Vol. 215, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 4, 1995 Pages 361-367

APO-DYSTROPHINS (DP140 AND DP71) AND DYSTROPHIN SPLICING ISOFORMS IN DEVELOPING BRAIN

G.E. Morris,*C. Simmons and Nguyen thi Man

MRIC Biotechnology Group, N.E. Wales Institute, Deeside, Clwyd, CH5 4BR, UK

Received August 25, 1995

PCR studies have shown that exons 71-74 are spliced out in most dystrophin mRNA transcripts in the brain. We have prepared new monoclonal antibodies against the syntrophin-binding region of dystrophin encoded by exons 73-74 and examined three protein products of the dystrophin gene in brain; the widely distributed Dp71, the recently discovered, brain-specific Dp140 and dystrophin itself. Exon 73-74 mAbs bound to all three proteins in brain and the extent of binding suggests that alternatively spliced dystrophins are less prominent at the protein level than predicted by PCR data. Dp140, unlike Dp71, was found to be present at much higher levels in foetal brain than in adult brain. If lack of functional Dp140 is the cause of the cognitive impairment in some Duchenne muscular dystrophy patients, this result suggests that the effects may occur early in development, which would reduce the options for therapeutic intervention. © 1995 Academic Press, Inc.

Dystrophin is the large, membrane-associated, cytoskeletal protein affected by mutations in the DMD gene in Duchenne and Becker muscular dystrophies (DMD and BMD) [1]. Dystrophin is found mainly in muscle, but utrophin, a closely-related protein from the DMDL gene [2, 3], and various short forms of dystrophin (apo-dystrophins: Dp71 [4, 5, 6], Dp116 [7], Dp140 [8] and Dp280 [9]) are found in a wide variety of tissues and cell lines and are also associated with membranes [10, 11]. An N-terminal domain interacts with actin filaments, while a C-terminal domain interacts with a transmembrane complex which also binds extracellular laminin; thus, dystrophin acts as a link between the cytoskeleton and the extracellular matrix [12, 13, 14]. In skeletal muscle, both utrophin and dystrophin are found at higher concentrations at postsynaptic neuromuscular junctions, where they may be involved in signal transduction [2, 15, 16].

In the brain, three full-length forms of dystrophin are produced from three different promoters and first exons and thus differ slightly at their N-termini [17]. They have different distributions in the brain, but some, at least, are associated with postsynaptic densities [18]. Dp71 is found in most tissues, including brain, but a brain-specific apo-dystrophin, Dp140,

*Correspondence to G.E. Morris, MRIC Biotechnology Group, N.E. Wales Institute, Deeside, Clwyd, CH5 4BR, UK. FAX: 1244-814305.E-mail: morrisge@newi.ac.uk.

0006-291X/95 $12.00
Copyright © 1995 by Academic Press, Inc.
All rights of reproduction in any form reserved.

has also been described recently [8]. The Dp140 promoter lies in a region of the dystrophin gene which is commonly deleted in MD patients. Effects of such deletions on Dp140 could account for the observation that about one-third of DMD patients have cognitive impairment [8]. Unlike Dp71, Dp140 includes a complete "hinge" region (hinge 4 [19]) containing a WW domain, which may be involved in cell signalling and/or interaction with other proteins [20, 21].

In addition to dystrophin isoforms produced from different promoters in the dystrophin gene, variants in the C-terminal domain produced by alternative splicing have also been identified, mainly by RT-PCR (reverse transcription-polymerase chain reaction) amplification of dystrophin mRNA [22, 23]. The nature of the alternative splicing varies between different tissues, but in brain mRNA, splicing out of exon 78 or exons 71-74 predominates [23]. The RT-PCR data do not show whether alternative splicing occurs in dystrophin mRNA itself or in one or other of the two apo-dystrophin mRNAs identified so far. Exon 78 splicing has, however, been shown to be characteristic of Dp71 and causes a frameshift which results in a novel hydrophobic C-terminus [4, 24]. Removal of exons 71-74 does not cause a frameshift but it would remove the binding site for syntrophin from the protein. Syntrophin, or 59DAP, is a 59kD dystrophin-associated protein, the function of which is still not known [25]. It binds to the part of dystrophin encoded by exon 74 or 73-74 within the alternatively-spliced region [26, 27]. Syntrophin binding appears not to be essential for dystrophin function in muscle, since dystrophic mdx mice have their muscle defects corrected by transgenic expression of a dystrophin lacking exons 71-74, but brain has not yet been studied [28].

