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Glycoprotein-binding site of dystrophin is confined to the cysteine-rich domain and the first half of the carboxy-terminal domain

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Dystrophin, a protein product of the Duchenne muscular dystrophy gene, is thought to associate with the muscle membrane by way of a glycoprotein complex which was co-purified with dystrophin. Here, we firstly demonstrate direct biochemical evidence for association of the carboxy-terminal region of dystrophin with the glycoprotein complex. The binding site is found to lie further inward than previously expected and confined to the cysteine-rich domain and the first half of the carboxy-terminal domain. Since this portion corresponds well to the region that, when missing, results in severe phenotypes, our finding may provide a molecular basis of the disease.

Dystrophin; Sarcolemma; Glycoprotein-binding site; Calpain; Peptide mapping

1. INTRODUCTION

Dystrophin, a protein product of the Duchenne muscular dystrophy (DMD) gene, is a spectrin-like protein with a molecular weight of 427 kDa [1–3]. Many immunohistochemical studies have established its membrane localization in skeletal and cardiac muscles, and its absence in muscles from DMD patients [4–6]. A linkage between dystrophin and the cell membrane is assumed to be mediated by a glycoprotein complex since, in detergent extracts of sarcolemma, dystrophin is found in a large complex with several other proteins containing wheat germ agglutinin (WGA)-binding glycoproteins [7–9].

On the basis of the deduced amino acid sequence, dystrophin is predicted to comprise four distinct domains: an amino-terminal domain, a central rod domain, a cysteine-rich domain and a carboxy-terminal domain [3]. Because the sequence of the last domain is highly conserved and shows no apparent resemblance to any previously reported protein except dystrophin-related protein (DRP) [10], and because deletions of this domain often result in loss of dystrophin from the membrane [11,12], this carboxy-terminal domain has been speculated to be the membrane-anchoring domain [3,13]. After the finding that the tightly associated complex of the sarcolemmal glycoproteins co-purified with dystrophin, this speculation has been naturally expanded to the idea that the glycoprotein-binding site exists in this domain [14]. However, there has been no direct biochemical evidence for association between this domain and the glycoprotein complex, and thus the precise glycoprotein-binding site remains speculative.

In this paper, by analyzing calpain fragments of dystrophin which still associate with the glycoproteins, we firstly determine an essential region for the binding. The binding site is confined to the cysteine-rich domain and the first half of the carboxy-terminal domain, and the last half of the carboxy-terminal domain is found not to be essential for the binding.

Abbreviations: DAPC, dystrophin and its associated protein complex; WGA, wheat germ agglutinin.

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2. EXPERIMENTAL

2.1. *Purification of DAPC*

Dystrophin and its associated protein complex (DAPC) was purified from rabbit skeletal muscles as previously described [8] except that 2 mM of dithiothreitol (DTT) was added during membrane preparation, and 0.5 mM during the following chromatographic procedure. Samples were confirmed not to contain a detectable amount of dystrophin-related protein (DRP) [10] through use of the DRP-specific polyclonal antibody [15] (data not shown).

2.2. *Antibody production*

All antibodies except Dy4/6D3 are polyclonal and raised against synthetic polypeptides corresponding to the amino acid sequences of human dystrophin [3]. The positions of amino acid residues against which each antibody was raised are as follows (see Fig. 4b): P00a, residues 11–60; P04d, 440–489; Dy4/6D3, 1,180–1,388; P22a, 2,360–2,409; P30b, 3,085–3,097; P31b, 3,186–3,200; P33c, 3,373–3,391; P34c, 3,495–3,544. The methods of preparation and the specificity of the antibodies, except P30b, P31b and P33c, were reported previously [8,16,17]. The new polyclonal antibodies, P30b, P31b and P33c, were generated in Japanese white rabbits using BSA-conjugated synthetic

