Dihydropyridine Receptor α Subunits in Normal and Dysgenic Muscle In Vitro: Expression of α_1 Is Required for Proper Targeting and Distribution of α_2

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Abstract. We have studied the subcellular distribution of the α_1 and α_2 subunits of the skeletal muscle dihydropyridine (DHP) receptor with immunofluorescence labeling of normal and dysgenic (*mdg*) muscle in culture. In normal myotubes both α subunits were localized in clusters associated with the T-tubule membranes of longitudinally as well as transversely oriented T-tubules. The DHP receptor-rich domains may represent the sites where triad junctions with the sarcoplasmic reticulum are being formed. In cultures from dysgenic muscle the α_1 subunit was undetectable and the distribution patterns of the α_2 subunit were abnormal. The α_2 subunit did not form clusters nor was it discretely localized in the T-tubule system. Instead, α_2 was found diffusely distributed in parts of the T-system, in structures in the perinuclear region and in the plasma membrane. These results suggest that an interaction between the two α subunits is required for the normal distribution of the α_2 subunit in the T-tubule membranes. Spontaneous fusion of normal non-muscle cells with dysgenic myotubes resulted in a regional expression of the α_1 polypeptide near the foreign nuclei, thus defining the nuclear domain of a T-tubule membrane protein in multi-nucleated muscle cells. Furthermore, the normal intracellular distribution of the α_2 polypeptide was restored in domains containing a foreign "rescue" nucleus; this supports the idea that direct interactions between the DHP receptor α_1 and α_2 subunits are involved in the organization of the junctional T-tubule membranes.

THE dihydropyridine (DHP)[1] receptor of skeletal muscle plays a major role in the transduction of membrane depolarization into muscle contraction, called excitation–contraction (E–C) coupling. Located in the membrane of the T-tubules, the DHP receptor is believed to function as voltage sensor in E–C coupling as well as slow, L-type Ca^{2+} channel (see reference 3 for review).

The purified DHP receptor isolated from T-tubule membranes is composed of at least four polypeptides. The α_1 subunit has a molecular mass of 170–200 kD, carries the DHP-binding site (5, 12, 41, 42) and reacts with a mAb that inhibits slow Ca^{2+} currents (28). The cDNA encoding this protein has been cloned and shown to contain the characteristics of a voltage-gated ion channel (43). The presence of a sequence similar to the putative voltage-sensing element of the Na channel (29) supports the idea that the α_1 subunit of the DHP receptor is the voltage sensor involved in E–C coupling. The expression of the cDNA encoding the α_1 polypeptide in L-cells was accompanied by the appearance of Ca^{2+} currents, indicating that this subunit alone is sufficient for channel activity (33).

Three other polypeptides copurify with the α_1 subunit of the DHP receptor: α_2, β, and γ. However, the functions of these polypeptides as well as their possible interactions with the α_1 polypeptide are unknown. The α_2 subunit is a glycoprotein of 140 kD molecular mass under reducing conditions and 170–200 kD without reduction (24, 26, 42, 45, 46). The differences in molecular mass values upon reduction of the disulfide bonds may be accounted for by the dissociation of a small polypeptide, referred to as the δ subunit. The primary structure of the α_2 polypeptide contains three putative membrane spanning domains and a large extracellular domain but it does not show any sequence similarities to other known proteins (10). Although the α_2 polypeptide is not required for DHP sensitive Ca^{2+} conductance (33), coexpression of the α_2 subunit with the cardiac α_1 subunit in Xenopus oocytes causes a doubling of the Ca^{2+} current (25). We have previously demonstrated the colocalization of the α_2 subunit and the α_1 subunit in the membranes of the junctional T-tubules of skeletal muscle in vivo (13). During development, however, the two α polypeptides are expressed differentially in that α_2 is abundant in muscle of newborn rats while levels of α_1 subunit expression are initially low but rise dramatically two weeks postnatally (27). These results suggest that the function of the α_2 subunit may not be limited to its association with the muscle DHP receptor.

1. *Abbreviations used in this paper*: DHP, dihydropyridine; E–C, excitation–contraction; HS, horse serum; *mdg*, muscular dysgenesis; SR, sarcoplasmic reticulum.

Muscular dysgenesis (*mdg*) in mice is a lethal recessive mutation characterized by the inability of skeletal muscle to contract (6, 30). Myotubes grown in culture from muscle of homozygous *mdg/mdg* mice lack E-C coupling (21, 36), the slow DHP-sensitive Ca^{2+} current (2), as well as the charge movement associated with voltage sensing in this process (1). Microinjection of an expression plasmid carrying the gene of the α_1 subunit of the skeletal muscle DHP receptor can restore E-C coupling and the slow Ca^{2+} current (1, 44). These results suggest that the primary defect of the *mdg* mutant is in the gene of the α_1 subunit. The mutation is believed to reside in the structural gene, resulting in a failure to express the α_1 polypeptide. The lack of the α_1 subunit does not seem to alter the expression of the DHP receptor α_2 subunit (22), however, the cytological distribution of the α_2 subunit in dysgenic muscle has not been described.

Co-cultures of dysgenic myotubes with spinal cord cells and fibroblasts from normal mice have also been shown to restore E-C coupling coupling (8, 23) and slow Ca^{2+} conductance (2, 40). This "rescue" of normal functions in dysgenic muscle cells has been explained by spontaneous fusion of normal non-muscle cells with dysgenic myotubes (7). Normal non-muscle nuclei that are incorporated into the defective myotubes are believed to be capable of expressing the α_1 polypeptide and thus reconstituting E-C coupling functions. However, direct evidence for the de novo expression of the α_1 polypeptide in rescued myotubes is still missing.

In the present study we have used mAbs against the α_1 and the α_2 subunit of the skeletal muscle DHP receptor to study the expression and distribution patterns of the DHP receptor α subunits in cultured myotubes from normal and dysgenic mice. We report the colocalization of both α subunits in clusters associated with the membranes of the developing T-tubules in normal myotubes. Furthermore, we show that the absence of α_1 in dysgenic myotubes causes an aberrant distribution of the α_2 subunit and that the lack of α_1 as well as the abnormal distribution of α_2 can be reversed by rescue with normal non-muscle cells. These results provide strong evidence that an interaction between the α_1 and the α_2 subunit of the DHP receptor is required for their normal organization in the junctional T-tubule membranes.

Materials and Methods

Animals

Newborn and embryonic mice, both homozygous mutant dysgenic, *mdg/mdg*, and their normal littermates, *+/mdg?* (+/+ or *+/mdg*) were obtained by dated pregnancies of heterozygous matings. No morphological, histological, or physiological differences between control +/+ of the strain or *+/mdg?* mice have been reported. Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) were mated overnight and dated pregnancies were assessed by remains of vaginal plugs. 15-d embryonic rats were used to obtain spinal cord cells.

