An Epitope Structure for the C-Terminal Domain of Dystrophin and Utrophin†

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ABSTRACT: The muscular dystrophy protein, dystrophin, and the closely related protein, utrophin, are large cytoskeletal proteins which link actin microfilaments to the plasma membrane. A panel of 38 monoclonal antibodies (mAbs) has been produced against the C-terminal domains of dystrophin and utrophin. This domain interacts with both dystrobrevins, via their "leucine zipper" coiled-coil helices, and syntrophins, adaptor proteins which also interact with nitric oxide synthetase and transmembrane sodium channels. The amino acid sequences recognized by the mAbs have now been identified using a variety of epitope mapping techniques, including fragmentation by transposon mutagenesis, synthetic peptides, phage-displayed peptide libraries, and mutant dystrophins expressed in transgenic mice. In addition to defining antibody recognition sites, mapping was sufficiently precise to provide structural information, since individual amino acids accessible on the surface of the native protein were identified in many cases. In two regions of the domain, short linear epitopes were found in proline-rich sequences which may form surface loops, turns, or linkers, but these were separated by a third region which contained mainly conformational epitopes. The results are consistent with a loose and flexible structure for much of the C-terminal domain, especially around the highly conserved second leucine zipper or coiled-coil helix (CC-H2), but there is evidence for denaturation-resistant tertiary structure in the syntrophin-binding region and the first coiled-coil helix (CC-H1).

The epitopes on protein antigens which are recognized by monoclonal antibodies (mAbs) can be identified by a variety of epitope mapping techniques (*1*). In addition to defining antibody recognition sites, epitope mapping can also provide information about protein structure since epitopes must be accessible to an antibody on the surface of the protein and not buried in the protein core. Proteolysis, which also depends on the accessibility of specific sites in the target protein, has been widely used to identify domains, linkers, and surface loops (*2*, *3*). However, compared with proteolysis methods, mAbs have seldom been used as structural probes. The main reason for this is that few panels of mAbs against different regions of the same protein exist and even fewer have been mapped with the required degree of precision, since epitope mapping can be a difficult and lengthy process.

We have prepared and mapped several large panels of mAbs against dystrophin, the protein defective in muscular dystrophy. These mapped mAbs were prepared primarily to study alternative forms of the proteins produced by genetic deletions (*4*), alternative promoters (*5*), and alternative splicing (*6*). These panels of mAbs were also used in combination with proteolysis to study the structure of dystrophin and its interactions in vivo (*7*). The latter studies supported the hinged rod structure proposed by Koenig and Kunkel (*8*), but not the antiparallel dimer model they suggested by analogy with spectrin. Recent studies with recombinant dystrophin in vitro also failed to detect dimers of any kind (*9*). The accepted model for the dystrophin (and utrophin) monomer is a central helical coiled-coil rod separating an actin-binding N-terminal domain from a cysteine-rich domain required for attachment to the membrane. Two sequence motifs have been identified in the cysteine-rich region, a WW domain (*10*) which interacts with the transmembrane protein, β-dystroglycan, and a zinc finger, ZZ domain (*11*). In the final C-terminal domain, a pair of coiled-coil helices likely to be involved in protein−protein interactions have been identified from the sequence data (*12*−*14*). Recent evidence suggests that these helices interact with equivalent helices on dystrobrevins (*15*, *16*). This C-terminal domain also interacts with the 59 kDa protein syntrophin (*17*−*19*) which, in turn, associates with nNOS (neuronal nitric oxide synthetase) (*20*) and sodium channels (*21*). The C-terminal sequence is very highly conserved between dystrophin and the dystrophin-related protein, utrophin, as well as between species. One aim of our present study was to characterize this C-terminal domain further by using mAbs as probes of surface structural features. This

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approach provides structural information which may be useful until the three-dimensional structures are determined by conventional means.

Two panels of antibodies were used, comprising 19 raised against dystrophin (*22*) and 19 against utrophin (*23*). Two important constraints were imposed on the epitopes recognized by the mAbs in this study. First, partially denatured, recombinant protein fragments after urea treatment were used as immunogens. Second, mAbs were selected which recognize both native dystrophin and utrophin (in immunofluorescence microscopy of frozen, unfixed muscle sections) and the partially unfolded proteins (on Western blots after SDS−PAGE). These constraints were imposed to limit the contribution of conformational epitopes, because such epitopes are usually difficult to map precisely, except by solving the crystal structure of antibody−antigen complexes. Hence, under these conditions, linear or sequential epitopes which remain flexible in the native protein would be selected and these can be mapped using fragmentation methods or synthetic peptides. Paradoxically, highly conformational epitopes which are resistant to denaturation and reform readily after urea or SDS treatment would also be selected (*24*). These may be associated with the folding "core" of a protein, or protein domain, which is often formed by hydrophobic interactions between rudimentary secondary structures in a folding intermediate.

EXPERIMENTAL PROCEDURES

Antibodies. We have described elsewhere panels of 19 mAbs against the last 329 amino acids of utrophin (MANCHO1−19) (*23*) and 19 mAbs against the last 500 amino acids of dystrophin (MANDRA1−19) (*22*). Some of these mAbs have been widely used in studies of muscular dystrophy and dystrophin and utrophin function, notably, MANDRA1 (*5*, *25*), MANCHO7 (*26*, *27*), and MANCHO3 (*28*).

Transposon Mutagenesis. This method of producing random truncations of a recombinant protein expressed from almost any plasmid vector in *Escherichia coli* has been described in detail elsewhere (*29*, *30*). The plasmid constructs used in this study were in pEX2 [utrophin; produced by D. R. Love and K. E. Davies, University of Oxford (*23*)] and pATH2 [dystrophin; produced by I. B. Ginjaar and G.-J. van Ommen, University of Leiden (*22*)]. An improved protocol, with a simpler transposition method using TnXR instead of Tn*1000*, has also been developed, but after the present studies were performed (*31*). The necessary bacterial strains are available from S. G. Sedgwick. Effective use of the method depends on selecting bacterial clones which react with some mAbs but not others; it is therefore best applied to a panel of several mAbs against different sites on the same protein.

Transgenic Mice. Transgenic *mdx* mice overexpressing full-length mouse dystrophin with deletions of either exons 71−74 or exons 75−78 have been described previously (*32*, *33*). The amino acids deleted are del71−74, P3402−R3511 in mouse dystrophin (equivalent to P3409−R3518 in the human sequence), and del75−78, G3528−R3675 in mice (equivalent to G3535−R3682 in humans). The first 16 amino acids of exon 75 are still present in the exon 75−78 deletion.

Peptide Methods. Synthetic peptides were obtained from Alta Bioscience (University of Birmingham, Birmingham, U.K.) or were synthesized on membranes using a SPOTS kit according to the supplier's instructions (Genosys, Cambridge, U.K.). Phage-displayed 6-mer and 15-mer peptide libraries in the pIII protein of fuse5 filamentous phage were a generous gift from G. P. Smith (University of Missouri, Columbia, MO) and were screened as described previously (*34*, *35*).