In order to study alternative splicing at the protein level, we have expressed a recombinant dystrophin fragment encoded by exons 71-74 and prepared monoclonal antibodies (mAbs) against it. We have mapped the mAbs to the syntrophin-binding region encoded by exons 73-74 and used them to show that this region is well-represented in all three brain products of the dystrophin gene separable on Western blots (full-length, Dp140 and Dp71). Furthermore, we have shown that Dp140 is much more abundant in foetal brain than in adult brain, a finding that may have implications for the possibility of therapy for the cognitive problems in muscular dystrophy.

MATERIALS AND METHODS

A dystrophin cDNA fragment encompassing exons 71-74 and part of exon 75 was produced by PCR of human dystrophin cDNA as previously described [29]. The primers were: exon 71 forward: 5'-ggggatccgttactctgatcaacttc and exon 74 reverse: 5'-cgccatgggcatcattcaggagg. PCR products of the appropriate size were cloned into the pT7Blue (Novagen Inc., Madison, WI, USA) T-tailed vector and subcloned into pMW172 (derived from the pET series [30]) after digestion with *BamH*I and *Stu*I (the *BamH*I site is in the PCR primer and the *Stu*I site is in the dystrophin cDNA). A sub-fragment containing exons 73 and 74 was obtained by PCR of this plasmid construct using primers: exon 73 forward: 5'-ggggatccgctagcagaaatggaa, exon 74 reverse: 5'-ggaaggcctgttttcttcctcaag. This was cloned directly into pET17b (Novagen) after digestion with *BamH*I and *Stu*I. The constructs were electroporated into *E. Coli.* BL21(DE3) and inducible clones expressing protein of appropriate size were selected. Identity of the dystrophin fragment and absence of PCR errors was confirmed by sequencing the pMW172 insert. Immunization of Balb/c mice and production of a mAb panel was performed as previously described [31].

Total protein extracts for SDS-PAGE were prepared from post-mortem brain samples (stored at -80°C) by homogenisation in 4 volumes of boiling SDS sample buffer [32]. Extracts were further dispersed by sonication before centrifugation at 10,000g for 5 min. The supernatants were centrifuged again at 100,000g for 20 min. SDS-PAGE on 3-12.5% gradient gels and Western blotting have been described elsewhere [2].

RESULTS

Two dystrophin cDNA subfragments encompassing exons 71-74 and exons 73-74 were generated by PCR and cloned into pET series vectors [30] for expression in E. Coli. A panel of 21 mAbs was generated using the proteins produced from exons 71-74 as immunogen. All 21 mAbs also recognised the smaller protein produced from exons 73-74 on Western blots. Four of the 21 mAbs cross-reacted with utrophin as defined by Western blotting of HeLa cell extracts, which contain utrophin but no dystrophin [10].

Fig.1a shows a dramatic decrease during human development in levels of the recently-discovered brain apo-dystrophin, Dp140 [8]. A faint dystrophin band is also detected in adult

