. Since proline residues tend to disrupt secondary structure, we have chosen to define the C-terminal boundary as residues 255–258 (Pro-Arg-Pro-Pro). Based on this, the constructs DMD233, DMD246 and DMD254 (containing the first 233, 246 and 254 residues of dystrophin, respectively) were synthesized and expressed in high yield (Fig. 1).

A clone, CF27, bp 410–2350 of the human dystrophin cDNA, in the *Bam*HI–*Eco*RI sites of pGEM3ZF+ was a generous gift from Drs. Kay Davies and Don Love (Molecular Genetics Group, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DU, UK). The missing DNA sequence corresponding to the first 66 amino acids of dystrophin was assembled from 5 oligonucleotides whose codon usage was optimised for *E. coli* expression. The resulting 207 bp fragment (DMD68) from the oligonucleotide annealing and ligation reaction has a 5′*Nde*I and a 3′*Bam*HI site. To ligate DMD68 to the 5′ end of CF27 it was necessary to re-engineer a *Bam*HI site that

**Present address:* The Whitehead Institute for Biomedical Research, Cambridge, MA 02142, USA.

***Present address:* Marie Curie Research Institute, the Chart, Oxted, Surrey RH8 0TL, UK.

Correspondence address: A.G. Weeds, MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK. Fax: (44) (223) 213556.

had been lost during the cloning of CF27 into pGEM3ZF+. A PCR reaction was used to generate a *Bam*HI site at the 5' end of CF27 and to insert a TAG stop codon and *Hin*dIII site after amino acid residue 254 in dystrophin. The product of this reaction was ligated simultaneously with DMD68 into the *Nde*I–*Hin*dIII sites of the pMW172 expression vector. Colonies containing the correct construct (DMD254) were identified by PCR and restriction digestion. An *Xba*I–*Hin*dIII fragment of DMD254 was cloned into M13mp19 to confirm the fidelity of the construct by sequencing. DMD246 and DMD233 were produced by the insertion of a stop codon after residues 246 and 233, respectively, by PCR using DMD254 as template. All DMD pMW172 constructs were sequenced in M13mp19 before transformation into BL21(DE3) and expression. Details of the cloning, expression and purification methods were based on those used for gelsolin mutants [17,18].

F-actin binding was assessed using sedimentation at 20°C in a Beckman Airfuge with 13.9 μM actin in 10 mM Tris–HCl, pH 8.0, 0.1 M NaCl, 1 mM $MgCl_2$, 0.1 mM ATP, 1.0 mM dithiothreitol and 3 mM NaN_3, with either 0.1 mM $CaCl_2$ or 0.2 mM EGTA as described previously [19]. Supernatant fractions were removed and the pellets redissolved in an equivalent volume of buffer for analysis on 15% SDS PAGE. Gels were scanned on a Molecular Dynamics computing densitometer. Control experiments using bovine serum albumin showed that 3.4% of the total albumin was trapped in the pellets. The bound dystrophin value was corrected for this level of trapping.

3. RESULTS AND DISCUSSION

DMD246 and DMD254 were purified from the soluble fraction on Whatman DE52 resin as described for Segment 1 of gelsonlin [17]. Gel filtration on S200 indicated that DMD246 was fully monomeric. DMD233 was expressed only in inclusion bodies. We could not analyze this construct, because it was not possible to obtain soluble material using procedures that had previously allowed gelsolin to be solubilized in a functional form [17,20].

Both DMD246 and DMD254 bound F-actin in a concentration-dependent manner, independent of calcium (Fig. 2). Binding was unaffected by pH in the range 6.5–8.0 and was specific for F-actin. There was no binding to G-actin nor any evidence for capping the barbed ends of filaments based on measurements of the critical concentration of actin in the presence or absence of 2 or 10 μM DMD254 (using pyrene-labelled actin [17] – data not shown). DMD246 shows maximal binding (1:1) at a 4–5-fold molar excess over actin (Fig. 3). Even at a 10-fold excess, we were unable to reach this stoichiometry with DMD254, showing that this larger construct bound more weakly.

Apparent dissociation constants were estimated based on the dystrophin concentration required to achieve 50% maximal binding. A value of ~44 μM was obtained for DMD246 (Fig. 3). This is about an order of magnitude higher than the K_d for the F-actin binding domain of filamin (K_d = 3 μM [21]). The actin-binding domain of α-actinin, which shows 62% amino acid sequence similarity with dystrophin [5], has also been expressed in *E. coli*, and binds F-actin with a K_d of 4 μM (Way et al. 1992, submitted), a value similar to that of the single actin-binding site of filamin. This gives us confidence that functional activity of these domains is preserved following expression in the bacterial system.