RESULTS

An epitope map of 19 mAbs raised against dystrophin (MANDRA1−19) and 19 mAbs raised against utrophin (MANCHO1−19) was produced by transposon mutagenesis (*29*, *31*). Transposon mutagenesis with Tn*1000* results in the random insertion of early stop codons into a cDNA sequence expressed as a recombinant protein in *E. coli*. The resulting library of randomly shortened antigen molecules is then screened with a panel of mAbs. Clones which discriminate between mAbs are selected for identification of the expressed sequence by DNA sequencing with Tn*1000* primers. A pEX2 plasmid expressing the last 329 amino acids of utrophin (*23*) and a pATH2 plasmid expressing the last 500 amino acids of dystrophin (*22*) were mutated with Tn*1000* in this way. The dystrophin clones were prescreened by *Pst*I digestion of plasmid mini-preps which give a 1.5 kb product in the nonmutated plasmid. The absence of this product in about 15% clones indicated transposon insertion into the dystrophin sequence. This is close to the theoretical value for a 500 bp target in a 3779 bp plasmid. Because of this prescreening, a high proportion (25/68) of the clones showed differential mAb reactivity. For many mAbs, a simple colony blot of the induced bacterial clone was sufficient to determine mAb specificity (data not shown), but in some cases, a Western blot after SDS−PAGE was necessary to eliminate ambiguity (Figure 1). Thirteen useful utrophin clones were also obtained by screening directly with mAbs. The results of DNA sequencing of all the transposed clones with differential mAb reactivity are shown in Table 1. Eleven mAbs which recognize both dystrophin and utrophin were tested with both sets of bacterial clones. Two mAbs, MANDRA2 and -9, were omitted from further study because they reacted too weakly to give consistent results.

The data from Table 1 were used to construct an epitope map (Figure 2). For each mAb, binding is lost when the antigen is shortened from the small arrow to the large arrow in Figure 2. For sequential epitopes, all or part of the epitope will be found in the sequence between the two arrows. This sequence is necessary, but may not be sufficient, for binding. Conformational epitopes may lie closer to the N terminus than a naive interpretation of the data might suggest since removal of C-terminal amino acids could cause conformational changes in the remaining protein. We therefore attempted to confirm and refine sequential epitope locations using synthetic peptides, overlapping peptide arrays synthesized as "SPOTS", or phage-displayed random peptide libraries. MANDRA1 is known to recognize a Dp71 isoform of dystrophin in which exons 78 and 79 are removed by alternative splicing (*5*), and this was used to place the epitope at exon 77 in Figure 2. The failure of MANDRA1 to recognize either human utrophin or *Torpedo* dystrophin suggests that Arg3771 may be involved. There is a four-

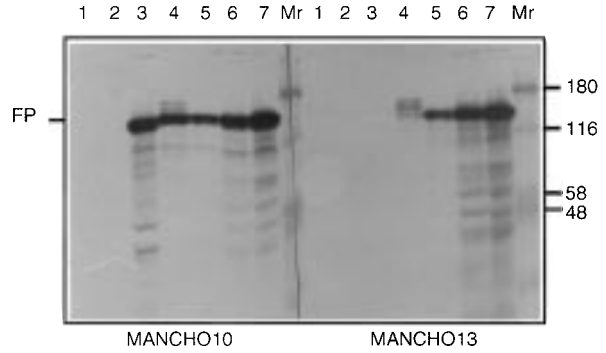

FIGURE 1: Mapping of two utrophin mAbs by transposon mutagenesis. A pEX2 construct in *E. coli* strain POP2136 expressing a fusion protein of β-galactosidase with the last 329 amino acids of utrophin was randomly transposed with Tn*1000*. Clones showing differential mAb reactivity on colony lift blots after induction by elevated temperature (42 °C) were selected, and induced cell pellets were prepared for SDS−PAGE (7% acrylamide) by boiling in SDS extraction buffer (*22*). After transfer to nitrocellulose by diffusion for 48 h, blots were developed with MANCHO10 or MANCHO13 mAbs. Diffusion blotting was used when screening several mAbs because two blots can be produced, one from each side of the gel. The clones shown are identified in Table 1b: lane 1, clone 27 (shortest fusion protein); lane 2, clone 28; lane 3, clone 29; lane 4, clone 30; lane 5, clone 31; lane 6, an unsequenced clone (not in Table 1); and lane 7, clone 32 (longest protein). There is little apparent difference in fusion protein size because transposition points are only 48 amino acids apart for lanes 3 and 7 (M_r range of 125−131 kDa). In lane Mr are prestained size markers (Sigma). FP represents the fusion protein.

amino acid deletion in the utrophin homologue at this site. Deletions in homologous proteins cannot generally be accommodated within the backbone structure and are therefore usually found in surface loops, or possibly a "loose tail" in the present case.

Table 2 lists five commercially prepared synthetic screening peptides based on the utrophin sequence. The epitopes for MANCHO19 and MANCHO10 were confirmed by their binding to 16-mer peptides 1 and 2, respectively, and these are underlined in the utrophin sequence (Figure 2). The 50% sequence difference between utrophin and dystrophin in peptide 2 explains why MANCHO10 is a utrophin-specific mAb, whereas MANCHO19 recognizes both proteins. The other three peptides in Table 2 also correspond to epitope regions predicted by transposon mutagenesis, but the expected antibodies did not bind, suggesting either that insufficient sequence is present or that the epitope(s) is conformational. Peptide 4 lies within a strongly predicted helical region consistent with the latter explanation.

A further means of defining epitopes uses mixtures of mAbs to select peptides from phage-displayed peptide libraries (*35*). Phage clones which were selected by each mAb mixture were then screened with individual mAbs as illustrated in Figure 3. This shows that MANDRA18 recognized one phage clone out of 40 after two rounds of biopanning of the 6-mer peptide library and five clones after the third round of panning. All six clones had the same sequence, (P)DLLSP, which is matched with the dystrophin sequence in Figure 4 and also shown in bold type in Figure 2. The result is consistent with transposon mutagenesis data, and MANDRA4 and -19 recognized the same phage clones (data not shown). Evidently, the last proline in the epitope sequence is essential since the transposon mutant A69 ending in DLLS did not bind these three mAbs (Table 1). SPOTS

Table 1: Truncations of Dystrophin and Utrophin by Transposon Insertion and the Effect on mAb Binding[a]