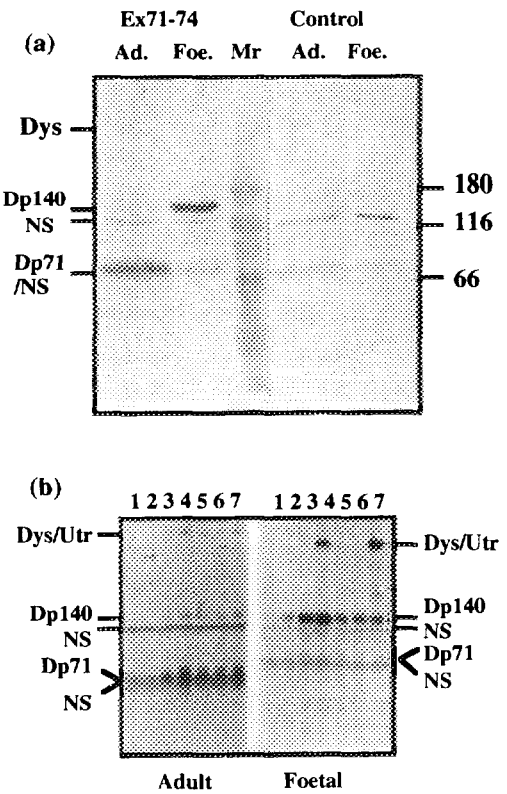

Figure 1. Comparison of three forms of dystrophin in foetal and adult brain.
Post-mortem tissues were from a 60-year-old adult and a 3.5 month foetus. Prestained Mr markers were from Sigma. (a) The control blot (without primary mAb) identifies two non-specific (NS) bands which are also present on the blot developed with dystrophin-specific exon 73-74 mAb to reveal dystrophin (Dys), Dp140 and Dp71. (b) Strip blots show that several different exon 73-74 mAbs detect the same dystrophin bands. Two of the mAbs also recognise utrophin which comigrates with dystrophin (Dys/Utr). Lane 1 is a control (no primary mAb) and lane 2 is a rather weak mAb which detects only the strongest band (Dp140 in foetal brain).

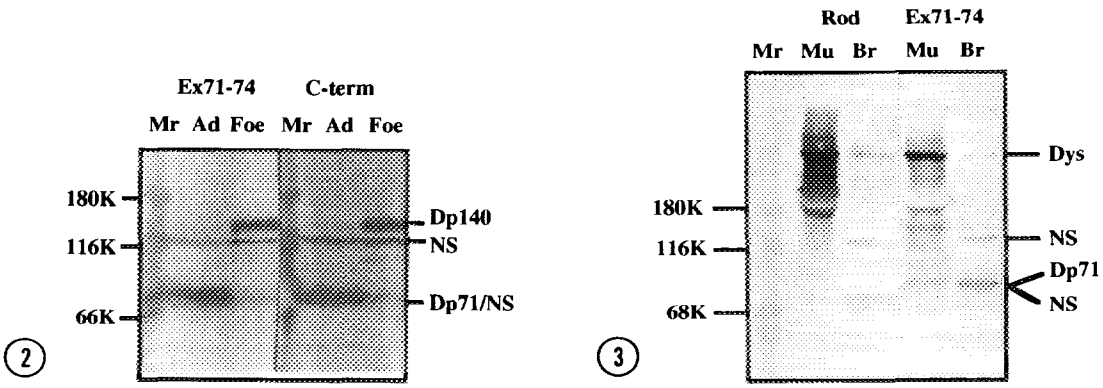

Figure 2. Developmental changes in Dp140 and Dp71 are detected by both a mAb against the syntrophin-binding region and a mAb which binds closer to the dystrophin C-terminus (MANDRA1).

Figure 3. Detection of dystrophin in adult human muscle and brain extracts by mAbs against the central helical rod domain (MANDYS1) and the syntrophin-binding region (ex 73-74). The muscle (Mu) lanes were overloaded in order to detect the low levels of full-length dystrophin in brain (Br), while maintaining equal amounts of total protein in all lanes.

human brain by exon 73-74 mAbs. In contrast to the decrease in Dp140, increased Dp71 levels are seen in adult brain (Fig. 1a). There are also two bands in brain (ca. 75kD and 125kD) produced by a non-specific (NS) reaction of the second antibody detection system and the smaller one almost co-migrates with Dp71. The distinction between Dp71 and the non-specific 75kD band can be seen more clearly on strip blots (Fig. 1b). Dp71 staining occurs within the lanes only, whereas non-specific staining is seen both within and between the lanes. Fig.1b also shows more clearly the large differences in Dp140 and Dp71 expression between adult and foetal brain and the fact that these differences are detected by several different dystrophin mAbs. Two of the exon 73-74 mAbs in Fig.1b cross-react with utrophin (lanes 4 and 7), so a clear decrease in utrophin during development can be seen, as well as an increase in dystrophin (lanes 5 and 6; see also Fig.1a). The absence of utrophin degradation products also shows that there has been little proteolysis of dystrophin/utrophin-like proteins in either extract. Equal amounts of total protein were loaded in each lane.

