Localization of Dystrophin COOH-terminal Domain by the Fracture-label Technique

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Abstract. The precise localization of dystrophin in the skeletal muscle cell should contribute to a better understanding of the yet unclear functional role of this protein, both in normal and in Duchenne muscular dystrophy. Immunocytochemical studies did not give conclusive results on the localization of dystrophin with respect to the sarcolemma and to the cytoskeletal components. To improve the reliability of the electron microscopic immunocytochemical localization of dystrophin, a mAb against the COOH-terminus of the molecule has been used in association with the fracture-label technique, which, causing a partition of the membrane in protoplasmic and exoplasmic halves, allows a more precise dystrophin localization. The results obtained indicate that dystrophin is associated with the protoplasmic half of the plasmalemma, and the observation that it does not randomly follow the partition of the membrane is consistent with a stable association with the cytoskeleton.

Duchenne and Becker muscular dystrophy (DMD and BMD)† are progressive muscular disorders which result from defects in a 2,000-kb X-gene at position Xp21. By reverse genetics it was possible, at first, to isolate the gene, and then to employ the DNA to identify the gene product (16, 10). Immunological studies with antisera raised in sheep led to the discovery of a new protein, the dystrophin, present in small quantities in normal skeletal muscle (0.002%), but absent or altered in DMD, mdx mouse, and BMD (12).

Dystrophin has a 427-kD molecular mass and, as it has been predicted from the amino acid sequence, it shares some structural features with the cytoskeletal proteins spectrin and alpha-actinin (14). According to these structural homologies, four domains have been identified: the NH2 terminal, with sequence homologies to the actin-binding domain of alpha-actinin; a long spectrin-like repeat domain, referred to as rod-like; a cysteine-rich domain with a weak homology to alpha-actinin; and finally a COOH-terminal domain which shows similarity to an autosomal analogue of dystrophin (18).

Cell fractionation studies suggested that dystrophin may be associated with triads and, in particular, with their T-tubule component (11), but subsequent immunocytochemical studies revealed that dystrophin is localized at the periphery of the muscle fiber close to the plasma membrane, with no correlation with the T-tubule system (6). A periodical distribution of dystrophin, was reported by using anti-rod-like fraction antibodies (6, 22), while a uniform labeling was found by using anti-NH2-terminal antibodies (4).

An electron microscopic study, carried out on cryosections, using a colloidal gold (c.g.) conjugated antibody against the rod-like domain of dystrophin, suggested that this portion of the protein is normally arranged close to the cytoplasmic face of the plasma membrane and that the dystrophin molecules form an interconnecting network (6).

It is thought that the NH2- and COOH-terminal domains may mediate the binding between the cytoskeleton and a membrane glycoprotein complex, composed of four or more subunits (3, 8, 9), some of which may be integral glycoproteins: in fact a reduction of membrane glycoprotein binding lectins was demonstrated in DMD muscle (9).

A considerable interest is now focused on the structure and function of the COOH-terminal domain: it has been shown by using a mAb against a peptide in the COOH-terminal end of the dystrophin molecule, that the COOH terminus is preserved in BMD and absent in DMD patients (1), supporting the hypothesis that in BMD the reading frame of the gene is maintained (17).

To investigate the function of dystrophin, efforts have been made to precisely localize the molecule with respect to the cytoplasmic membrane. Morphological investigations, based on the use of isolated sarcolemmal vesicles, showed at the

1. Abbreviations used in this paper: BMD, Becker muscular dystrophy; c.g., colloidal gold conjugated antibody; CPD-FDL, critical point drying fracture label; DMD, Duchenne muscular dystrophy; E, exoplasmic; P, protoplasmic; TSFL, thin section fracture label.
light microscope level, specific labeling for both NH₂- and COOH-terminal domains localized at the cytoplasmic face of the plasma membrane (23). On the other hand, it has been hypothesized that the COOH-terminal fraction is localized in the plasma membrane, closer to the outer than to the inner membrane half, by means of calculations on the statistical distribution of the label on muscle cryosections, using a polyclonal antibody (7). A model of the dystrophin-associated glycoproteins, based on immunochemical methods, has been recently proposed in which the dystrophin is localized in the subsarcolemmal region where it could mediate the binding between cytoskeletal F-actin and membrane glycoproteins (at the COOH- and NH₂-terminal ends, respectively) (8).

The aim of this study is to localize the COOH-terminal fraction, at the electron microscopic level, on the whole muscle cells. For this purpose we used a mAb (raised against a synthetic polypeptide corresponding to the last 17 amino acids of the COOH-terminal end of dystrophin) for pre-embedding labeling of cryosections and frozen fractured samples (fracture-label). The latter technique allows one to split the membranes along a middle plane, obtaining two distinct membrane halves (19) to which the observed label can be definitely ascribed.