Fig. 1. Lanes 1–3 show a representative purification of DMD254. Lane 1, total cell protein; lane 2, soluble fraction; lane 3, DMD254 pooled fractions after chromatography on Whatman DE52. Lanes 4 and 5 show the supernatant and pellet fractions from control airfuge assays of DMD254 alone as described in Fig. 2 (there is negligible protein in the pellet).

It is evident from the sigmoidal nature of the binding plot that DMD246 associates with F-actin in a strongly co-operative manner. Using non-linear regression to fit these data with the Hill Equation (with Enzfitter, dis-

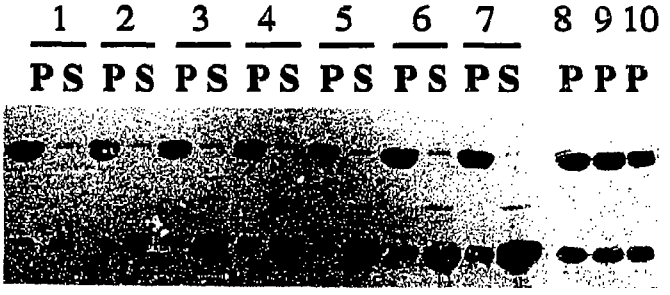

Fig. 2. Supernatant (S) and pellet (P) fractions after centrifugation of mixtures of DMD246 with F-actin. The upper band is actin and the lower, DMD246. Proteins were mixed at molar ratios of DMD246:actin as follows: 1 = 0.6, 2 = 0.9, 3 = 1.7, 4 = 2.6, 5 = 3.5, 6 = 4.3, 7 = 5.2, 8 = 6.0, 9 = 7.0, 10 = 7.8. Similar results were obtained in calcium.

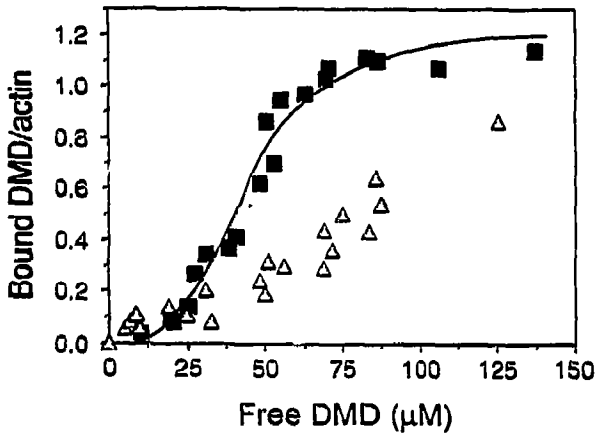

Fig. 3. Binding of DMD246 (■) and DMD254 (△) to F-actin in EGTA. Bound actin has been expressed as a molar ratio to F-actin subunits. Non-linear least squares fitting gives a Hill constant of 3.48.

tributed by Biosoft, Cambridge, UK) gave a Hill constant of 3.48 ± 0.15. Co-operativity of binding to F-actin has been noted previously for both tropomyosin [22] and α-actinin [23]. In the former case this is due to end-to-end association of the tropomyosin molecules and in the latter to bundling of the actin filaments. In the case of the dystrophin, the co-operativity suggests self-association, possibly induced by binding to actin filaments. As stated above, there was no evidence for self-association of DMD246 from gel filtration (at least at concentrations up to ~10 μM). Furthermore, the K_d of intact dystrophin may be even lower, if, as indicated from electron microscopy, distrophin dimerizes [15]. For example, the apparent K_d for dissociation of the α-actinin dimer from actin is 0.4 μM, 10-fold lower than our K_d for the actin-binding domain on its own. This shows the extent to which binding affinity is increased by cross-linking filaments. Finally, as in the case of the spectrin dimer whose affinity for actin is enhanced by several orders of magnitude in the presence of Band 4.1 [24], additional components that bind to dystrophin, such as the glycoprotein complex [9], may also affect interaction with actin.

Our results demonstrate conclusively that the putative F-actin binding site of dystrophin indeed binds F-actin*. Although dystrophin constitutes only 0.002% of total muscle protein [25], its concentration in the membrane cytoskeleton fraction is very much greater (about 5% [26]). Thus attachment of dystrophin to membrane sites in the sarcolemma increases its effective concentration over 1,000-fold to > 100 μM. This high affinity for membranes may account for the lack of any association of dystrophin with stress fibres when expressed in COS cells [27]. From this it is clear that the local concentrations of both dystrophin and actin are sufficiently great that high levels of binding are expected to occur in vivo.