Clone No.	DNA sequence at Tn1000 insertion	Last amino-acid of truncated protein	mAbs bound
a) Dystrophin.			
1	5'(ccc c)tt ttc cat ttc3' **E M E**...	E3446	MANCHO11-14, 19
2	5'(c ccc) cat tac tgc 3' **H Y C**...	H3474	
3	5'(c ccc) gac tcc ccc 3' **D S P**...	D3482	
4	3' (cc cc)g act acg att5' 5' cct cgt agt c(gg 3' ....P R **S**	S3490	
5	5'(cc cc)t agt cct gca 3' **S P A**...	S3490	
6, 7	5'(ccc c)at ctt gag gaa3' **L E E**...	L3513	
8	3'(ccc c)ta ttc tgc ttg 5' 5'caa gca gaa ta(g3' ...Q A **E**	E3523	MANDRA17
9	5' (c ccc) tta cac agg 3' **L H R**...	L3590	MANCHO15, 17 MANDRA6-8,10-16
10	5'(ccc c)aa ccc cag gcx gag3' **P Q A E**...	P3600	MANDRA3
11	5'(ccc c)ag gca gag gcc 3' **A E A**...	A3602	
12-15	5'(cc cc)t cct tct acc3' **P S T**...	P3614	MANCHO18
16	5'(cc cc)t tct acc tct3' **S T S**...	S3615	
17	3'(cc cc)a agt ttg act 5' 5' agt caa act t(gg 3' ....S Q **T**	T3634	
18	3'(c ccc) atc ttc ctc 5' 5' gag gaa gat 3' ....E E **D**	D3644	MANDRA5
19	3'(cc cc)g act gag aag 5' 5' ctt ctc agt c(gg 3' ...L L **S**	S3647	
20	5'(c ccc) gac aca agc 3' **D T S**..	D3651	MANDRA4,18,19
21, 22	3'(ccc c)tt gag ttg ctc5' 5'gag caa ctc aa(g 3' ...E Q **L**	L3663	
23	3'(ccc c)tt gtt gag ttg 5' caa ctc aac aa(g 3' ....Q L **N**	N3664	
24	5'(c ccc) ttc cct agt 3' **F P S**....	F3667	
25	5' actacatgt 3'	3' UTR	MANDRA1
b) Utrophin.			
26	3'(cc cc)a act tgt tct taa 5' ctt aag aac aag tt(g ....L K N **K**	K3136[3379][b]	None
27	3'(cc cc)c tgt ctg gac agg 5' cct gtc cag aca g(gg ....P V Q **T**	T3156[3399]	MANCHO19
28	3'(ccc c)gt ctc taa gtt gtc 5' gac aac tta gag ac(g ....D N L **E**	E3164[3407]	
29	5'(cccc) ct caa ctg ttt **Q L F**...	Q3184[3427]	MANCHO10
30	3'(cccc)ttgaatgggtgtc 5' gac acc cat tca a(gg ....D T H S **R**	R3193[3436]	
31	5'(cccc) act aat ggg tct **T N G S**...	T3207[3450]	MANCHO11-14
32	5'(cccc) ag tat tgc caa **Y C Q**...	Y3232[3475]	
33	3'(c ccc) ttc ttc ctc cag 5' ctg gag gaa gaa (ggg ....L E E **E**	E3273[3516]	MANCHO1-6
34	3'(ccc c) ag ttc tga atc ctc 5' gag gat tca gaa ct(g ....E D S E **L**	L3314[3558]	MANCHO7-9 MANDRA17
35	5'(cccc) tc agg cag cac aaa **R Q H K**..	R3322[3565]	
36	5'(cccc) aa gat cac aat aaa **D H N K**..	D3337[3578]	
37	3'(ccc c)tt att gtg atc ttc 5' gaa gat cac aat aa(g ....E D H N **K**	K3340[3581]	
38	3'(cccc)ctg tcg gag gcg gtg 5' cac cgc ctc cga cag (ggg ....H R L R **Q**	Q3351[3595]	MANCHO15-17 MANDRA16
INTACT		M3433[3684]	MANCHO18

[a] The position of insertion of Tn*1000* was determined by DNA sequencing. Since insertion orientation is random, the Tn*1000* primer reads the coding strand from 5′ to 3′ in some cases and the antisense strand from 3′ to 5′ in others; in both cases, (cccc) marks the end of the transposon sequence. In the latter case, the last amino acid sequenced is the last in the truncated protein (though up to 36 "nonsense" amino acids may be added from the transposon sequence). In the former case, the first amino acid downstream of the transposon is also the last amino acid of the truncated protein because of a 5 bp duplication on insertion (*29*, *31*). mAbs are shown alongside the shortest bacterial clone which they recognize. [b] Utrophin is 251 amino acids shorter than dystrophin because of several deletions, so the number of the corresponding dystrophin amino acid is given in parentheses.