Fig.2 shows that very similar results were obtained when an exon 73-74 mAb was compared with a C-terminal dystrophin mAb (MANDRA1; exons 76-79 [31]). This shows that at least some Dp140 and Dp71 molecules contain the syntrophin-binding site in both foetal and adult brain. We cannot conclude that all apo-dystrophin molecules have the syntrophin binding site, however, because mAbs vary in their intensity of staining (cf. Fig.1b). This is a problem that arises only when comparing two different mAbs and not when comparing two different tissues with the same mAb as in Fig. 1a.

Fig.3 shows a Western blot of muscle and brain extracts developed with mAbs against either the dystrophin rod domain or the exon 73-74 encoded, syntrophin binding region. Dystrophin in brain is detected both by the rod domain mAb, MANDYS1, and by the dystrophin-specific exon 73-74 mAb, whereas if most brain dystrophin lacked exons 71-74,

staining by MANDYS1 would be much stronger. The MANDYS1 staining of brain dystrophin is slightly stronger, but MANDYS1 also appears to have a higher avidity for skeletal muscle dystrophin than the exon 73-74 mAb (Fig.3). Quantitation by microdensitometry of the photographic negative gave values for the ratio of dystrophin in brain to dystrophin in muscle of 0.088 for MANDYS1 and 0.066 for the exon 73-74 mAb, only 25% lower. Similar results were obtained with mouse extracts (not shown). Dp71 was detected by the mAb against the exon 73-74 region but not by the rod domain mAb (exons 31/32) because the Dp71 promoter in the dystrophin gene is 3' to these exons (Fig.3). The two mAbs used in Fig. 2 were also used to stain dystrophin in skeletal muscle (in which alternative splicing of exons 71-74 does not occur) and, in this case, MANDRA1 was slightly weaker by 25 to 50%. Taken together, the results suggest that splicing out of exons 71-74, if it is manifested at all at the protein level, is found in less than 50% of all dystrophin protein molecules and may occur in all three forms of dystrophin.

DISCUSSION

Changes in Dp140 during brain development have not been described previously and the decrease we have found is at least as dramatic, if not even greater, than the decrease in utrophin levels which occurs over the same period (Fig.1b and [33]). The Dp140 sequence begins with exon 51 of dystrophin but the promoter and first exon are found in intron 44 [8]. A minority of Duchenne muscular dystrophy patients show cognitive impairment and correlations have been made with genetic deletions in the exon 40-52 region [8]. These deletions would particularly affect synthesis or function of Dp140, which may therefore have a role in preventing cognitive impairment [8]. If, as seems likely from our results, Dp140 functions early in development, prospects for therapeutic intervention must be more remote. The full-length dystrophin gene and primary RNA transcript may be too large for expression in dividing cells and tissues [34], so some of the functions of dystrophin may have to be performed in foetal tissues by utrophin or Dp140. Dystrophin and Dp71 levels were higher in adult brain; in our earlier work, Northern blot data suggested a decrease in Dp71 mRNA levels over the same developmental period [5] but Blake et al [6] also found higher Dp71 protein levels in adult tissues. Splicing out of exon 78, a characteristic of Dp71 [4], also decreases during development [23], supporting the Northern blot data. There is no obvious explanation for this discrepancy, unless Dp71 protein is turned over more rapidly in foetal tissues. The changes in Dp140 and Dp71 observed with exon 73-74 mAbs are not due to splicing differences between adult and foetal brain because the same results were obtained with a mAb which binds outside the spliced region of dystrophin (Fig.2).

PCR studies predict a mixture of alternatively-spliced "dystrophin" RNAs in brain, some lacking exons 71-74 while others are intact or lack exon 78 only [22, 23]. It has not been established whether these "deletions" occur in dystrophin, apo-dystrophins or both, although exon 78 is known to be absent from Dp71 [4]. In theory, it should be possible to detect the

partial absence of exons 71-74 using mAbs against this region, provided the protein products of alternate splicing are present in excess over "normal" proteins. Bies et al [23] found little or no unspliced dystrophin mRNA in human brain and the major mRNA species lacked exons 71-74. A similar pattern was seen in mouse brain, except that a band of unspliced dystrophin mRNA was also present. If the ratio of these mRNA species were reflected in the ratio of dystrophin protein products, we would expect our exon 73-74 mAbs to detect much less of these than other dystrophin mAbs (mAbs with different avidities for the same antigen will produce bands of different intensity on Western blots, but we took this account by comparing their binding to skeletal muscle dystrophin.).