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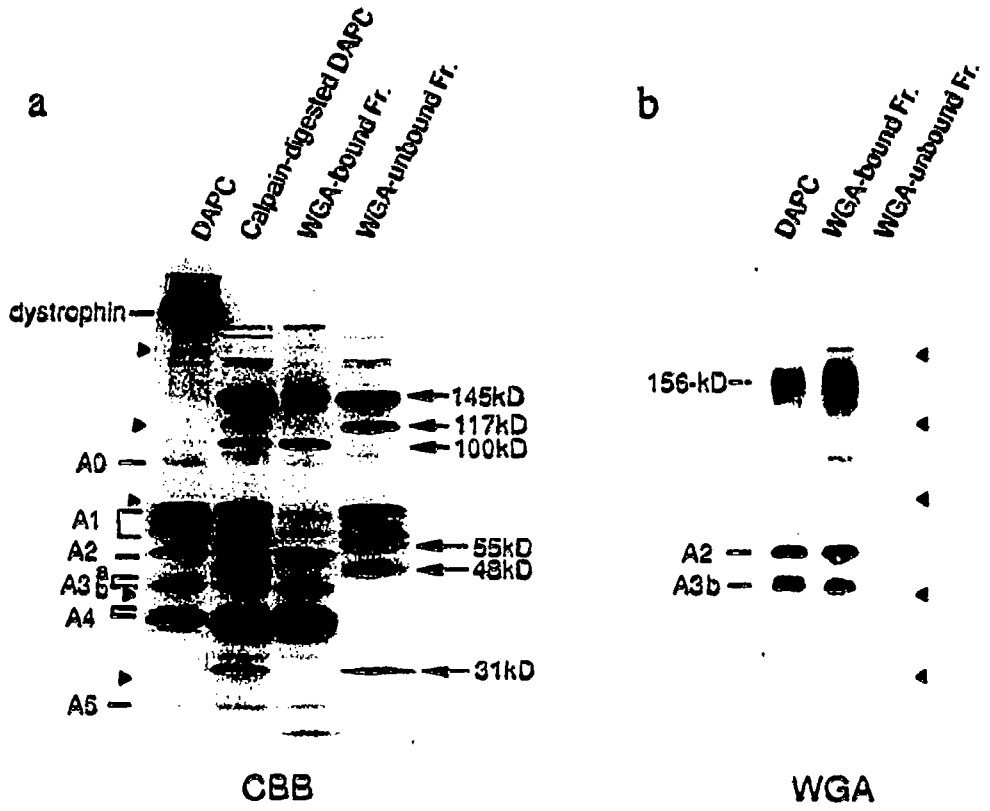

Fig. 1. Calpain digestion of DAPC and separation of the resultant fragments using WGA-affinity chromatography. Shown are a Coomassie blue-stained gel (a) and a biotinyl-WGA-stained blot (b). Arrows indicate molecular weights of the main dystrophin fragments produced by calpain digestion. A0–A5 and 156-kDa represent dystrophin-associated proteins. Positions of molecular weight standards are indicated by arrowheads on the left or right of each panel: from top to bottom, 200, 116, 66, 42 and 30 kDa. The lowest band in lane 3 of (a) is contaminated WGA subunits.

polypeptides. All antisera were passed through a BSA-Sepharose column to remove antibodies against epitopes on BSA. Monoclonal antibody Dy4/6D3 [18] was a gift from Professor L.V.B. Nicholson.

2.3. *Calpain digestion of DAPC and the following WGA-affinity chromatography*

Digestion was performed at 25°C for 30 min in buffer C (20 mM HEPES, 270 mM NaCl, 2 mM $CaCl_2$, 0.5 mM DTT, 0.1% digitonin, pH 7.5) using *m*-calpain (Sigma) at a ratio of 100 DAPC/1 calpain (w/w). The reaction was stopped by adding 5 mM EGTA, and then the solution was loaded on WGA-Sepharose equilibrated with binding buffer (20 mM HEPES, 0.5 mM NaCl, 3 mM $MgCl_2$, 2 mM EGTA, 0.5 mM DTT, 10 μg/ml leupeptin, pH 7.5). After collection of the unbound fraction and extensive washing of the gel with binding buffer, bound fragments were eluted with *N*-acetyl-D-glucosamine (NAG)-containing buffer (20 mM HEPES, 0.3 M NAG, 0.5 mM DTT, 0.1% digitonin, 10 μg/ml leupeptin, pH 7.5). KI treatment was performed as previously described [8] immediately after the cessation of calpain digestion.

2.4. *PAGE and immunoblotting*

SDS-PAGE was performed on 4–15% gradient gels as described by Laemmli [19]. For non-denaturing PAGE, Davis' buffer system [20], containing 0.1% digitonin, was used. Two-dimensional PAGE was performed, using non-denaturing gels instead of isoelectric-focused gels, by the method of O'Farrell [21]. The second dimension was a 4–15% SDS gel described above. Proteins separated in SDS or non-denaturing polyacrylamide gels were either stained with Coomassie blue or transferred electrophoretically onto polyvinylidene difluoride membranes [22]. The membranes were processed for Western blotting with antibodies to dystrophin or biotinyl-WGA as described previously [8].