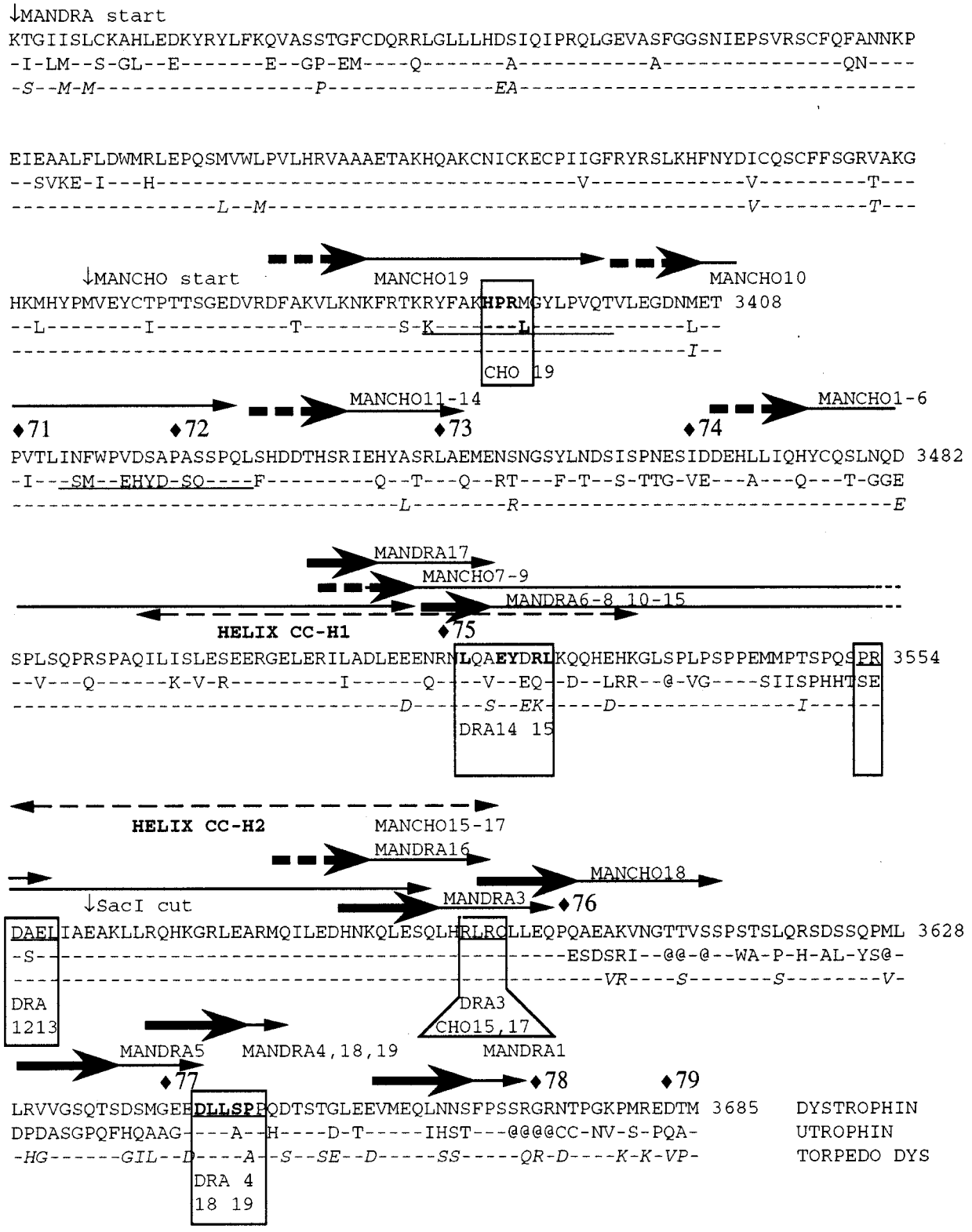

FIGURE 2: Epitope map of the C-terminal domains of dystrophin and utrophin obtained by transposon mutagenesis and additional methods. The three sequences aligned are human dystrophin, human utrophin, and dystrophin of *T. marmorata* (italics). Identities with human dystrophin are indicated by − and gaps by @. Exon boundaries are indicated by diamond symbols for dystrophin exons 71−79. MANDRA start and MANCHO start indicate the first amino acid for the recombinant dystrophin and utrophin fragments used as immunogens, respectively. For each mAb (or group of mAbs), a large arrow indicates the longest transposon mutant to which the mAb does not bind and a smaller arrow shows the shortest mutant to which the mAb does bind; in many cases, the epitope is likely to lie between the large and small arrows (except conformational epitopes; see the text for discussion). Transposon mapping with utrophin fragments (Table 1b) is shown as broken lines on the large arrows and dystrophin fragments (Table 1a) as continuous lines. Epitopes identified using synthetic peptides are underlined, and the three identified using phage-displayed peptide libraries are shown in bold type; the best-defined epitopes are boxed with their corresponding mAbs. A C-terminal subfragment produced by restriction enzyme digestion is shown as SacI cut, and the positions of two predicted coiled-coil helices (CC-H1 and CC-H2) are shown by broken lines.

Table 2: Confirmation of Transposon Mapping Data Using Synthetic Peptides[a]

Peptide sequence.	mAb binding	
	Predicted	Actual
1) KYFAKHPRLGYLPVNT	MANCHO19	MANCHO19
2) ISMWPEHYDPSQSPQL	MANCHO10	MANCHO10
3) SRIEQYATRLAQMERT	MANCHO11-14	None.
4) IIADLEEEQRNLQVEY	MANCHO7-9 MANDRA17	None
5) LEDHNKQLESQLHRLR	MANCHO15-17 MANDRA16	None

[a] Synthetic 16-mer peptides for antibody screening were obtained commercially (Alta Bioscience, University of Birmingham, Birmingham, U.K.). Utrophin sequences were chosen from the transposon mapping data from Figure 2 (sequences between the large and small arrows). Peptides were allowed to attach to a microtiter plate, and mAb binding was assessed by ELISA using a peroxidase-labeled rabbit anti-mouse Ig second antibody. The failure of the last three peptides to bind mAbs probably indicates that insufficient sequence is present in the peptide (e.g., conformational epitope), but the possibility that these three peptides do not bind well to the plate (PVC; Dynatech Labs) has not been ruled out.

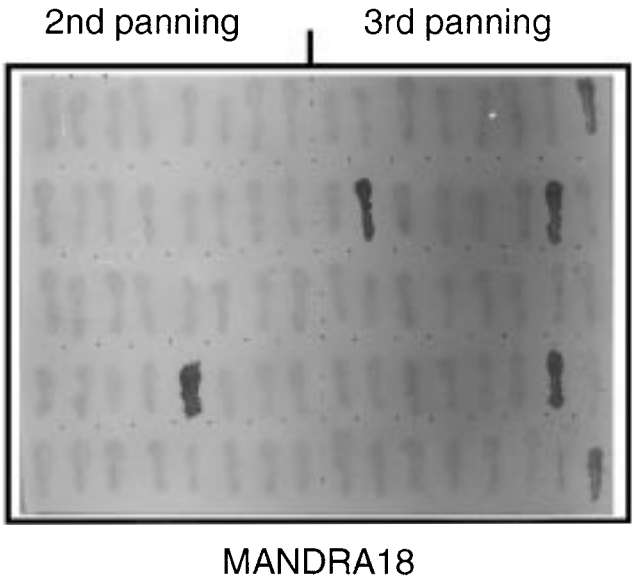

FIGURE 3: Enrichment of a phage-displayed random peptide library after two or three rounds of biopanning with mAb MANDRA18. A random 6-mer peptide library in the pIII protein of bacteriophage fd-tet was selected by biopanning against MANDRA18 attached to a Petri dish coated with rabbit anti-mouse Ig as described previously (*35*). The selected phage population was then amplified in *E. coli* strain K91Kan for the next round of biopanning. Individual *E. coli* colonies after phage infection were grown on nitrocellulose overnight and developed with MANDRA18 as described previously (*35*). Results after the second and third pannings are shown here.

synthesis of 10-mer overlapping peptides in this region (with a two-amino acid overlap) showed that DLLS3647 was sufficient for MANDRA19 binding (Figure 5a); recognition of DLLS in SPOTS but not in transposon mutants may be due to the very high peptide concentrations that are achieved on SPOTS (>10 μg per SPOT). MANDRA4, however, showed stronger staining of peptides 6 and 7 in Figure 5a, consistent with a requirement for the proline in this case (results not shown). The corresponding sequence in utrophin is DLLAP so MANDRA4 must require S3647 since it is dystrophin-specific. Although MANDRA18 and -19 will bind utrophin, the cross-reaction is rather weak, suggesting some preference for Ser at 3647. This weak cross-reaction is easily observed with Western blots of recombinant utrophin with high protein levels but is almost undetectable in *mdx* mouse lung blots (*22*). Figure 6a shows the cross-reaction of all dystrophin mAbs, MANDRA1−19, with dystrophin from the *Torpedo marmorata* electric organ, and the negative reaction of MANDRA4 is explained by the replacement of the important proline residue by alanine (Figure 2). MANDRA18 and -19 do recognize *Torpedo* dystrophin, and this is also consistent with the SPOTS results.

MANCHO19 was selected from a phage-displayed 15-mer library of a peptide containing the sequence HPRL which matches utrophin and lies within the synthetic peptide region (Figures 2 and 4). This mAb also reacts with dystrophin which has the sequence HPRM, so replacement of the final leucine by methionine is tolerated. Another phage-displayed 15-mer peptide recognized by MANDRA15 matched the dystrophin sequence at LXXEYXRL3527 (Figure 4). This interrupted matching is consistent with a helical structure in this region, and a helical wheel analysis shows that all five implicated amino acids are found on one side of the helix (not shown). The dystrophin specificity of the mAbs suggests that R3526 is important since this is replaced by Gln in utrophin and by Lys in *Torpedo* dystrophin which MANDRA15 does not recognize (Figure 6a).

Two additional epitopes were mapped using overlapping SPOTS peptides. Using 10-mer peptides with a three-amino acid overlap, MANDRA12 and -13 were shown to recognize SPQSPRDAEL3558 at one end of the linker between the two coiled-coil helices (Figure 5b), while MANCHO17 showed strong binding to RLRQ3595 using 10-mers with a two-amino acid overlap, with weaker binding of MANCHO15 and MANDRA3 to the same peptides (data not shown). A synthetic 16-mer peptide ending with Arg3594 was not recognized by MANCHO15−17, suggesting that the final Gln3595 may be required (Table 1). This region is highly conserved between dystrophin and utrophin, and all the mAbs cross-react with both proteins.