Cell Cultures

Primary muscle cultures were prepared from 18-d embryonic or newborn *mdg/mdg* or *+/mdg?* mice. Myoblasts were harvested using a modification of previously published methods (8, 21). Hindlimb muscle was digested at 37°C for 15 min with intermittent aspiration in a solution of 0.125% trypsin and 0.05% pancreatin in Ca^{2+} and Mg^{2+} free HBSS. Digestion was stopped by adding an equal volume of complete medium, consisting of DME with 10% horse serum (HS; Gibco Laboratories, Grand Island, NY), 10% FBS (Gibco Laboratories), and 2% chick embryo extract. The cell suspension was filtered through gauze and centrifuged. The pellet was resuspended in plating medium which contains three parts complete medium to one part conditioned medium reserved from 5-d muscle cultures. Dysgenic cultures were preplated for 1 h on plastic Petri dishes to enrich for myoblasts which were plated onto carbon-coated coverslips with 0.1% gelatin at 5×10^3 cells per 13-mm coverslip (two coverslips per 35-mm dish). Normal cultures, which tended to have fewer myoblasts and form fewer myotubes if plated by the same method, were preplated at 3.5×10^5 cells per 1% gelatin-coated 60-mm Primaria dishes. After 48 h, as an additional myoblast enrichment step, the normal cultures were treated with Dispase and replated onto coverslips prepared as above (9). Dysgenic cultures treated with Dispase were similar to untreated cultures, so the conventional plating method was followed. Once myotubes had formed and cultures were near confluency, the cultures were treated with 10 μM 1 β-D-arabinofuranosylcytosine hydrochloride, Ara-C, (Sigma Chemical Co., St. Louis, MO) to prevent fibroblastic overgrowth. Cultures were maintained in contraction medium (DME, 10% HS, 1.25% chick embryo extract) in a humidified 37°C incubator. Quiescent cultures (both dysgenic and normal) were fed medium containing 12 mM K^+. Our experience over many years (37) in microscopic and electrophysiological studies of normal and dysgenic muscle proves that spontaneous action potentials (in dysgenic) and contraction in normal myotubes are completely absent at this K^+ concentration. Cultures were fixed two to three weeks following plating.

For "rescued" co-cultures, dysgenic myoblasts were plated as above. Rat cells (spinal cord or fibroblasts) were added at the onset of fusion, 3-5 d after initial plating. The degree of rescue, indicated by the onset of spontaneous contractions, was similar in co-cultures of dysgenic muscle with either spinal cord cells or fibroblasts (8). Co-cultures were treated with Ara-C and maintained in contraction medium as above.

Rat spinal cord cells were prepared from spinal cords of 15-d embryos by a method similar to that used for harvesting embryonic mouse spinal cord cells (8) except that most of the spinal ganglia were retained. Cells were mechanically dissociated in contraction medium and added to fusing dysgenic myoblasts at 1×10^6 cells per 35-mm dish.

Rat fibroblasts were obtained from two cell lines. FR cells (American Type Culture Collection, Rockville, MD) were maintained in DME with 10% HS. Alternatively, fibroblast cells (JRF) were derived from a spontaneous transformation within a culture prepared from newborn rat sciatic nerve sheath. These cells were maintained in DME, 10% HS, 5% FBS. Both fibroblast lines were equally fusible with dysgenic myotubes and were equivalent in their ability to effect rescue. Cells were routinely passed once a week and were added to fusing dysgenic myoblasts at 2×10^4 cells per 35-mm dish. Rescued cultures were fixed 11 to 18 d following addition of rat cells.

Immunofluorescence Labeling of Cultured Muscle Cells

The co-cultures used in the "rescue" experiments were incubated in 10 μg/ml Hoechst nuclear dye #33342 (Polysciences, Inc., Warrington, PA) for 45 min at room temperature and rinsed several times in PBS, pH 7.3, before fixation. All cultures were fixed at −20°C in methanol for 10 min and then rinsed in several changes of PBS. Subsequently, the cultures were incubated with 10% normal goat serum in PBS. 0.1% BSA (PBS/BSA) for 30 min or longer and then incubated in primary antibodies for at least 2 h at room temperature or overnight at 4°C. After washing in five changes of PBS/BSA the cultures were incubated in fluorochrome-conjugated secondary antibodies for 1-2 h at room temperature and washed again. Finally, they were mounted in 90% glycerol, 0.1 M Tris, pH 8.0, with 5 mg/ml ρ-phenylene diamine to retard photobleaching.

Quantitative Analysis of Rescued Cultures

To quantitate the expression of the α_1 subunit in rescued co-cultures, coverslips were screened for myotubes which were free of fibroblastic overgrowth. The analyzable myotubes were scored as positive or negative with respect to expression of the α_1 subunit. Then coverslips were rescreened for myotubes which contained at least one foreign nucleus. Quantitation of normally distributed α_2 subunit in rescued myotubes required more stringency. Myotubes were screened segment by segment (one segment extending ~150 μm) and scored for normal or abnormal α_2 distribution patterns. A few segments with ambiguous staining patterns were not counted. Subsequently, myotubes were rescreened for the presence of foreign nuclei.

Table I. Specificity of Antibodies Used in This Study

Antigen	Code	Type	Tissue sections (in vivo)		Cell culture (in vitro)			Reference
			Rat	Mouse	Rat	Mouse	C_2 Cell	
DHP, α_1	1A	mouse mAb	+*	+	+	+	+	13, 26
DHP, α_2	20A	mouse mAb	+*	+	+	+	nd	13, 27
T-tubule	αTT	rabbit AP	+*	+	+	+	+	14
Sarcolemma	αPM	rabbit AP	+	nd	+	+	nd	14
α-Actinin	–	rabbit AP	+	+	+	+	+	4, 13

The antibodies have been previously used in immunofluorescence and immunogold (*) studies on tissue sections and cultures from rat as well as from mouse skeletal muscle. *AP*, affinity purified antibody.

Antibodies

The following primary antibodies were used: mouse mAb 1A (specific for the α_1 subunit of the DHP receptor) (26) and mAb 20A (specific for the α_2 subunit) (27) both used at a concentration of 0.1 μM IgG. Rabbit affinity purified antibody αTT (against T-tubule proteins), αPM (against plasma membrane proteins) both described in reference 14, and an affinity purified antibody against α-actinin (4). As secondary antibodies, rhodamine-conjugated goat anti-mouse IgG (BCA/Cappel Products, Organon Technika, Malvern, PA) and fluorescein-conjugated goat anti-rabbit IgG (Miles Laboratories, Inc., Elkhart, IL) were used at dilutions of 1:500 and 1:400, respectively. For specificity of antibodies, see Table I.

Results

We have studied the distribution and association of the DHP receptor α_1 and α_2 subunits in muscle cultures of normal and dysgenic mice using mAbs against both DHP receptor α subunits as well as an affinity purified antibody against T-tubule membranes.

Normal Myotubes

Normal mouse muscle cells were cultured for approximately three weeks under conditions permitting a high degree of spontaneous contractile activity. Cultures immunolabeled with antibodies against several myofibrillar components (α-actinin, myosin, actin, and titin) showed that the myotubes in any culture dish reach varying degrees of sarcomeric organization (Flucher et al., unpublished results). Some myotubes showed no cross striation of any of the myofibrillar components. Many myotubes achieved cross-striated organization of myofibrillar components that assemble early in development but showed no cross striations under phase contrast optics. A smaller number of cells were cross-striated with respect to all examined myofibrillar proteins as well as under phase contrast optics. The transverse organization of the T-tubule system, which occurs late in sarcomere formation (14) was achieved only in a small proportion of the myotubes.

Immunofluorescence labeling of myotubes with the antibodies against the α_1 and α_2 subunits of the DHP receptor resulted in punctate labeling patterns, suggesting that both subunits are concentrated in small clusters rather than diffusely distributed in the membranes (Fig. 1). These DHP receptor clusters were located throughout the cytoplasm. Double labeling of either α subunit antibody with the T-tubule antibody (αTT) showed that the DHP receptor clusters in the cytoplasm coincide with the tubules of the developing T-tubule system. In poorly differentiated myotubes containing randomly oriented T-tubules the DHP receptor-rich domains were lined up along the tubules at irregular intervals (Fig. 1, *a* and *c*). In myotubes that had achieved a higher degree of differentiation in their contractile apparatus, T-tubules were longitudinally oriented in between the myofibrils and the DHP receptor-rich domains were first seen periodically aligned along the T-tubules (Fig. 1 *d*). Only after this alignment of DHP receptor-rich domains (presumably representing developing triads) had occurred did the T-tubules themselves rearrange into a transverse orientation. In well-developed regions of myotubes the transverse component of the T-system became prevalent and the longitudinal tubules disappeared (Fig. 1 *b'*; see also Fig. 5, *c* and *d*).