Our general conclusion is that we did not find the large differences in mAb binding that we expected from the PCR data; we could clearly detect exon 73-74-encoded protein in full-length dystrophin and in the short forms, Dp71 and Dp140, but alternatively spliced proteins might be present at levels of 25-50% of the total. Furthermore, mouse brain extracts gave very similar results to human brain (results not shown) in spite of species differences in the PCR data [23]. One possible explanation for our results is that dystrophin lacking the syntrophin-binding region could be less stable and turned over more rapidly than "normal" dystrophin. Another example of decreased protein levels due to protein instability, rather than lack of mRNA, is the loss of dystroglycan from the muscle membranes of mdx mice and DMD patients [35, 36]. In this case, dystroglycan mRNA levels are unchanged and the absence of dystrophin in mdx and DMD is thought to lead to more rapid turnover of dystroglycan [35]. Rafael et al [28] showed that dystrophin lacking exons 71-74 could be over-expressed in a transgenic mouse and that it brought normal muscle function back to mdx mice, which lack dystrophin. Levels of this internally-deleted dystrophin and levels of membrane dystrophin-associated proteins (dystroglycan) were even higher than in normal mice. This does not suggest that the spliced dystrophin is particularly unstable in muscle, though differential stability cannot be ruled out since a direct comparison with full-length, over-expressed dystrophin was not made. Also the brain was not studied in this transgenic mouse, nor in mice over-expressing Dp71 [37, 38].

To summarise, we have detected exon 73-74-encoded sequences in all three forms of dystrophin in brain (full-length, Dp71 and Dp140) and such unspliced forms of dystrophin appear to be more abundant than the PCR data on mRNA predicted. Whether alternatively-spliced forms are concentrated in particular areas of the brain remains to be investigated by immunolocalisation studies. Unfortunately, mAbs cannot detect the internally-deleted protein directly, but only as a reduction in mAb staining intensity. Production of a mAb which specifically recognises the "abnormal" exon 70/75 junction in the deleted protein would solve this problem, but such an Ab may be technically difficult to produce.

ACKNOWLEDGMENTS

We thank the Muscular Dystrophy Group of Great Britain and Northern Ireland and HEFC(Wales) DevR for grant support and Marian James for microdensitometry.