The remaining epitopes were not confirmed with peptides, so a conformational element cannot be ruled out. A transposed dystrophin clone ending in Asp3644 was recognized by MANDRA5, but the loss of nine more amino acids (to Gln 3635) abolished binding. The sequence in this region is not shared by utrophin, and MANDRA5 is indeed dystrophin-specific, consistent with a linear epitope, possibly around GSQTS3637 since this is also conserved in *Torpedo* dystrophin which MANDRA5 recognizes. Several different clones ending at Pro3614 or Ser3615 bind MANCHO18, but binding was abolished when the antigen was further truncated to Ala3602 (Table 1). Although MANCHO18 binds both dystrophin and utrophin, there is little sequence evidence for a conserved linear epitope between Ala3602 and Pro3614. An alternative to a linear epitope is that truncation is disrupting an upstream conformational epitope, with the implication that some amino acids after Ala3602 are required for the conformation. The localization of this epitope is supported by the transposon mutagenesis of utrophin which showed that truncation to Leu3593 abolished binding of MANCHO18 (Table 1).

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HKMHYPMVEYCTPTTSGEDVRDFAKVLKNKFRTKRYFAKHPRMGYLPVQTVLEGDNMET  DYSTROPHIN
--L--------I-----------T--------S-K--------L-------------L--  UTROPHIN
                                  YSALRFHPRLSVFQG  MANCHO19    PHAGE

PVTLINFWPVDSAPASSPQLSHDDTHSRIEHYASRLAEMENSNGSYLNDSISPNESIDDEHLLIQHYCQSLNQD
-I---SM--EHYD-SQ----F---------Q--T---Q--RT---F-T--S-TTG-VE---A---Q---T-GGE


SPLSQPRSPAQILISLESEERGELERILADLEEENRNLQAEYDRLKQQHEHKGLSPLPSPPEMMPTSPQSPR
--V---Q------K-V-R---------I------Q----V--EQ--D--LRR--@-VG----SIISPHHTSE
                                  VDSQLFVEYFRLRG  MANDRA15

DAELIAEAKLLRQHKGRLEARMQILEDHNKQLESQLHRLRQLLEQPQAEAKVNGTTVSSPSTSLQRSDSSQPML
-S-----------------------------------------ESDSRI--@@-@--WA-P-H-AL-YS@-


LRVVGSQTSDSMGEEDLLSPPQDTSTGLEEVMEQLNNSFPSSRGRNTPGKPMREDTM    DYSTROPHIN
DPDASGPQFHQAAG----A--H----D-T-----IHST---@@@@CC-NV-S-PQA-    UTROPHIN
               PDLLSP  MANDRA18, 4, 19                         PHAGE
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FIGURE 4: Three epitopes identified using phage-displayed peptides. Dystrophin and utrophin C-terminal sequences are shown together with two peptide sequences obtained with a 15-mer library and one sequence obtained with a 6-mer library. The important amino acids for antibody binding (shown in bold) are those shared by the peptide and the authentic protein.

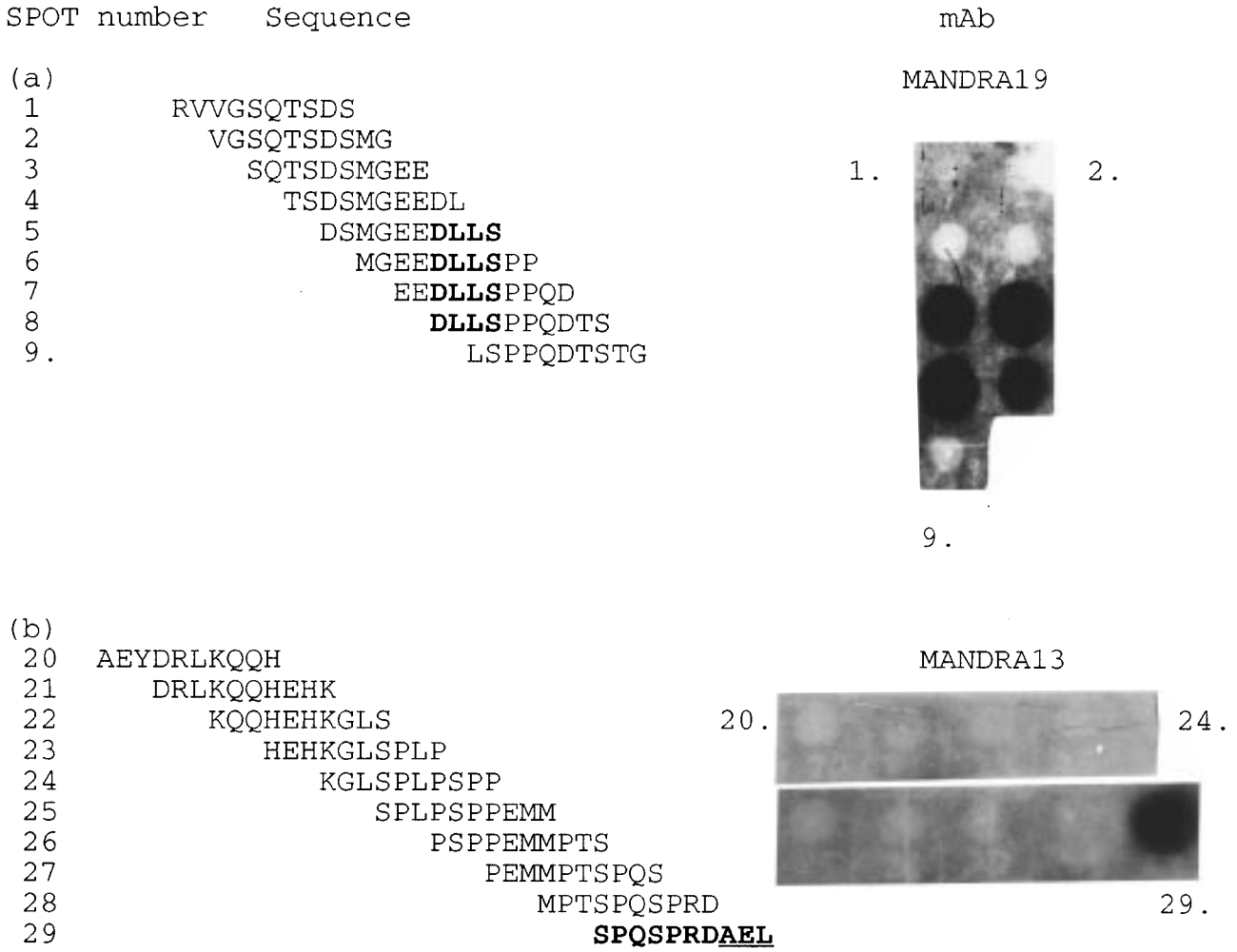

FIGURE 5: Epitope mapping using overlapping peptides synthesized as SPOTS. Peptides were chemically synthesized as SPOTS (*46*) on cellulose membranes and reacted with mAb (a) MANDRA19 or (b) MANDRA13. The membranes were then developed with peroxidase-labeled rabbit anti-mouse Ig and diaminobenzidine substrate. The membranes could be regenerated once or twice using a urea−SDS treatment (*46*), but they were always checked by development without primary mAb and with a negative mAb, to control for complete regeneration, before performing the second round. In this way, peptide 29 in part b was not recognized by MANDRA11 after the first regeneration, but was subsequently recognized by MANDRA12.