Dysgenic Myotubes

Muscle cultures from homozygous dysgenic mice (*mdg/mdg*) and their normal siblings (+/*mdg*?) were compared with respect to the expression and distribution patterns of the DHP receptor α subunits. Dysgenic myotubes were noncontractile and the rate at which they developed following fusion was slower than that of normal myotubes. A smaller number of myotubes achieved as high a degree of organization in their contractile elements than that found in normal cultures (Flucher et al., manuscript in preparation).

Immunolabeling with the antibody against the α_1 subunit of the DHP receptor under conditions that labeled normal myotubes, resulted in no immunolabeling of dysgenic myotubes (Fig. 2 *a*). The absence of α_1 label was complete in all cells of dysgenic cultures, supporting the idea that the *mdg/mdg* mutation results in the complete absence of α_1 subunit expression in skeletal muscle cells. Double labeling for α_1 and T-tubules (with the αTT antibody) demonstrated the presence of an extensive T-tubule network in α_1 negative cultures (Fig. 2 *b*), suggesting that the development of the T-tubules is not dependent upon expression of the α_1 polypeptide. Immunolabeling of the α_2 subunit of the DHP receptor yielded a strong fluorescent signal in dysgenic myotubes. However, the labeling patterns were different from the α_2 distribution in normal myotubes (compare Fig. 1 *c* and *d* to Fig. 2 *c*). Myotubes that had an apparently normal T-system (Fig. 2 *d*) did not show the normal punctate distribution of DHP receptor-rich domains but showed patches of α_2 label colocalizing only with parts of the T-tubules, whereas much of the T-system contained no α_2 (Fig. 2, *c* and *d*). In many myotubes in which these patches coincided with the T-system, the α_2 patches appeared wider than the typical T-tubules suggesting that they represent distortions of the T-system or of its precursor. In addition to cytoplasmic structures labeled with α_2, diffuse or patchy α_2 labeling of the

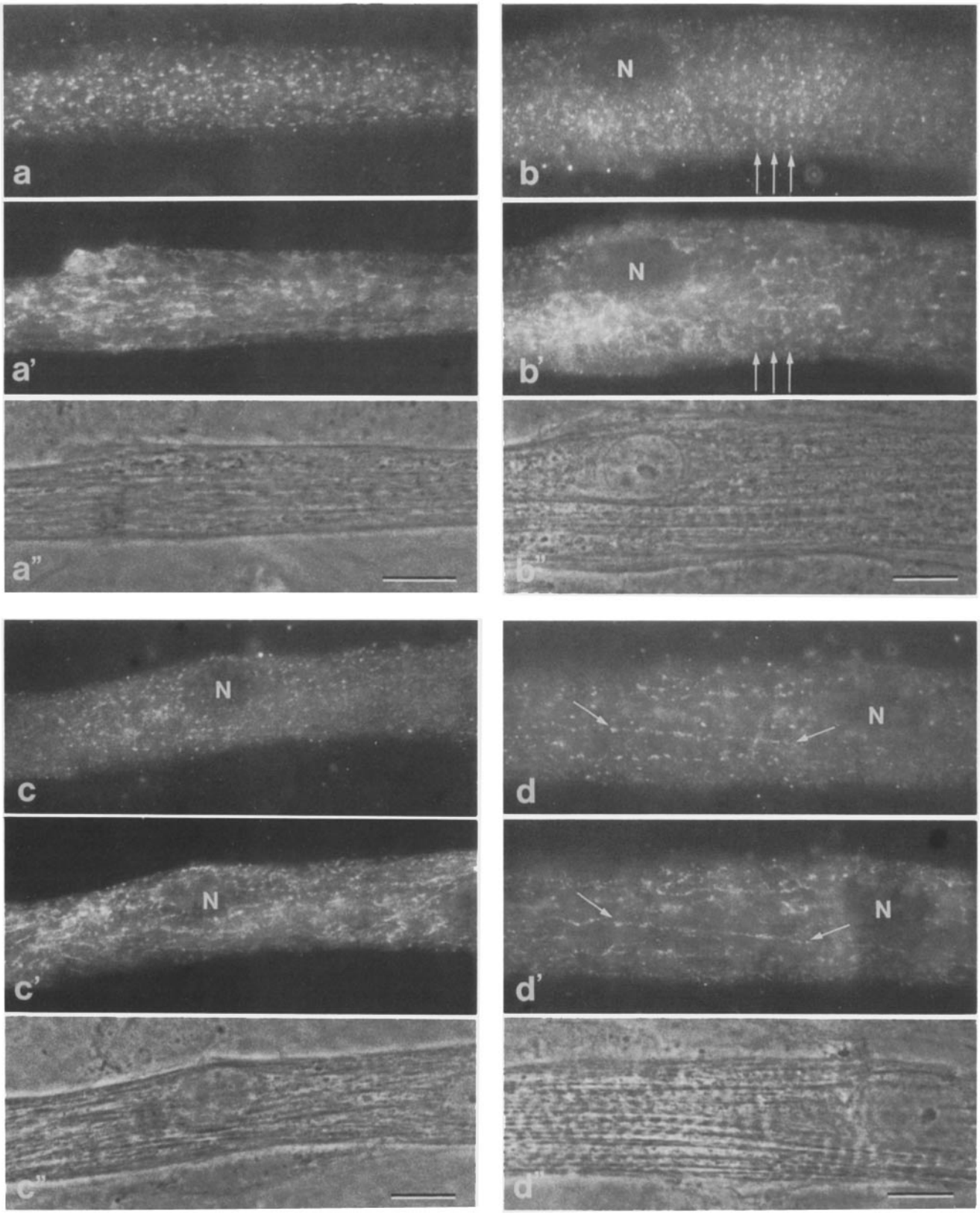

Figure 1. Normal mouse myotubes were double immunofluorescence labeled with antibodies against the α subunits of the skeletal muscle DHP receptor (*a*, *b*, *c*, and *d*) and against T-tubules (*a'*, *b'*, *c'*, and *d'*). The corresponding phase contrast images are shown in *a"*, *b"*, *c"*, and *d"*. Both the α_1 (*a* and *b*) and the α_2 (*c* and *d*) subunits of the DHP receptor appeared in punctate staining patterns, which colocalized with T-tubules at different stages of development: randomly oriented tubules (*a'* and *c'*) as well as longitudinal oriented tubules (between arrows in *d'*) showed no periodicity of the DHP receptor clusters, whereas in more highly differentiated myotubes with beginning transverse orientation of the T-tubules (examples indicated with arrows in *b'*) the DHP receptor clusters were aligned in rows across the myotube (*b*; *arrows*). *N*, nuclei. Bars, 10 μm.