REFERENCES

1. Hoffman E.P., Brown R.H. & Kunkel L. M. (1987) Cell 51, 919-928.
2. Nguyen thi Man, Ellis, J. M., Love, D. R., Davies, K. E., Gatter, K. C., Dickson, G. & Morris, G. E. (1991) J. Cell Biol. 115, 1695-1700.
3. Love, D.R., Hill, D.F., Dickson, G., Spurr, N.K., Byth, B.C., Marsden, R.F., Walsh, F.S., Edwards, Y.H. & Davies, K.E. (1989) Nature 339, 55-58.
4. Lederfein, D., Levy, Z., Augier, N., Mornet, D., Morris, G.E., Fuchs, O., Yaffe, D. & Nudel, U. (1992) Proc. Natl. Acad. Sci. USA 89, 5346-5350.
5. Hugnot, J. P., Gilgenkrantz, H., Vincent, N., Chafey, P., Morris, G. E., Monaco, A. P., Berwaldnetter, Y., Koulakoff, A., Kaplan, J. C., Kahn, A. & Chelly, J. (1992) Proc. Natl. Acad. Sci. USA 89, 7506-7510.
6. Blake, D. J., Love, D. R., Tinsley, J., Morris, G. E., Turley, H., Gatter, K., Dickson, G., Edwards, Y. H. & Davies, K. E. (1992) Hum. Molec. Genet. 1, 103-109.
7. Byers, T. J., Lidov, H. G. W. & Kunkel, L. M. (1993) Nature Genet. 4, 77-81.
8. Lidov, H. G. W., Selig, S. & Kunkel, L. M. (1995) Hum. Molec. Genet. 4, 329-335.
9. D'Souza, V. N., Nguyen thi Man, Morris, G. E., Karges, W., Pillers, D. & Ray, P. N. (1995) Hum. Molec. Genet. 4, 837-842.
10. Nguyen thi Man, Le Thiet Thanh, Blake, D. J., Davies, K. E. & Morris, G. E. (1992) FEBS Lett. 313, 19-22.
11. Rapaport, D., Greenberg, D. S., Tal, M., Yaffe, D. & Nudel, U. (1993) FEBS Lett. 328, 197-202.
12. Ervasti, J. M. & Campbell, K. P. (1991) Cell 66, 1121-1131.
13. Suzuki, A., Yoshida, M., Hayashi, K., Mizuno, Y., Hagiwara, Y. & Ozawa, E. (1994) Eur. J. Biochem. 220, 283-292.
14. Ahn, A. H. & Kunkel, L. M. (1993) Nature Genet. 3, 283-291.
15. Bowe, M. A., Deyst, K. A., Leszyk, J. D. & Fallon, J. R. (1994) Neuron 12, 1173-1180.
16. Gee, S. H., Blacher, R. W., Douville, P. J., Provost, P. R., Yurchenco, P. D. & Carbonetto, S. (1993) J.Biol. Chem. 268, 14972-14980.
17. Gorecki, D. C., Monaco, A. P., Derry, M. J., Walker, A. P., Barnard, E. A. & Barnard, P. J. (1992) Human Molec. Gen. 1, 505-510.
18. Lidov, H.G.W., Byers, T.J., Watkins, S.C. & Kunkel, L.M. (1990) Nature 348, 725-727.
19. Koenig, M. & Kunkel, L.M. (1990) J. Biol. Chem. 265, 4560-4566.
20. Hofmann, K. & Bucher, P. (1995) FEBS Lett. 358, 153-157.
21. Bork, P. & Sudol, M. (1994) Trends Biochem. Sci. 19, 531-533.
22. Feener, C.A., Koenig, M. & Kunkel, L.M. (1989) Nature 338, 509-511.
23. Bies, R. D., Phelps, S. F., Cortez, M. D., Roberts, R., Caskey, C. T. & Chamberlain, J. S. (1992) Nucleic Acids Res. 20, 1725-1731.
24. Fabbrizio, E., Nudel, U., Hugon, G., Robert, A., Pons, F. & Mornet, D. (1994) Biochem. J. 299, 359-365.
25. Kramarcy, N. R., Vidal, A., Froehner, S. C. & Sealock, R. (1994) J. Biol. Chem. 269, 2870-2876.
26. Suzuki, A., Yoshida, M. & Ozawa, E. (1995) J. Cell Biol. 128, 373-381.
27. Ahn, A. H. & Kunkel, L. M. (1995) J. Cell Biol. 128, 363-371.
28. Rafael, J. A., Sunada, Y., Cole, N. M., Campbell, K. P., Faulkner, J. A. & Chamberlain, J. S. (1994) Hum. Molec. Genet. 3, 1725-1733.
29. Le Thiet Thanh (1995) Amer. J. Hum. Genet. 56, 725-731.
30. Way, M., Pope, B., Cross, R. A., Kendrick-Jones, J. & Weeds, A. G. (1992) FEBS Lett. 301, 243-245.
31. Nguyen thi Man, Ginjaar, I. B., van Ommen, G. J. B. & Morris, G. E. (1992) Biochem. J. 288, 663-668.
32. Nguyen thi Man, Cartwright A.J., Morris G.E., Love D.R., Bloomfield J.R. & Davies K.E. (1990) FEBS Lett 262, 237-240.
33. Clerk, A., Morris, G. E., Dubowitz, V., Davies, K. E. & Sewry, C. A. (1993) Histochem. J. 25, 554-561.
34. Tennyson, C. N., Klamut, H. J. & Worton, R. G. (1995) Nature Genet. 9, 184-190.
35. Ibraghimov-Beskrovnaya, O., Ervasti, J. M., Leveille, C. J., Slaughter, C. A., Sernett, S. W. & Campbell, K. P. (1992) Nature 355, 696-702.
36. Helliwell, T. R., Nguyen thi Man & Morris, G. E. (1994) Neuromusc. Disord. 4, 101-113.
37. Greenberg, D. S., Sunada, Y., Campbell, K. P., Yaffe, D. & Nudel, U. (1994) Nature Genet. 8, 340-344.
38. Cox, G. A., Sunada, Y., Campbell, K. P. & Chamberlain, J. S. (1994) Nature Genet. 8, 333-338.