2.5. *Peptide sequencing*

Dystrophin fragments in the WGA-bound or unbound fraction (125 μg) were separated on 4–15% gradient SDS gel according to Moos Jr., et al. [23] and transferred electrophoretically onto a PVDF membrane. The corresponding region of the membrane to each fragment was used as samples for analysis. For sequencing, the gas phase sequenator 477A, with on-line phenylthiohydantoin amino acid analyzer, 120A (Applied Biosystems), was used.

3. RESULTS AND DISCUSSION

As reported previously, dystrophin can be enriched from detergent extracts of the sarcolemma by using WGA-affinity chromatography, because it forms a large protein complex containing WGA-binding glycoproteins [7–9]. Coomassie blue-stained gels of the purified dystrophin and its associated protein complex (DAPC) shows six proteins other than dystrophin, A0–A5, whose molecular weights are 94, 62, 52, 43, 36 and 24 kDa, respectively [8] (Fig. 1a, lane 1). The 156-kDa glycoprotein [9] is difficult to detect with protein stain-

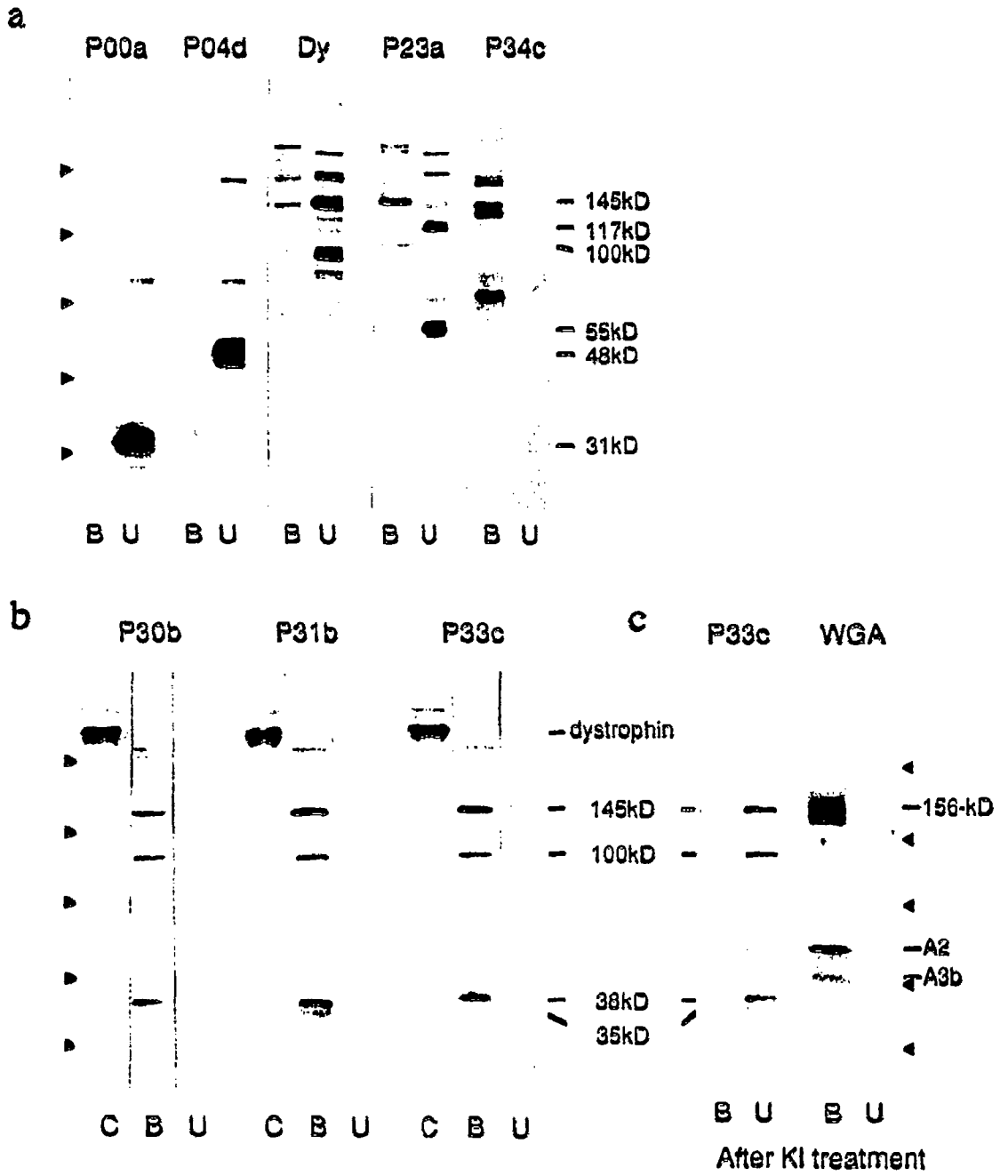

Fig. 2. Immunoblot analysis showing the origin of WGA-binding fragments (a and b) and the effect of KI treatment on WGA binding of these fragments (c). Lanes B, WGA-bound fraction; lanes U, WGA-unbound fraction; lanes C, DAPC (control for the specificity of dystrophin detection of newly prepared antibodies). Antibodies used for each set of strips are indicated at the top of lanes (Dy4/6D3 is abbreviated as Dy). WGA in (c) means biotinyl-WGA staining. The positions of the main fragments of dystrophin and the three glycoproteins are indicated. The molecular weight standards (arrowheads) are the same as those used in Fig. 1.

ing, but can be detected with biotinyl-WGA staining (see Fig. 1b). When this purified DAPC was digested with calpain, dystrophin was rapidly attacked and finally degraded to mainly 145, 117, 100, 55, 48 and 31 kDa fragments, while major dystrophin-associated proteins, except A0 and A3a, appeared to be much less sensitive to calpain digestion (Fig. 1a, lane 2). The resistance of the three WGA-binding glycoproteins, the 156-kDa protein, A2 and A3b, was confirmed by blotting analysis using biotinyl-WGA: the molecular weights of these proteins changed little after the treatment, and they still bound strongly to WGA (Fig. 1b). These results raised the possibility that some dystrophin fragments retain the ability to bind to WGA through glycoproteins. We fractionated the calpain-digested DAPC by WGA-affinity chromatography, and ana-