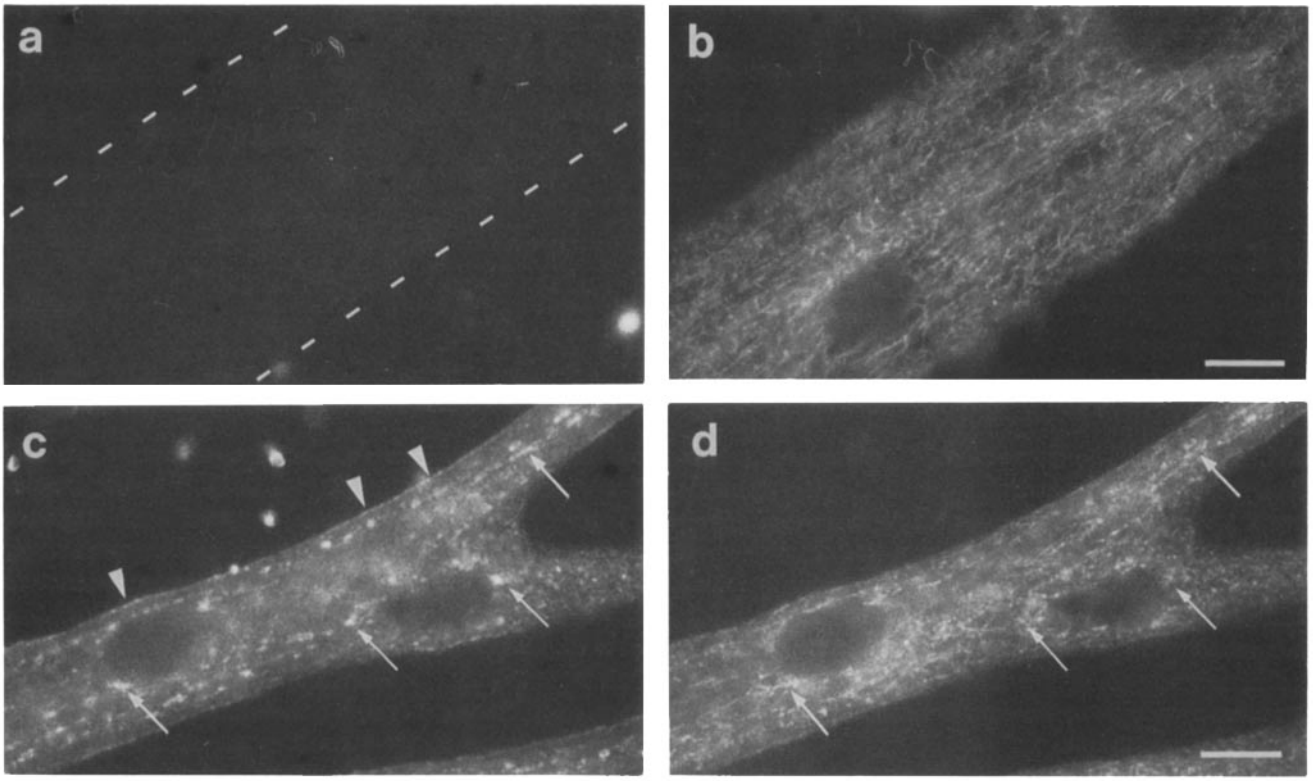

Figure 2. Dysgenic (*mdg/mdg*) muscle cultures double labeled with antibodies against the α subunits of the DHP receptor and with a T-tubule antibody. Dysgenic myotubes showed no α_1 label (*a*; myotube outlined with dashed line) but often labeled intensely for the α_2 subunit (*c*). The structure of the T-tubule system appeared normal (*b* and *d*) but the distribution patterns of the α_2 subunit were abnormal (*c*) in dysgenic myotubules: label for the α_2 subunit was patchy (*arrows*) and colocalized only with parts of the T-system (*c* and *d*). In addition, α_2 label could be observed in the plasma membrane (*c*; *arrowheads*). Bars, 10 μm.

plasma membrane was commonly observed in dysgenic myotubes (Fig. 2 *c*).

The aberrant distribution patterns of α_2 immunolabel varied widely in dysgenic cultures. Two more examples of typical α_2 labeling patterns in dysgenic myotubes with the corresponding fluorescence images of the T-tubule system are shown in Fig. 3 to demonstrate that the normal differentiation of the T-tubule system is not hindered by the lack of organization in the DHP receptor complex. In many cells the T-tubule system appeared normal whereas the α_2 label was restricted to compartments which were frequently located in the perinuclear region and contained α_2 as well as T-tubule antigens (Fig. 3 *a*). These compartments appeared dilated, and more like cisternae than tubular structures. Henceforth these structures will be called α_2/TT-containing compartments. Normal looking T-tubules that did not label with the antibody against α_2 could often be observed in close proximity to the abnormal α_2/TT-containing structures (Fig. 3 *a'*). In some regions of dysgenic myotubes α_2 label was completely absent from cytoplasmic structures. However, even in such extreme cases of α_2 deficiency the T-system developed normally, as seen by the transverse arrangement of the T-tubules (Fig. 3 *b'*). Phase contrast images of dysgenic myotubes show that their general appearance was normal (Fig. 3 *b''*; Fig. 4 *b''*). Double labeling of normal and dysgenic myotubes with antibodies against the α_2 subunit and against the myofibrillar component α-actinin (Fig. 4, *a* and *b*) shows that the myofibrillar development was similar in myotubes of the normal and the mutant phenotype of α_2 distribution. Thus, distorted α_2 distribution patterns do not result from a lack of differentiation or from degeneration of dysgenic myotubes.

We attempted the further characterization of the α_2/TT-containing compartments in dysgenic cells. The perinuclear location of these structures was reminiscent of ER or Golgi apparatus staining and suggested the possibility that the α_2 subunits were partially retained in compartments of the biosynthetic pathway. However, double labeling of myotubes with antibodies against α_2 and markers for the ER or fluorescent WGA showed no colocalization of α_2 with the ER or Golgi apparatus, respectively (not shown). Our observation that α_2 in dysgenic myotubes occurs diffusely distributed or in patches in the plasma membrane, suggests the possibility of faulty targeting of this polypeptide to the plasma membrane. Indeed, double labeling of α_2 with an affinity-purified antibody against plasma membranes that did not cross-react with T-tubules in normal myotubes (14), revealed the existence of specific plasma membrane components in the α_2/TT-containing compartments (Fig. 5, *a* and *b*).

Since *mdg/mdg* myotubes are noncontractile and contractile activity seems to promote differentiation of cultured mouse muscle cells (37), we compared the aberrant α_2 distribution patterns in dysgenic myotubes with the distribution patterns of "quiet" normal (+/*mdg?*) cultures (Fig. 5, *c* and *d*). The lack of contractile activity achieved by elevating the KCl concentration in the culture medium to 12 mM (37) had