Materials and Methods

Normal human quadriceps femoralis fragments, obtained from biopsies of non-dystrophic patients, and rat soleus samples were fixed in 0.01% glutaraldehyde—2% paraformaldehyde in 0.1 M sodium phosphate buffer (PB) for 1 h at 4°C.

Cryoultramicrotomy

The samples were cryoprotected in 2.3 M sucrose for 90 min, frozen in liquid nitrogen (LN₂), and sectioned at ~90°C in a Reichert Ultracut microtome equipped with FC4 cryosectioning unit. After immunolabeling (see paragraph), the sections were impregnated with Epon and stained with uranyl acetate and lead citrate.

Fracture-label

The samples were cryoprotected with 30% glycerol in 0.1 M PB for 20 min at room temperature. One group of them underwent critical point drying fracture label (CPD-FL) (19): for this purpose they were mounted on gold supports, frozen in freon 22-LN₂, fractured in a Balzers BAF 400 D apparatus, and thawed in 30% glycerol in PB. After immunolabeling (see paragraph), they were dehydrated with ethanol, dried at the critical point in a Balzers CPD 030 apparatus, replicated with 2 nm platinum at 45° and 20 nm carbon at 90° in Balzers BAF 400 D, and digested in sodium hypochlorite.

A second group underwent thin section fracture label (TS-FL) (19). After freezing in freon 22-LN₂, they were fractured in LN₂ with a precooled metal pestle; thawing, labeling, and dehydration were the same as for CPD-FL. Then the samples were embedded in Epon and sectioned with a Reichert Ultracut ultramicrotome. The sections were stained with uranyl acetate and lead citrate. After the labeling, some debris of fractured tissue was used for the following technique.

Sectioned Replica

Debris of fracture-labeled tissue was embedded in 4% gelatin in PB, cross-linked with 2.5% glutaraldehyde for 1 h, cryoprotected in 30% glycerol-0.1 M PB, frozen in freon 22-LN₂, fractured, and shadowed with 2 nm Pt at 45° in Balzers BAF 400 D (carbon coating was deliberately omitted). These samples were then processed with the sectioned replica technique (20): thawing in 30% glycerol-0.1 M PB, dehydration in ethanol, and embedding in Epon. To obtain broad replicated areas immersed in the thickness of the sections, the plane of cutting was parallel to the replicated surface.

Immunolabeling

All samples were washed in PBS, pH 7.2, containing 0.5% BSA (Sigma Chemical Co., St. Louis, MO) and 0.15% glycine, then in PBS-BSA, and incubated with 5% normal goat serum (Sigma Chemical Co.) in PBS-BSA for 30 min at room temperature. The samples were then incubated for 1 h with undiluted primary antidystrrophin mAb Dys 2 (raised in the mouse against a synthetic polypeptide corresponding to the COOH-terminal 17 amino acids of dystrophin [Novocastra, Newcastle-Upon-Tyne, UK]) at room temperature, washed in PBS-BSA, and incubated with the secondary antibody-collodial gold complex (goat-antimouse conjugated with 10 nm c.g.) (Janssen, Belgium), 10% in PBS-BSA, and finally washed with PBS-BSA. Controls were incubated in PBS-BSA instead of the first antibody. In these conditions the label was completely absent (data not shown). The Dys 2 antibody, tested in immunohorror analysis, recognizes a single band of ~400 kDa, as reported by Clerk et al. (5) (data not shown).

After labeling the samples were fixed in 2.5% glutaraldehyde in 0.1 M PB and processed for the different techniques mentioned above. On some cryosections the reaction was amplified with IntenSE EM Silver Enhancement Kit (Amersham International, Amersham, UK) for 7 min.

Results

Cryoultramicrotomy

No background label is present in cryosections, neither in the outer space, nor in the myofilaments and in the sarcoplasmic reticulum membranes. The labeling is present exclusively along the sarcolemma, while the endomysium is free of granules (Fig. 1 a). In those areas where the endomysium has been detached, the sarcolemma is much more intensely labeled (Fig. 1 c) than in the areas where the endomysium is still present (Fig. 1 b). In this case, a mechanical damage of the membrane probably occurs during cutting, causing a situation similar to TS-FL described below. However, the membrane damage cannot be controlled on cryosections; moreover, the localization of the c.g. granules with respect to the membrane cannot be finely determined because of the larger size of the Ab-gold complex in comparison with the membrane thickness.