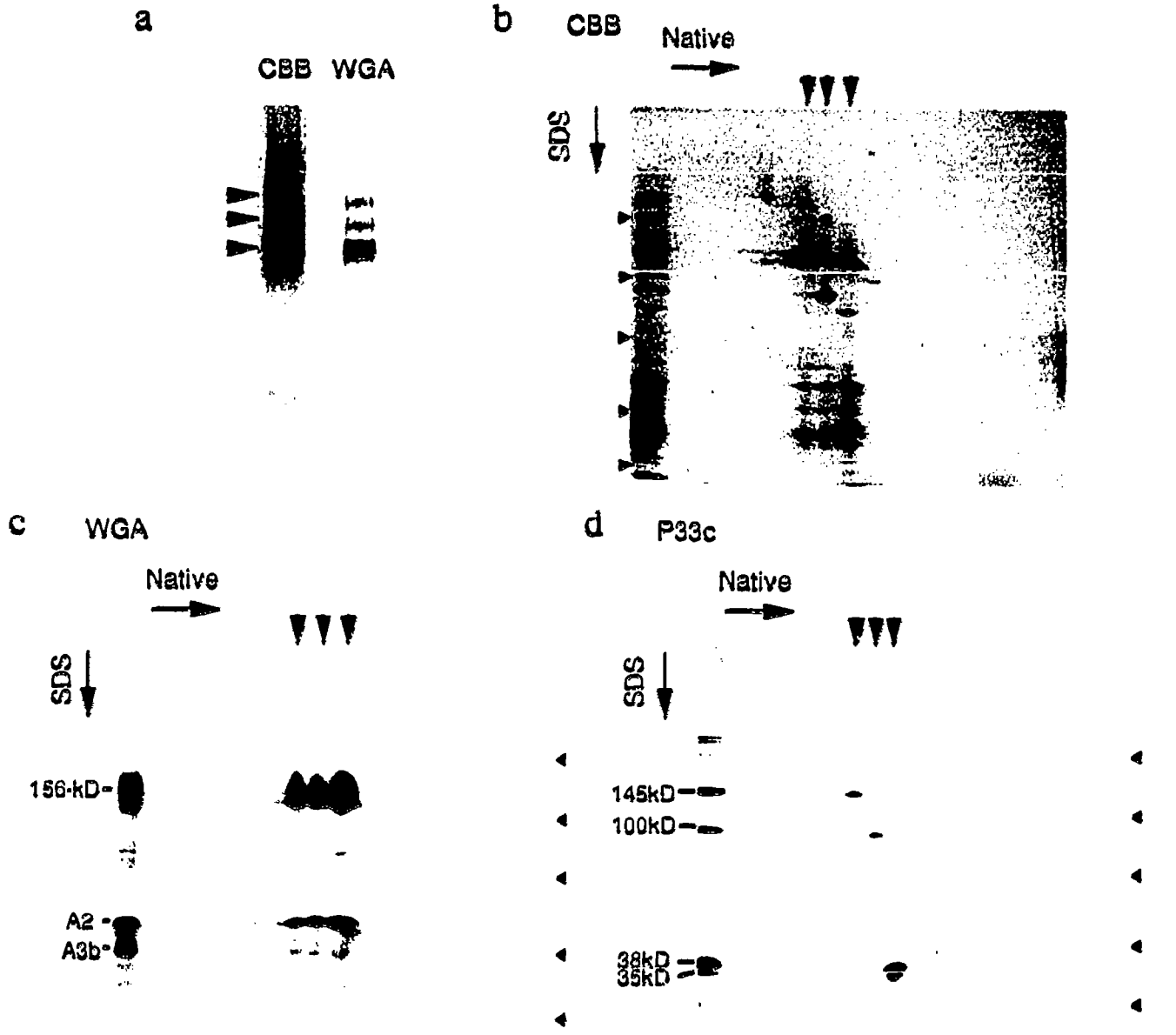

Fig. 3. Two-dimensional polyacrylamide gel electrophoresis of WGA-bound fraction. (a) The first dimension of 3.5–6% non-denaturing PAGE. (b,c and d) The second dimension of 4–15% SDS-PAGE. Shown are Coomassie blue-stained gels (lane 1 in a and b) and blots of identical gels stained with biotinyl-WGA (lane 2 in a and c) or P33c (d). Large arrowheads in (a) indicate positions of three large protein complexes observed in the first-dimensional gel, and those in b–d are corresponding positions of these complexes on the second-dimensional gels. In b–d, staining patterns of samples not subjected to the first electrophoresis are also shown on the left edge of the gels. The molecular weight standards (small arrowheads) are the same as those used in Fig. 1.

lyzed the bound and unbound fractions on SDS-PAGE (lanes 3 and 4 in Fig. 1a). Besides low-molecular-weight proteins, which should be A2, A3b, A4 and A5, one of the main fragments of dystrophin, the 100-kDa fragment, totally bound to WGA, whereas four of the other main fragments, 117, 55, 48 and 31 kDa, were found exclusively in the WGA-unbound fraction. The 145-kDa fragment was separated into two fractions: WGA-binding and -non-binding fragments. The former, 145b, remained on the resin even after extensive washing. Because dystrophin itself is not a WGA-binding glycoprotein, the above results show that 145b and 100-kDa fragments contain the glycoprotein-binding site.

To confine the glycoprotein-binding site on the dystrophin molecule, the WGA-bound and -unbound fractions were analyzed by immunoblotting using several anti-dystrophin antibodies which recognize different epitopes spread over the entire dystrophin molecule (Figs. 2a and 4b). Polyclonal antibodies, P00a and P04d, and monoclonal antibody, Dy4/6D3, raised against amino acid residues in the amino-terminal half of human dystrophin, did not specifically recognize any