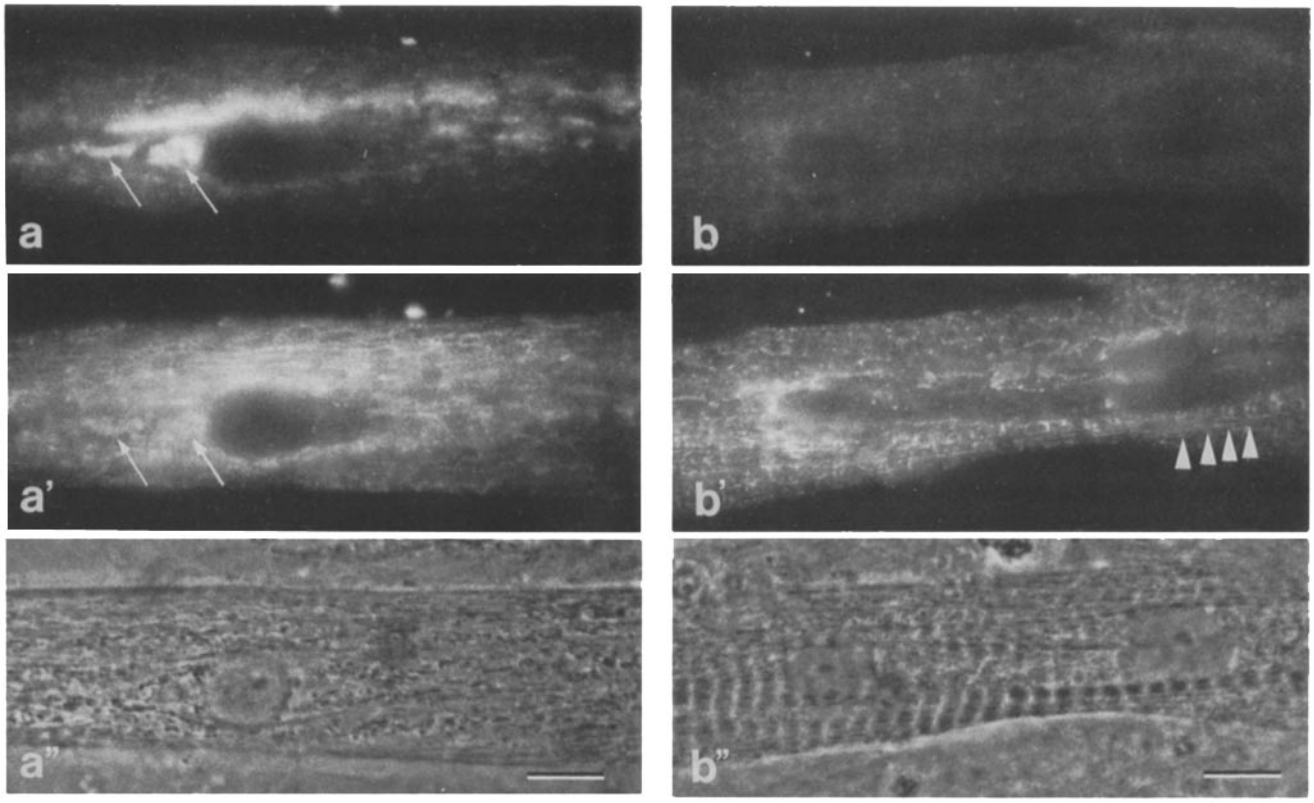

Figure 3. Two examples of abnormal α_2 labeling patterns in dysgenic myotubes with poorly (*a*) and highly developed T-tubules (*b*). Myotubes were double labeled for the DHP receptor α_2 subunit (*a* and *b*) and T-tubules (*a'* and *b'*); the corresponding phase contrast images are shown (*a"* and *b"*). Immunolabel for the α_2 subunit did not occur in punctate patterns as observed in normal cultures. Instead, α_2 was frequently colocalized with T-tubule specific antigens in structures preferentially located in the perinuclear region (*a*; *arrows*) while normal, randomly oriented T-tubules (upper portion of the myotube) were devoid of α_2 label. Little or no α_2 was expressed in the myotube shown in *b*, however, the T-tubules achieved the highest degree of organization reached in culture, as indicated by their transverse orientation at the I-A interfaces (*arrowheads*). Bars, 10 μm.

no apparent effect on the distribution of the DHP receptor α_2 subunits. Normal quiescent cultures did not exhibit as many cross-striated myotubes as active cultures. However, in many of the normal myotubes rendered quiescent for 14 d myofibrillar cross striations were apparent (not shown) and the α_2 subunits occurred in normal clusters colocalized with the T-tubules of longitudinal as well as cross-striated organization (Fig. 5, *c* and *d*). The α_2/TT-containing compartments seen in dysgenic myotubes were not found in silent normal cultures, suggesting that the lack of contraction is not responsible for the loss of normal α_2 distribution in dysgenic cells.

Rescued Myotubes

Dysgenic muscle cells are capable of spontaneously fusing with normal muscle as well as with non-muscle cells (7) and such fusion has been shown to restore slow Ca^{2+} currents as well as E–C coupling to dysgenic myotubes (8). Fusion of *mdg/mdg* myotubes with rat non-muscle cells created heterokarya of mouse myotubes with a small proportion of rat nuclei, which could be reliably distinguished from the host cell nuclei by the lack of heterochromatin that characterizes the mouse nuclei (see Figs. 6 and 7).

Immunolabeling of dysgenic myotubes co-cultured with normal non-muscle cells with the antibody against the DHP receptor α_1 subunit resulted in regionally confined labeling of some myotubes (Fig. 6, *a* and *c*). Double labeling with the fluorescent nuclear dye (Hoechst #33342) showed that these α_1-positive regions always coincided with at least one rat nucleus (Fig. 6). However, not all rat nuclei incorporated into dysgenic myotubes displayed α_1 expression. Quantitation of α_1 expression with respect to the number of rat nuclei incorporated into dysgenic cultures showed that 32.7% ($\pm$3.2) of all myotubes (n = 149) possessed foreign nuclei and 10.9% ($\pm$1.3) of all myotubes expressed the α_1 polypeptide in regions where at least one foreign nucleus was located. Thus, a third of the rat non-muscle nuclei incorporated into *mdg/mdg* myotubes were expressing the α_1 subunit of the skeletal muscle DHP receptor and could therefore be considered "rescue" nuclei.

The distribution patterns of α_1 in rescued myotubes were punctate (Fig. 6, *a* and *c*) and the DHP receptor clusters colocalized with the T-tubules (not shown). This organization resembled that of the DHP receptor-rich domains of normal myotubes. Furthermore, in rescued myotubes the α_1 antibody did not label any cytoplasmic structures similar to the α_2/TT-containing compartments found in dysgenic myotubes. The segment of the myotube in which α_1 was expressed could be as short as 40 μm or up to 500-μm long with an average of 270 μm in length. Typically it would con-