Fracture-label

For defining the fractured surfaces, the nomenclature proposed by Branton et al. (2) has been followed. In thin-sectioned-fractured-labeled samples the labeling is found only along the profiles of fractured sarcolemma, while contiguous membranes of endothelial cells are completely negative (Fig. 2). In the muscle cells the label is localized in close association with the profile of the fractured membrane, specifically on the protoplasmic (P) half (2), while the exoplasmic (E) half as well as the myofilaments and the collagen fibers (Fig. 3, a and b) are free of c.g. granules. The

Figure 1. Human muscle, cryosectioning. In two adjacent muscle cells (a, silver enhancement), the c.g. granules are detectable only along the sarcolemma (arrows) of the cell in which the endomysium (En) has been detached; no background is visible. The intensity of labeling is greatly dependent on the presence of the endomysium: where it is present only a few granules (arrows) are visible (b), while numerous granules are present all along the sarcolemma after the endomysium detachment (c). M, myofilaments.
membrane often appears rearranged (Fig. 3, c and d) as usual in frozen-fractured and thawed samples (19). In the samples processed with the CPD-FL method, the fractured areas are not only seen as profiles, but also as "en face" surfaces; the c.g. granules are adhering to the replica and are seen superimposed on it. More precisely, the c.g. granules were localized on the tissue and covered with the platinum layer (replica). The digestion in sodium hypochlorite removes the tissue, but the c.g. granules remain attached under the replica, making them appear superimposed on the platinum layer. The general aspect of this kind of replica is quite different from routinely freeze-fractured and replicated samples, due to the fact that in the CPD-FL method the samples are replicated after thawing, labeling, and chemical dehydration-CPD steps. The cell morphology, however, is quite well preserved: myofilaments are easily recognized, as well as the membrane P halves at the myofiber border (Fig. 4, a and c). The E half of the membrane is difficult to identify; therefore, the absence of labeling on it relies on the observation of analogous fields in the TS-FL method.

The labeling pattern in these samples (Fig. 4, a and b) is comparable with that of cryosections: when the membranes are cross fractured together with the endomysium, the c.g. granules are localized at the periphery of the myofibers, in the plasma membrane zone. When the fracture has gone in the membrane plane, splitting away the external half and the endomysium, the P half is intensely labeled (Fig. 4, c and d). The cytoplasm, collagen, and endomysium show no background labeling.

**Sectioned Replica**

The sectioned replica technique is a useful method for comparing, in the same picture, the morphology obtained by two different techniques: freeze-fracture replication and sectioning, i.e., in our experiment, CPD-FL and TS-FL. For this purpose we replicated some labeled tissue fragments, obtained from the TS-FL method before the dehydration. Also the embedding of samples processed for the CPD-FL, in place of the tissue digestion step, should give analogous results but with a worse replica quality. The labeling shown on these samples is comparable (for the sectioned portion) with that of the TS-FL, while on the replicated portion only few c.g. granules are visible, due to the limited extension, in the section thickness, of the replicated rim. The c.g. granules are present along the profile of the P membrane half either on the sectioned portion or on the replicated portion (Fig. 5 a); and, if the second fracture has gone in the same plane of the first one, some granules appear shadowed (Fig. 5 b).

**Discussion**

The Dys 2 mAb, which in Western blot identifies a single band of ~400 Mr, in the experimental conditions for immunocytochemistry presents a high specificity since only the sarcolemma is labeled, while the membranes of the other cells and the cytoplasmic structures are completely negative.

The cytochemical reaction produces, on cryosections, a
Figure 3. Rat muscle, TS-FL. In a, the c.g. granules (arrows) are concentrated along the protoplasmic membrane half profile (P) of the lower fiber; fragments of the exoplasmic membrane half (E) adhering to the endomysium (En) are unlabeled. The membrane of the upper fiber (arrowheads) has not been fractured and is, therefore, unlabeled. In b (incomplete fracture), both the unlabeled exoplasmic (E) and the labeled protoplasmic (P) profiles of the sarcolemma are visible. Details of the P half are visible in c and d. In all figures, membrane discontinuities and blebs are visible, which derive from postfracture rearrangement of the half membrane layers.
Figure 4. Human muscle, CPD-FL. (a) Low magnification of a cross fractured muscle fiber. M, myofilaments; C, collagen. b shows detail of the squared area in a; c.g. granules are visible along the membrane profile (arrows). (c) Low magnification of the "en face" fractured membrane; the protoplasmic membrane half (P) has been partially exposed by the fracture which then has gone through the muscle cell; M, myofilaments. d shows detail of the squared area in c; groups of c.g. granules are present on the protoplasmic layer; the labeling is quite intense, no background is present inside the cytoplasm.