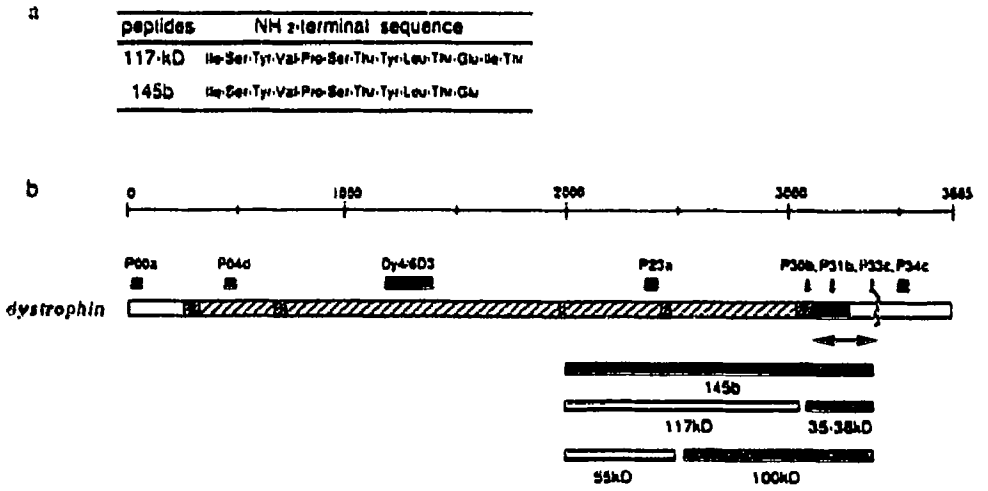

Fig. 4. Peptide mapping of WGA-binding fragments. (a) The results of peptide sequencing of 145b and 117-kDa fragments. Initial yields were 25 and 34 pmol, and average repetitive yields, 90 and 89%, respectively. (b) Schematic diagram showing locations of some dystrophin fragments on the molecule. The largest bar represents the dystrophin molecule showing the domain organization predicted by Koenig et al. [3]: from left to right, the N-terminal domain (open box), the central rod domain (lightly hatched box), the cysteine-rich domain (black box), and the carboxy-terminal domain (dotted box). Densely hatched boxes represent five potential non-repeat segments which were predicted to be targets for proteinase digestion because of their flexible structures [30]. The corresponding portions of the peptides used to generate the antibodies in this study are indicated by black blocks above the dystrophin diagram, and the highly conserved region of the dystrophin sequence [24] is indicated below the diagram by a bi-directional arrow. A jagged line at the carboxy-terminus indicates the main alternative splicing site of dystrophin [31]. Locations of some dystrophin fragments produced by calpain digestion are shown by smaller bars below the diagram on the basis of the results presented here. Cross hatched bars represent WGA-binding fragments, and open ones, WGA-non-binding fragments. The amino acid scale is given at the top.

fragments smaller than 250 kDa in the WGA-bound fraction (195 and 145 kDa bands faintly observed in lane B for Dy4/6D3 are presumably non-specific remains of WGA-non-binding fragments stained due to high titer of this antibody). On the other hand, P34, the polyclonal antibody against the carboxy-terminal amino acid residues (3,495–3,544), recognized five minor fragments, all of which bind to WGA. The minimum size (64 kDa) of the WGA-binding fragments recognized by P34c implies that the glycoprotein-binding site is confined to carboxy-terminal ~750 amino acids. However, P34c detected neither 145b nor the 100-kDa fragment. The former was recognized exclusively by P23a, whose epitope resides in the carboxy-terminal half of the central rod domain (2,360–2,409), while the latter did not show strong affinity to any of the antibodies. Interestingly, P23a also recognized the 117-kDa fragment, one of the major WGA-non-binding fragments. Therefore, we consider the glycoprotein-binding site to precede the epitope of P34c and reside within a portion of 145b which does not overlap with the 117-kDa fragment. By microsequence analysis, we found that these two fragments share the same amino-terminal sequence, which shows an identical match to the published amino acid sequence of human dystrophin [3] from residue 1,993 (Fig. 4a). Thus we estimated that the glycoprotein-binding sit is present around the cysteine-rich domain (3,115–3,269) [3]. This also explained why the 100-kDa fragment did not react with any antibodies.

To confirm this, we prepared polyclonal antibodies, P30b, P31b and P33c, which were directed against amino acid residues 3,085–3,097, 3,186–3,200 and 3,373–3,391 of human dystrophin, respectively (see Fig. 4b). Immunoblot analysis of the calpain fragments using these antibodies revealed that all of them reacted with both 145b and the 100-kDa fragment (Fig. 2b). On the other hand, no WGA-non-binding fragments, including the 117-kDa fragment, were stained. Furthermore, these antibodies detected two additional WGA-binding fragments, 35 and 38 kDa, which were not discerned from A4 by Coomassie blue staining in Fig. 1. These fragments should correspond to the non-overlap region between 145b and the 117-kDa fragment. The 35-kDa fragment should be the smallest WGA-binding fragment produced by calpain digestion, and its amino- and carboxy-termini may be around the epitopes of P30b and P33c, respectively, because these antibodies had very low affinity to this fragment.