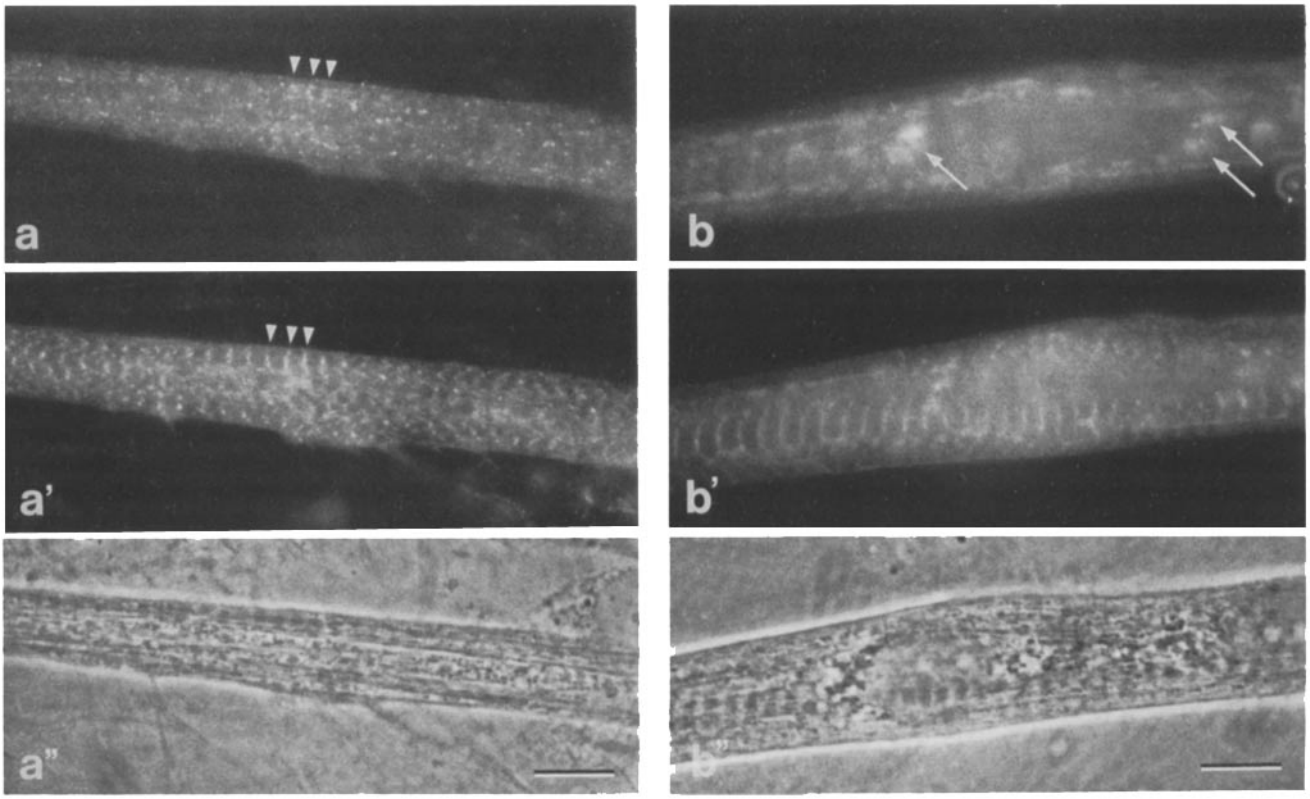

Figure 4. Normal and dysgenic myotubes double labeled for the DHP receptor α_2 subunit (*a* and *b*) and the myofibrillar protein α-actinin (*a'* and *b'*), with the corresponding phase contrast images (*a"* and *b"*). Punctate (*a*) and dysgenic α_2 labeling patterns (*b*) can be observed in normal and *mdg/mdg* myotubes, respectively, which show the same degree of myofibrillar differentiation. Whereas in normal myotubes α_2 clusters begin to align transversely in regions of α-actinin cross-striations (*arrowheads*), no relationship between the α_2 patches (*arrows*) and α-actinin cross striations can be observed in dysgenic myotubes. Bars, 10 μm.

tain three to four mouse nuclei in addition to the rat rescue nucleus (Fig. 6 *b*). The position of the rescue nucleus within this α_1-positive domain was highly variable and in some cases the rat nucleus was located at the very edge of the labeled region.

Triple labeling with antibodies against α_2, αTT and the fluorescent nuclear dye showed that regions of normally clustered α_2 distribution (Fig. 7, *a* and *d*) always occurred at or adjacent to a rat nucleus (Fig. 7). Quantitation of these results revealed 36.4% (±2.4) of analyzable myotube segments (n = 118) contained a rat nucleus, but only 16.9% (±0.9) of the segments displayed a normal labeling pattern for the α_2 subunit. Thus 46% of the segments containing a rat nucleus were rescued with respect to the α_2 subunit. The T-tubules appeared normal in these rescued regions and no α_2/TT-containing compartments were observed (Fig. 7, *a*, *c*, *d*, and *f*). However, the plasma membrane in these domains commonly displayed diffuse α_2 staining (Fig. 7 *d*) suggesting that normal organization of α_2 was not completely restored in the region of the rescue nuclei.

Discussion

Normal Distribution of the α Subunits of the DHP Receptor

In primary cultures of normal skeletal muscle the α_1 and the α_2 subunit of the DHP receptor were localized in clusters. This distribution pattern is similar to that observed in the myogenic cell line C2 using the same antibody (M. E. Morton, Holy Cross College, Worcester, MA; personal communication). The clusters of DHP receptors colocalized with the tubules of the developing T-tubule system, a finding that is consistent with the localization of the DHP receptor in the T-tubule membranes of mature muscle (13, 16, 20, 24). The non-uniform distribution of DHP receptors in the developing T-tubules suggests that the T-tubule membranes consist of at least two membrane domains: DHP receptor-rich domains, which may be the sites where triad junctions with the SR form, and unspecialized membrane regions in between the DHP receptor clusters. Electron microscopic studies show that junctions between T-tubules and SR (diads, triads, and other constellations) are formed in myotubes of comparable developmental stages in vivo (17, 34) and in vitro (8, 40). Presently we do not have evidence as to whether DHP receptor-rich domains exist in the absence of a close contact with the SR membranes. It is conceivable that DHP receptor clustering precedes the first interactions with the SR and that these specialized domains of the T-tubule membranes subsequently form junctions with the SR. However, reports that triads can be found in rare cases in the absence of the DHP receptor α_1 subunit in dysgenic muscle cells (8, 34) and more frequently in dysgenic myotubes treated with calcitonin gene-related peptide (18) suggest that DHP receptor α_1 subunit clustering is not a prerequisite for triad formation.

Our finding that both α subunits appeared as clusters with

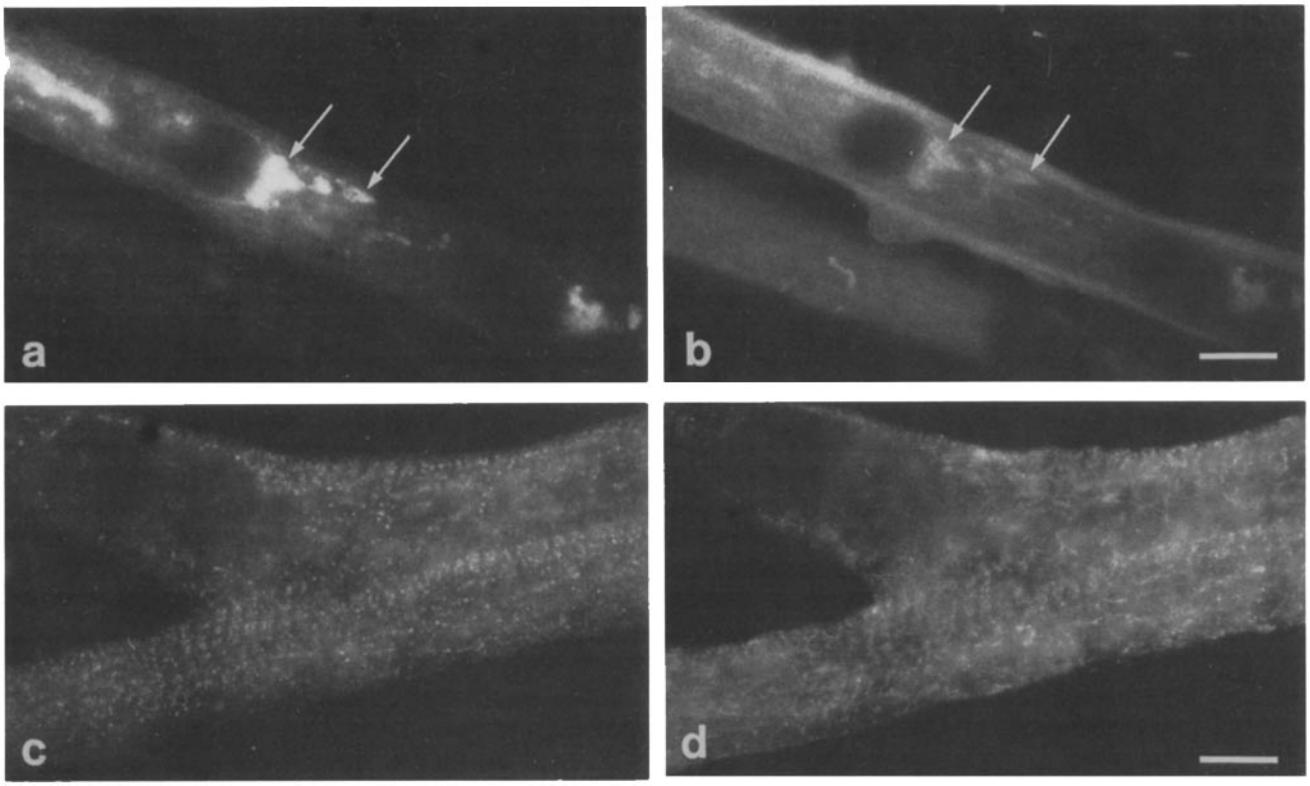

Figure 5. Further characterization of α_2 distribution (control experiments). Dysgenic myotubes double labeled with α_2 and an antibody against plasma membrane proteins (*a* and *b*, respectively) showed both antigens colocalized in a structure in the perinuclear region (*arrows*). This colocalization was found in myotubes with and without diffuse α_2 label in the plasma membrane. Normal myotubes were grown under conditions prohibiting muscle contraction and were double labeled with α_2 (*c*) and T-tubule antibodies (*d*). The distribution patterns of both antigens were normal and showed a high degree of differentiation in the E–C coupling system, suggesting that inactivity did not impale the normal distribution of the α_2 subunit or the differentiation of the T-system. Bars, 10 μm.