The mapping of MANDRA mAbs up to this point is consistent with earlier mapping using a *Dra*I−*Sac*I subfragment of dystrophin cDNA expressed as Leu3558−Met3685 since MANDRA1, -3, -4, -5, -16, -18, and -19 all recognize

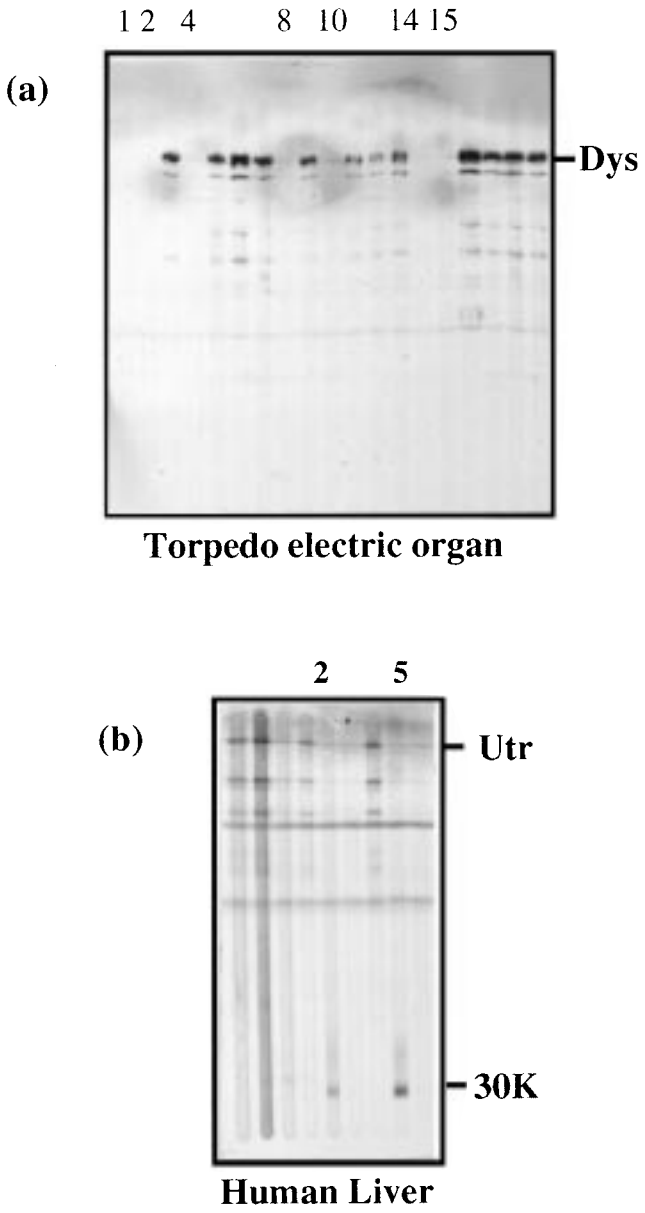

FIGURE 6: Specificity of monoclonal antibodies and epitope characterization. (a) Reaction of 19 MANDRA mAbs with dystrophin from *T. marmorata*. The total protein extract from the *Torpedo* electric organ was subjected to SDS−PAGE on 3 to 12.5% acrylamide gels, and the Western blot was analyzed with 19 mAbs in the sequence MANDRA1−19 using a miniblotter. The mAbs which fail to recognize *Torpedo* dystrophin are in lanes 1, 2, 4, 8, 10, 14, and 15 as shown. This result is consistent with epitope mapping data (see the text) except that the amino acid sequence at the MANDRA14 epitope is the same in human and *Torpedo* dystrophins. The lower-M_r bands are probably degradation products of dystrophin. (b) Binding of nine MANCHO anti-utrophin antibodies to proteins in a human liver extract. A Western blot of adult human liver proteins, as in part a, was probed with nine different mAb culture supernatants (1:20 dilution) using a miniblotter. The mAbs are MANCHO7, -8, -9, -1, -2, -4, -3, -5, and -6, and a 30 kDa cross-reacting protein is recognized by MANCHO2 and -5 only (fifth and eighth lanes). The two broad bands across and between all lanes are cross-reactions of the secondary antibody system. The bands with an intensity similar to that of utrophin, but with a lower M_r, are probably degradation products (though the possibility of C-terminal short forms or "apoutrophins" cannot be ruled out).

this C-terminal subfragment (*22*). When 67 amino acids after Glu3523 were removed by transposon mutagenesis of dystrophin, binding of nine dystrophin-specific mAbs was lost (MANDRA6−8 and -10−15 inclusive; Figure 2). The utrophin fragment ending in DSEL3314 (DAEL3558 in dystrophim) was recognized by all nine utrophin-specific mAbs (MANCHO1−9). Binding of MANCHO7−9 was abolished by removal of a further 41 amino acids, and further shortening by 42 amino acids abolished binding of the remaining six (Figure 2). The binding of MANDRA17, which cross-reacts with utrophin, was also abolished when 10 amino acids were removed from the dystrophin fragment ending in LQAE3523.

As further support for the epitope map of the MANCHO mAbs in Figure 2, we may compare the results with an earlier study in which the mAbs were divided into seven different epitope groups on the basis of their reaction with spontaneous proteolytic subfragments of the recombinant utrophin immunogen on Western blots (*23*). This method placed three mAbs, MANCHO10, -18, and -19, in a group of their own (groups 2, 7, and 1, respectively) and defined four additional groups: group 4 (MANCHO1−6), group 5 (MANCHO7−9), group 3 (MANCHO11−14), and group 6 (MANCHO15−17), which is perfectly consistent with the transposon mutagenesis grouping shown in Figure 2. Our inability to resolve group 4 further by epitope mapping does not necessarily mean that the mAbs in this group recognize exactly the same epitope. Figure 6b shows that MANCHO2 and MANCHO5 both cross-react with a protein of about 30 kDa in adult human liver extracts, and this clearly implies that they recognize an epitope different from those of other members of this group.

Epitope mapping can also be performed using dystrophins with precisely defined deletions expressed in transgenic *mdx* mice. The *mdx* mouse does not produce any endogenous dystrophin. Figure 7 shows that all mAbs bind to normal dystrophin; however, mAbs 19−23 do not bind to dystrophin with the exon 71−74 deletion, and mAbs 1−16 do not bind to dystrophin with the exon 75−78 deletion [after allowing for cross-reaction of some mAbs with utrophin which is upregulated in the *mdx* mouse (*23*); see the legend of Figure 7]. There is a short sequence of 16 amino acids encoded by the start of exon 75 which is present in both deleted dystrophins, and this has enabled us to map MANDRA14 and -15 which bind to both (lanes 17 and 18 in Figure 7). We had already established with phage-displayed peptides (Figure 4) that MANDRA15 recognizes this sequence. These results have thus enabled us to locate the MANDRA14 epitope and to confirm the locations of the other epitopes.