*Qualitative demonstration of dystrophin binding to F-actin has recently been reported using a fusion protein containing the first 233 residues of human dystrophin and glutathione-S-transferase (Hemmings, L., Kuhlman, P.A. and Critchley, D.R., J. Cell Biol., in press).

Acknowledgements: The authors would like to thank Dr. D.R. Critchley for a preprint of his paper received when this work was being written up, and for helpful discussions.

REFERENCES

[1] Moser, H. (1984) Hum. Genet. 66, 17–40.
[2] Hoffman, E.P., Fischbeck, K., Brown, R.H., Johnson, M., Medori, R., Loike, J.D., Harris, J.B., Waterston, R., Brooke, M., Specht, L., Kupsky, W., Chamberlain, J., Caskey, C.T., Shapiro, F. and Kunkel, L.M.N. (1988) N. Engl. J. Med. 318, 1363–1368.
[3] Hammonds, R.G. (1987) Cell 51, 1.
[4] Davison, M.D. and Critchley, D. (1988) Cell 52, 159–160.
[5] Blanchard, A., Ohanion, V. and Critchley, D.R. (1989) J. Muscle Res. Cell Motil. 10, 280–289.
[6] Koenig, M., Monaco, A.P. and Kunkel, L.M. (1988) Cell 53, 219–228.
[7] Cross, R.A., Stewart, M. and Kendrick-Jones, J. (1990) FEBS Lett. 262, 87–92.
[8] England, S.B., Nicholson, L.V.B., Johnson, M.A., Forrest, S.M., Love, D.R., Zubrzycka-Gaarn, E.E., Bulman, D.E., Harris, J.B. and Davies, K.E. (1990) Nature 343, 180–182.
[9] Ervasti, J.M., Ohlendieck, K., Kahl, S.D., Gaver, M.G. and Campbell, K.P. (1990) Nature 345, 315–319.
[10] Mandel, J.L. (1989) Nature 339, 584–586.
[11] Monaco, A.P. and Kunkel, L.M. (1988) Advances in Human Genetics, vol. 17 (Harris, H. and Hirschorn, K., eds.) pp. 61–98, Plenum Press.
[12] Love, D.R. and Davies, K.E. (1989) Mol. Biol. Med. 6, 7–17.
[13] Bonilla, E., Samitt, C.E., Miranda, A.F., Hays, A.P., Salviati, G., DiMauro, S., Kunkel, L.M., Hoffman, E.P. and Rowland, L.P. (1988) Cell 54, 447–452.
[14] Sealock, R., Butler, M.H., Kramarcy, N.R., Gao, K.-X., Murname, A.A., Douville, D. and Froehner, S.C. (1991) J. Cell Biol. 113, 1133–1144.
[15] Pons, F., Augier, N., Heilig, R., Leger, J., Mornet, D. and Leger, J.J. (1990) Proc. Natl. Acad. Sci. USA 87, 7851–7855.
[16] Levine, B.A., Moir, A.J.G., Patchell, V.B. and Perry, S.V. (1990) FEBS Lett. 265, 159–162.
[17] Way, M., Pope, B., Gooch, J., Hawkins, M. and Weeds, A.G. (1990) EMBO J. 9, 4103–4109.
[18] Way, M., Pope, B. and Weeds, A.G. (1992) J. Cell Biol. (in press).
[19] Pope, B. and Weeds, A.G. (1986) Eur. J. Biochem. 161, 85–93.
[20] Way, M., Gooch, J., Pope, B. and Weeds, A.G. (1989) J. Cell Biol. 109, 593–605.
[21] Gorlin, J.B., Yamin, R., Egan, S., Stewart, M., Stossel, T.P., Kwiatkowski, D.J. and Hartwig, J.H. (1990) J. Cell Biol. 111, 1089–1105.
[22] Wegner, A. (1979) J. Mol. Biol. 131, 839–853.
[23] Meyer, R.K. and Aebi, U. (1990) J. Cell Biol. 110, 2013–2024.
[24] Ohanian, V., Wolfe, L.C., John, K.M., Pinder, J.C., Lux, S.E. and Gratzer, W.B. (1984) Biochemistry 23, 4416–4420.
[25] Hoffman, E.P., Brown, R.H. and Kunkel, L.M. (1987) Cell 51, 919–928.
[26] Ohlendieck, K. and Campbell, K.P. (1991) FEBS Lett. 283, 230–234.
[27] Lee, C.C., Pearlman, J.A., Chamberlain, J.S. and Caskey, C.T. (1990) Nature 349, 334–336.