identical distribution patterns in cultured muscle suggests that the α_1 and α_2 subunits are colocalized in the DHP receptor-rich domains developing in muscle in vitro. Using the same antibodies as in the present study, Western blot analysis revealed that the expression of the α_1 and α_2 subunits during development is differentially regulated (27). Skeletal muscle of newborn rats expresses the α_2 subunit in substantial amounts and its concentration increases steadily over a period of three weeks. In contrast, expression of the α_1 subunit is very low at birth but increases dramatically two weeks later. In the present immunocytochemical study on cultured muscle, however, we have found no evidence for a pool of the α_2 subunit apart from the α_1 subunit. On the contrary, the localization of both subunits seemed to be closely associated in normal myotubes of all developmental stages observed.

Localization of the DHP Receptor α Subunits in Dysgenic Muscle

Immunolabeling of dysgenic myotubes with the antibody against the α_1 subunit gave no detectable signal in any of the myotubes. The *mdg* mutation is believed to reside in the structural gene, resulting in the lack or severe underexpression of the α_1 polypeptide (22, 44). Our results support this idea by demonstrating the complete absence of the α_1 subunit on the cellular level. If there were still a low level of α_1 expression and the remaining subunits behave normally, one could expect them to form DHP receptor clusters, which would be detectable with immunofluorescence, in reduced quantities. However, the total lack of α_1 label shown in our experiments provides further evidence that the α_1 subunit is not expressed in muscles of dysgenic mice.

The α_2 subunit of the DHP receptor was expressed in dysgenic myotubes, confirming existing evidence from immunoblot analysis of dysgenic muscle (22). However, the distribution patterns of α_2 were grossly altered in *mdg/mdg* cultures. Normal DHP clusters were not found. Parts of the T-tubule system were devoid of α_2 and wherever α_2 and the T-tubule antigens were colocalized in the cytoplasm, the α_2/TT-containing structures were distinct from normal T-tubule profiles. In electron microscopic studies of dysgenic muscle in vivo and in vitro swollen membrane compartments have been noted (35; Powell, unpublished results), and it is possible that some of the membrane compartments described as swollen SR may actually correspond to the α_2/TT-containing structures seen with immunofluorescence. The frequent observations of diffuse α_2 label in the plasma membrane of dysgenic myotubes and the finding that the plasma membrane specific components are colocalized with the perinuclear α_2/TT-containing structures suggests that the lack of the α_1 subunit may cause problems with the targeting of specific components to the plasma membrane and T-tubules. The completely aberrant distribution of α_2 in dysgenic myotubes was not due to a general state of degener-

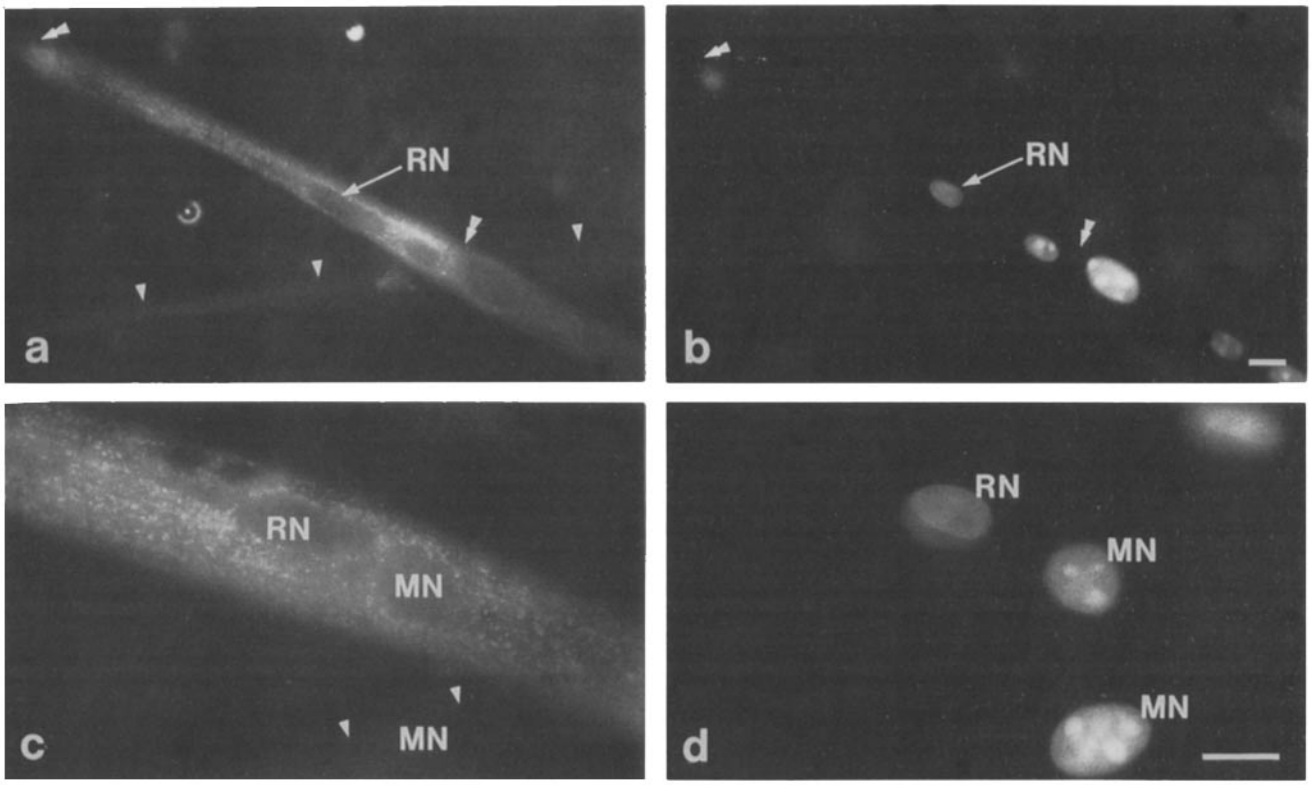

Figure 6. Restoration of DHP receptor α_1 expression in dysgenic myotubes by fusion with normal cells. Co-cultures of mdg/mdg myotubes and normal non-muscle cells from rat were double labeled with the α_1 subunit antibody (*a* and *c*) and the nuclear dye (Hoechst #33342) (*b* and *d*). The chromatin of rat nuclei (*RN*) appears homogeneous; in contrast, the mouse host cell nuclei (*MN*) exhibit heterochromatin. Immunolabel for the α_1 subunit could be seen in regions of dysgenic myotubes containing at least one rat nucleus. Expression of α_1 was restricted to a nuclear domain that usually contained several mouse nuclei in addition to the rat nucleus (*a* and *b*; between double arrowheads). The regions outside the "rescued" domain and "non-rescued" myotubes (*arrowheads*) show no α_1 expression. In the rescued regions α_1 appeared in its normal punctate distribution pattern (*c*). Bar, 10 μm.