Figure 8 is a graphical representation of predicted secondary structures in the C-terminal domain and is not intended to represent any tertiary folding or any higher level of structure. The two predicted coiled-coil α-helices (CC-H1 and CC-H2) are shown, together with likely flexible linker regions which contain all the proline residues (shown as "P") characteristic of turns and loops, often in association with serine and glycine residues. Furthermore, utrophin is eight amino acids shorter than dystrophin in this domain, and the "gaps" all occur in these linker regions where the core structure is unlikely to be affected. Some of the regions between these linkers have sequences predicted by the PHD method (*36*) to form amphipathic helices with one hydrophobic face (shown as "helix?"). Helix predictions are consistent with physical studies which suggest a 41% helical

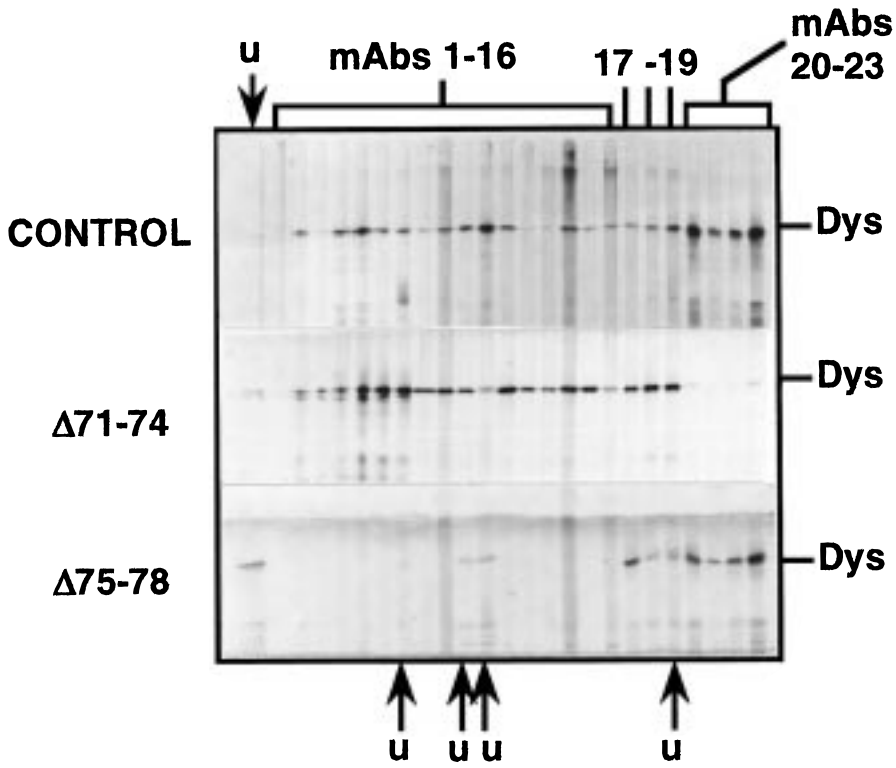

FIGURE 7: Epitope mapping with transgenic *mdx* mice which express dystrophins with defined deletions in the C-terminal domain. Western blots of control muscle and muscle from *mdx* mice overexpressing mouse dystrophin without exons 71−74 or exons 75−78 were developed on a miniblotter with 23 mAbs which recognize dystrophin. The first two lanes show a utrophin-specific antibody, as a control for utrophin upregulation in the *mdx* mouse (*23*), and a blank lane (PBS only). Subsequent lanes contain the following mAbs in epitope order: MANDRA1, -4, -18, -19, and -5, MANCHO18, MANDRA3 and -16, MANCHO15 and -17, MANDRA6, -7, -8, -10, -11, -12, -14, -15, and -17, and MANCHO11−14. Two mAbs in lanes 17 and 18 (MANDRA14 and -15, respectively) recognize dystrophin in both transgenic mice. The first 16 amino acids of exon 75 are not deleted in the del75−78 mouse, so MANDRA14 and -15 must recognize this sequence, N3519−K3534 (human dystrophin numbering). MANDRA17 (lane 19) is one of the four mAbs indicated (u) which are known to cross-react with utrophin (*22*). The last four mAbs also recognize utrophin, which explains the weak reaction on the del71−74 blot.

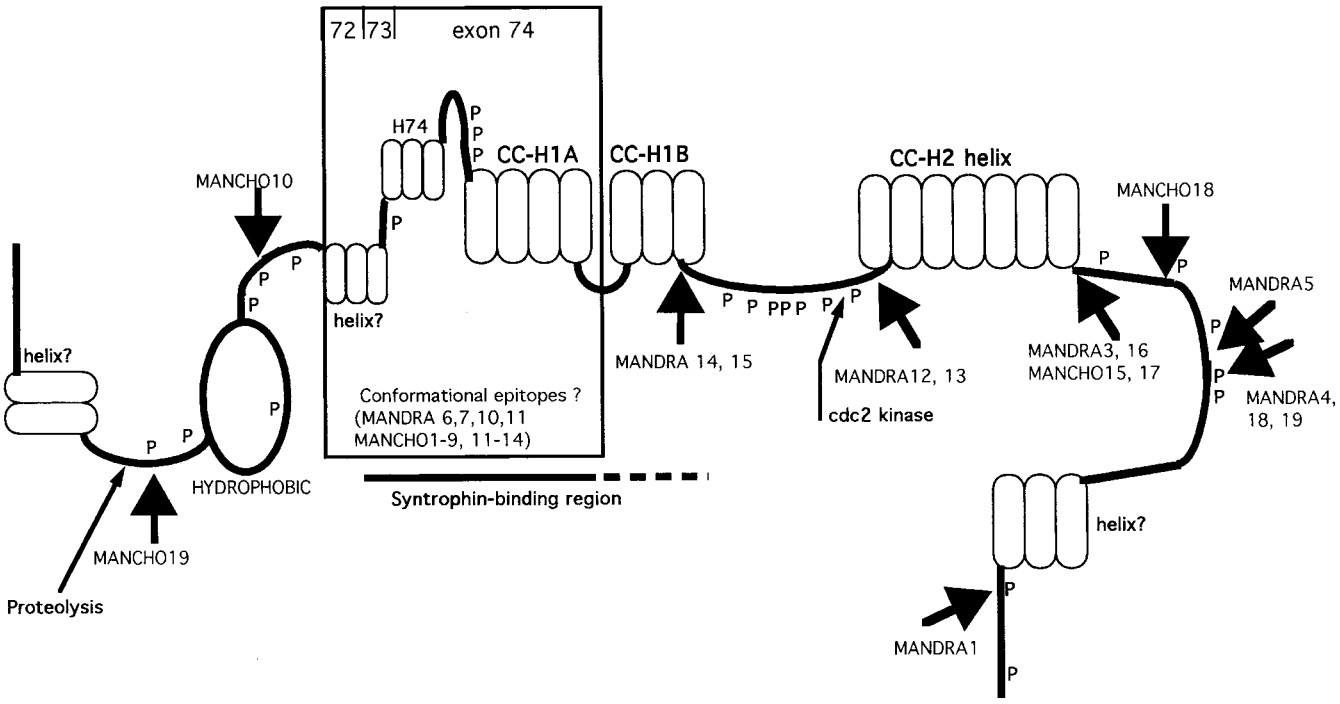

FIGURE 8: Linear representation of epitopes and predicted helices in the dystrophin and utrophin C-terminal domain. The dystrophin C-terminal region is represented as a string of possible secondary structures (e.g., helices) separated by linkers containing proline (P) residues (see the text). No prediction of tertiary folding is intended. In addition to the two coiled-coil helices (CC-H1 and CC-H2), other potential amphipathic helices (helix?) and two sites accessible to protease or kinase are shown. The box encloses a region with conformational epitopes and contains sequences encoded by the alternatively spliced exons 71−74.

content for this region (*37*). Most of the epitopes that were difficult to map and are possibly conformational are associated with the boxed area in Figure 8.