Figure 5. Rat muscle, sectioned replica. The label is present only on the surfaces exposed by the first fracture (analogous to the TS-FL samples); the replicated areas have been exposed by the second fracture. Where the two fracture planes converge, some c.g. granules are visible superimposed to the replica (a and b, arrowheads). On the sectioned areas the profiles of the P membrane half are labeled as in TS-FL (a, arrows). Mi, mitochondrion; M, myofilaments; G, gelatin. In b a small area of labeled P membrane half (shown at lower magnification in the insert) has been intersected by the second fracture plane, exposing some c.g. granules to the platinum shadowing (arrows); Mi, mitochondrion.
constant labeling along the sarcolemma. It is noteworthy that where the endomysium has been detached from the plasmalemma, the labeling on the latter is markedly increased, suggesting that specific antigens could have been exposed allowing more c.g. granules to be bound and all of them to be viewed, even those which are localized on the membrane surface in the section thickness. The probable mechanism of the splitting of the endomysium, which could involve the membrane, could be the mechanical stress during sectioning (13), analogous to the fracturing process in the freeze-fracture technique. The precise localization of the label with respect to the membrane is not possible on cryosections, unless using theoretical calculations, by which Cullen et al. (7) has recently proposed an intramembrane localization of dystrophin. In fact, the possibility that the c.g. granules may be localized in the range of 40 nm from the epitope (6) makes the cryosections, whose thickness is rarely smaller than 100 nm, of little reliability for determining the localization of dystrophin over a structure (the membrane) whose thickness is \( \sim 8 \) nm.

For localizing the dystrophin COOH-terminal fraction we used preembedding labeling and the fracture-label technique. This method enabled us to carry out the reaction on two distinguishable membrane halves, as well as to minimize the manipulation of the sample, because the most denaturing steps are carried out only after the immunolabeling. Moreover, it is possible, by the sectioned replica technique, to localize the c.g. granules, present in the section thickness, with respect to the replicated structures at the sample fracture surface, and to directly compare the two different FL methods. The main drawbacks of the sectioned replica are, however, the limited extension of the replica (proportional to the section thickness) and the scarce probability of finding c.g. granules at the replica surface.

The label in TS-FL samples is present only on the profiles of the P membrane half. The resolution of the cell substrutures is increased in comparison with cryosections, but the number of visible c.g. granules is smaller due to the section thickness. The E membrane half is distinctly visible and appears devoid of c.g.; this suggests that dystrophin does not remain attached to the external layer of the plasmalemma during fracture and agrees with the reported internal localization of dystrophin in isolated membrane vesicles (23). The localization of c.g. on the P membrane half is confirmed by the CPD-FL technique, which allows us to look at the fractured membrane from above: small groups of c.g. granules appear on the membrane layer still attached to the protoplasm; in cross fractured membranes the localization of c.g. granules resembles that of cryosections and TS-FL. Since the identification of E membrane halves by CPD-FL is very difficult in muscle samples, the absence of labeling on them can be determined only on TS-FL samples. The presence of the c.g. marker on the P membrane half should not be misunderstood as a localization on a true protoplasmic fracture face which is normally isolated from the aqueous environment. In frozen-fractured and thawed tissues the observation of labeled hydrophilic groups on an apparently hydrophobic membrane surface is likely to be allowed by the local rearrangement of the membranes after the fracture. During this process, in fact, it is possible that membrane proteins facing the protoplasm are shifted towards the external environment, where they can be labeled as well as the proteins which were immersed in the inner lipid layer, exposed by the fracture. This process could also involve membrane-associated proteins, such as dystrophin, which although it is not a membrane protein, is known to be tightly attached to the membrane protoplasmic surface (9, 23).

We found, in fact, a particularly evident increase in labeling on cryosections, where the sarcolemma has been mechanically damaged following the detachment of the endomysium; moreover, in TS-FL we could directly find traces of the rearrangement as blebs and discontinuities of the membrane.

We determined the localization of the COOH-terminal fraction of dystrophin by labeling after thawing the fractured sample, i.e., after the rearrangement has happened. Since we split up the membrane before labeling and find c.g. granules only on the P membrane half, our data suggest that the COOH-terminal domain is associated with the internal membrane layer, probably with the COOH hydrophilic end originally on the protoplasmic surface, and that it is not possible to distinguish between protoplasmic fracture face and protoplasmic surface localization by the FL technique. According to previous hypothesis on membrane protein partition (21), the observation that dystrophin does not randomly follow the partition of the membrane lipid layers should suggest that it is maintained attached to the protoplasm by stable interactions, probably with the cytoskeleton, and that the reported binding to membrane glycoproteins (which should have a transmembrane domain) (8) does not affect this preferential partition.

The authors would like to thank Dr. L. Merlino (Ist. Ortopedici Rizzoli) for consenting to the use of human biopsy material, and Mr. A. Valmori (Ist. Citomorfologia N.P. Consiglio Nazionale Ricerche) for the excellent photographic work.

This work was partially supported by grants from Ministero Pubblica Istruzione 1992 and “Ricerca Corrente 1992” Istituti Ortopedici Rizzoli, Italy.

Received for publication 20 February 1992 and in revised form 22 June 1992.

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