To exclude the possibility that the detected fragments remained on the resin due to non-specific binding, we carried out the following experiments and confirmed direct association between the fragments and the glycoproteins. (i) The association of dystrophin with the glycoprotein complex is sensitive to molar concentrations of potassium iodide [7–9]. All fragments also lost their WGA-binding activity after KI treatment, whereas the glycoproteins still remained on the resin (Fig. 2c). (ii) The WGA-binding fraction of the digest was further analyzed by non-denaturing polyacrylamide gel electrophoresis followed by SDS-PAGE in a second dimen-

sion. The first electrophoresis revealed three major bands (arrowheads in Fig. 3a), each corresponding to a large protein complex with one of the WGA-binding fragments of dystrophin and a set of glycoproteins (Fig. 3b,c,d). (The complex containing the 35-kDa fragment and that containing the 38-kDa fragment were not separated under this resolution.)

Fig. 4b summarizes the results presented here. We identified the 35- and 38-kDa fragments of dystrophin which should contain the essential region for glycoprotein binding. Based on the immunoblot analysis using several antibodies (P30b, P31b, P33c and P34c) raised against the amino-acid sequences showing no intramolecular homology [3], we concluded that these fragments mainly cover the cysteine-rich domain and the first half of the carboxy-terminal domain [3], but not the last half of the carboxy-terminal domain. This region corresponds well to the highly conserved sequence (3,123–3,418) between chicken and human (>99%) [24]. The carboxy-terminal sequence of dystrophin-related protein (DRP), an autosomal gene product with high homology to dystrophin observed on the skeletal muscle membrane [10,15], exhibits 82% amino acid identity with a large part of (3,187–3,400) this portion, suggesting that DRP may also utilize this membrane glycoprotein complex. One might imagine a role for Ca ions in glycoprotein binding of dystrophin since the cysteine-rich domain contains two potential EF-hand-like sequences [3]. However, it should be noted that, in our experiments, the chelation of Ca^{2+} with EGTA did not dissociate the dystrophin fragments from the glycoproteins (see section 2).

In this report, we firstly demonstrated direct association between the glycoprotein complex and the carboxy-terminal region of dystrophin, and thus established membrane anchoring of this region. We also found that the essential region for binding lay further inward than previously expected [13,14]. Our conclusion is based on the analysis of the calpain-digested fragments of dystrophin. Therefore, we cannot completely rule out the possibility that calpain digestion disrupted other interactions between the glycoproteins and dystorphin, especially in the last half of the carboxy-terminal domain which is highly sensitive to proteolysis (see Fig. 2a). Actually, by using the immunogold labeling technique, Cullen et al. showed that the last 17 amino acids of the dystrophin carboxy-terminus are found in a narrow band enclosing the plasma membrane [25]. However, we believe that the region we confined is essential, because the complete WGA-binding activity of the 35- and 38-kDa fragments, and their dissociation after KI treatment, explain the main features of the glycoprotein binding of intact dystrophin [8,9]. High sequence conservation of this region also supports this idea.

Recently, some patients were found to produce truncated dystrophin lacking the carboxy-terminal region but show almost normal localization of the protein at the sarcolemma [26–28]. Surprisingly, in the most extreme case, one of the patients had a deletion which removed the carboxy-terminal half of the protein [28]. In light of our findings, the defined carboxy-terminal region is not the sole membrane-attaching site, and is not necessarily indispensable for the membrane localization. However, more importantly, in spite of the apparent normal membrane localization of the truncated dystrophin, their phenotypes were severe (one case [27] is too young to make a final diagnosis). This is still very consistent with the results of Koenig et al. [11] that the region which, when missing, leads to severe phenotypes is limited to the cysteine-rich domain and the first half of the carboxy-terminal domain. This is the region defined here to be essential for the glycoprotein binding. Recently, Ervasti and Campbell [14] and Ibraghimov-Beskrovnaya et al. [29] showed that the 156-kDa protein is an extracellular protein which binds to laminin. Hence, together with those results, our finding strongly supports the idea that the essential cause of the disease is the lack of interaction between this dystrophin carboxy-terminal region and the extracellular matrix, by way of the glycoprotein complex.

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