DISCUSSION

The distribution of epitopes in the dystrophin and utrophin C-terminal domains is quite striking (Figure 8). The first two epitopes are associated with proline-rich linkers, and both could be mapped with peptides, consistent with a flexible and accessible structure in this region. This is supported by proteolysis studies (*14*, *38*); a calpain-sensitive site in native dystrophin was identified near an immunogenic peptide, p33c, which corresponds to the MANCHO19 epitope. All the epitopes in the following region up to the end of the first coiled-coil helix (CC-H1) appear to show significant conformation dependence since they were difficult to map precisely and could not be mapped by peptide methods, even though potential proline-containing turns and linkers are present. This implies a local tertiary structure which readily refolds after SDS treatment (since the mAbs recognize both native dystrophin and dystrophin on Western blots). After CC-H1, 12 epitopes are associated with all the proline-rich linkers and most of them could be mapped precisely by peptide methods (Figure 8), consistent with a flexible structure around CC-H2. The presence of a cdc2 kinase phosphorylation site immediately adjacent to the MANDRA12 and -13 epitope (*39*) supports the surface accessibility of this site. The overall structure suggested is a flexible linker to the preceding cysteine-rich domain, followed by a highly structured region which includes CC-H1 and, finally, another flexible region containing CC-H2.

It is important to emphasize that the mAbs used in this study were selected for their ability to recognize native dystrophin or utrophin in situ at the sarcolemma in frozen sections of human muscle (*22*, *23*). There is no evidence that any significant denaturation occurs during the freezing and thawing of muscle sections, so dystrophin in situ is as close as we are likely to come experimentally to a native dystrophin molecule. As direct evidence for native dystrophin in situ, when we selected mAbs against denatured dystrophin by Western blotting only, several mAbs failed to detect dystrophin in frozen sections unless the section was treated with SDS or urea (*22*). Epitopes that are detectable on both sections and Western blots must therefore be accessible (or become accessible easily) on the surface of the native proteins and must also be retained in the partially folded proteins recovering from SDS treatment. Many "globular" proteins regain much of their secondary structure when SDS is removed, but most only partially regain tertiary folding [some enzymes show partial or complete recovery of activity, and at least one protein can even retain subunit associations (*40*)]. There are two types of epitopes that fulfil these conditions: first, a linear, sequential epitope that is flexible and accessible in the native state (e.g., a surface loop or a turn between helices), and second, an assembled, conformational epitope formed by secondary folding or, possibly, by local tertiary folding. Only the former type of epitope is likely to be mimicked by short synthetic peptides. We have observed both types of epitopes in folding intermediates of creatine kinase (*24*, *41*), a globular enzyme, the C-terminal domain of which has a rapidly refolding β-sheet structure (*42*).

Results from this study support this interpretation of the nature of epitopes. Thus, six of the epitopes located by transposon mutagenesis were confirmed using synthetic peptides, and in two cases further confirmation and refinement of the epitope was possible using phage-displayed peptide libraries (Figure 4). Five of these six sequences contain, or are closely associated with, amino acids which are often found in turns and loops, such as prolines. None of these precisely defined epitopes is present in human dystrobrevin sequences (*15*). In our original characterization of the MANDRA mAbs, we found that six mAbs were significantly weaker on sections than on Western blots (*22*), and all of these have now been found to be associated with the coiled-coil region (MANDRA8 and -11−15 in Figure 2). In this case, the weaker reaction in situ could be due either to a conformational difference or to partial masking of the epitopes in situ by dystrobrevins (note that complete masking is not possible because mAbs which do not bind at all to muscle sections would not have passed our hybridoma selection process). A clue to the disposition of the coiled-coil helices may be found in the MANDRA15 epitope on CC-H1. The observation that the important amino acids in these epitopes lie on one face of the helix (Figures 4 and 7a) is likely significant and not merely coincidental, since selection of such helical epitopes from phage libraries has been reported previously (*43*). It implies not only that the epitopes are formed by the helical secondary structure but also that this face, which includes the leucine residues, is partially accessible on native dystrophin.

A number of epitopes were difficult to locate accurately or confirm with peptides, and nearly all of these were associated with the first coiled-coil helix (CC-H1 in Figure 8) and the sequences preceding it. Furthermore, a panel of 25 mAbs against the same region of dystrophin encoded by exons 73 and 74 (*6*) has so far been resistant to epitope mapping attempts using peptide and fragmentation methods (unpublished data). This area, boxed in Figure 8, may be more tightly folded or conformationally stable than the rest of the domain. This is an interesting region of the dystrophin molecule because in vitro experiments suggest that it is involved in interaction with other proteins, such as syntrophin and dystrobrevins. Isoform mRNAs lacking exons 71−74 have been identified in human brains by RT-PCR methods (*44*), although the corresponding proteins appear to be minor species at best (*6*). One might have speculated that exons 71−74 could encode a discrete functional subdomain, but the exon 71−74 deletion removes half of the first coiled-coil helix (CC-H1) which might be expected to cause considerable disruption of the local structure. Both Suzuki et al. (*19*) and Ahn and Kunkel (*18*) raised the possibility of a short linker at the exon 74−exon75 boundary which would divide the first helix (CC-H1A and CC-H1B in Figure 8). Their attempts to define the syntrophin-binding sites on dystrophin in vitro produced rather different results. Thus, Suzuki et al. (*19*) argued on the basis of gel overlay studies that CC-H1 is the β1-syntrophin binding site and the α-syntrophin binding site precedes it. Ahn and Kunkel (*18*) used a more direct approach with recombinant protein fragments and found that the CC-H1 sequence would not bind β1-syntrophin, though the sequence encoded by exon 74, which includes the proposed α-syntrophin binding site, would. Recently, yeast two-hybrid protein interaction studies

have clearly implicated CC-H1 in dystrobrevin binding (*16*), and because of the nature of coiled-coil interactions and the lack of "leucine zipper" helices in syntrophins (*13*), simultaneous direct binding of syntrophin by CC-H1 seems unlikely. Paradoxically, dystrophin lacking exons 71−74 appears to function in a manner identical to that of full-length dystrophin, producing a normal phenotype in a transgenic *mdx* mouse, with normal syntrophin localization (*32*) and dystrobrevin localization (*45*). This apparent paradox may be partly explained, however, if syntrophins (and hence dystrobrevins) can also be placed at the sarcolemma via transmembrane sodium channels (*21*).

These studies suggest a loose open structure around CC-H2, but a more conformational and denaturation-resistant association between CC-H1 and preceding dystrophin and utrophin sequences. Since these preceding sequences include a syntrophin binding site, one can speculate that a more rigid three-dimensional structure in this region might contribute to the spatial organization of syntrophins and dystrobrevins in the functional complex which also includes nNOS and sodium channels. Since dystrobrevins appear to be structurally homologous to dystrophin, sharing coiled-coil helices and cysteine-rich domains, determining whether they have a similar epitope structure will be of interest.

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