ation, since myofibrillar organization was intact and, more significantly, T-tubules in dysgenic myotubes were for the most part normally developed and were able to achieve a degree of differentiation similar to that in normal myotubes (Flucher et al., manuscript in preparation). Thus, the altered α_2 distribution patterns in *mdg/mdg* myotubes suggest that the α_2 subunit requires the presence of the α_1 subunit for its aggregation into DHP receptor-rich domains as well as for its specific localization in the T-tubule system. Alternatively, the aberrant α_2 distribution might be explained by indirect effects of the mutation on muscle morphology. One possibility is that the lack of contractile activity during development in vitro could prevent the proper organization of the DHP receptors in the T-tubules. However, normal myotubes grown under conditions that inhibited spontaneous contraction did not show any signs of adverse effects of this treatment on the clustering or the normal organization of the DHP receptor subunits. Furthermore, inactivity in vivo does not reduce the number of triads (32). Other secondary causes such as altered regulation of intracellular Ca^{2+} levels, cannot be completely ruled out. However, we believe that the aberrant distribution of the α_2 subunit is most directly explained by the disruption of the interactions between the α_1 and α_2 subunits that normally may lead to the aggregation of the DHP receptor complexes into clusters in the T-tubule system.

Rescue of the Expression and Distribution of the DHP Receptor α Subunits

If the described aberrations in the expression and distribution patterns of the DHP receptor α_2 subunit are caused by the lack of the α_1 subunit of the DHP receptor, reconstitution of dysgenic myotubes with the α_1 polypeptide should restore normal structures and functions. Expression and normal distribution of the α_1 subunit was restored in dysgenic myotubes fused with normal cells. This finding is in agreement with results from electrophysiological studies on rescued *mdg/mdg* muscle showing that the slow Ca^{2+} current and voltage gating are restored (1, 2). Our immunocytochemical localization of the α_1 subunit in myotubes with foreign nuclei showed that a third of the normal (rat) nuclei in rescued myotubes expressed the α_1 subunit. Pavlath and her colleagues (31) reported that not all heterokaryons of mouse myoblast and human non-myogenic cells expressed the foreign protein, e.g., 49% expressed human sarcomeric myosin heavy chain and 87% expressed human sarcolemmal neuronal cell adhesion molecule. In the present study, those foreign nuclei that apparently did not express the α_1 polypeptide may reflect a population of the non-muscle cells which was capable of fusing with the muscle cells, but was insensitive to the regulatory signals inducing the expression of muscle specific gene products. Alternatively, the coexis-

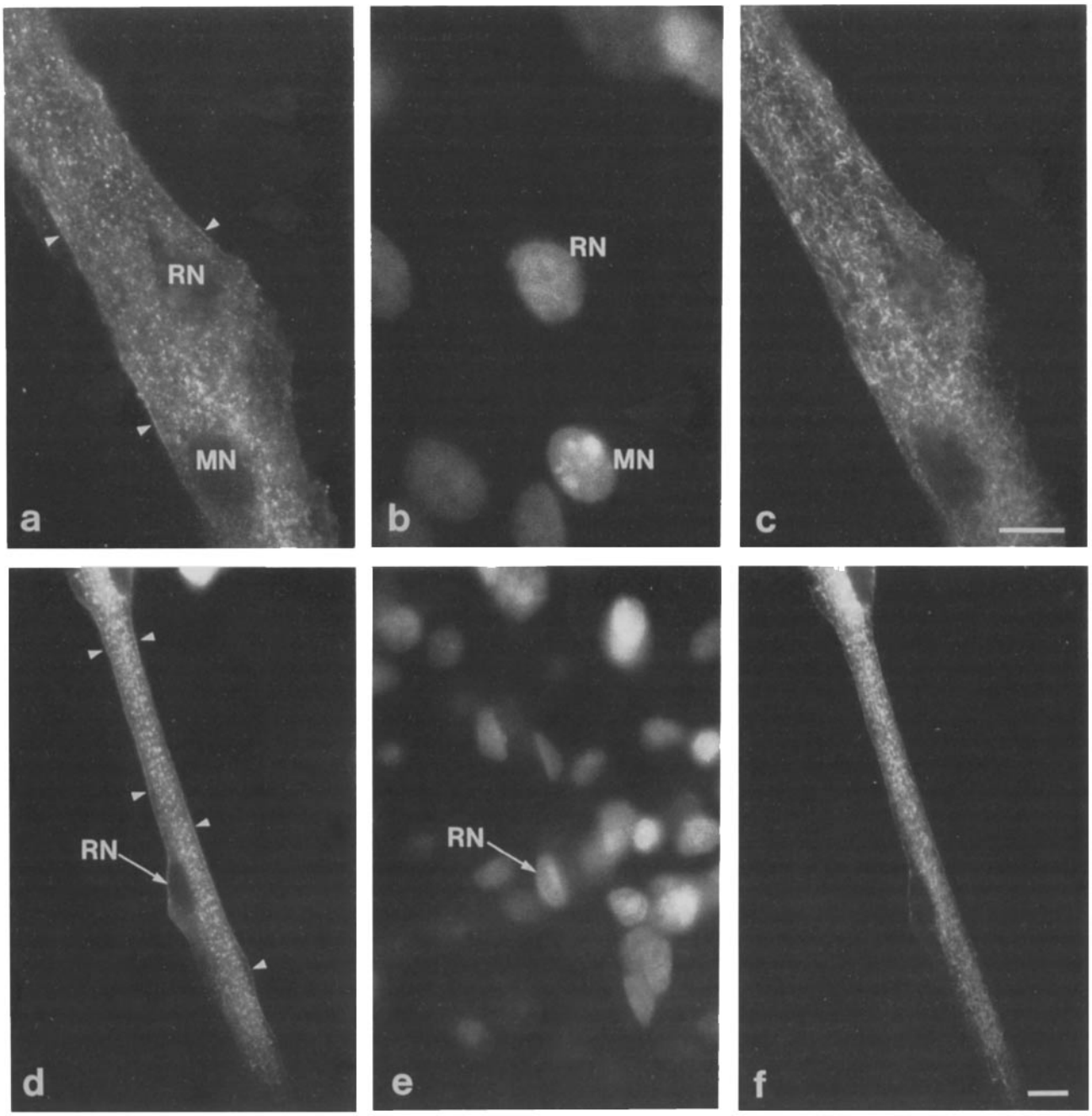

Figure 7. Restoration of normal distribution patterns of the DHP receptor α_2 subunit in regions of dysgenic myotubes containing normal foreign nuclei. Co-cultures of *mdg/mdg* myotubes and normal non-muscle cells were triple labeled with antibodies against the α_2 subunit (*a* and *d*) and T-tubules (*c* and *f*) as well as with the nuclear dye (*b* and *e*). The distribution patterns of both antigens in the cytoplasm were normal in rescued domains of poorly differentiated (*a–c*) as well as highly differentiated myotubes (*d–f*). Immunolabel of α_2 was punctate and coincided with the T-tubules. Whereas aberrant α_2/TT-containing structures (as shown in Figs. 2 and 3) were absent from these regions, diffuse α_2 label in the plasma membrane, unaccompanied by T-tubule staining could be observed (*a* and *d*; *arrowheads*). *RN*, rat nucleus; *MN*, mouse nucleus. Bars, 10 μm.

tence of foreign nuclei with different α_1 expression patterns may be indicative of nuclear specializations within the multinucleated muscle cells, as has been reported for acetylcholine receptor transcripts (15).

Immunolabel for α_1 was always found associated with a foreign rat nucleus, covering an area (i.e., "nuclear domain") of the myotube that often included several host mouse nuclei. Nuclear domains appear to vary in size depending on the nature of the protein and the target organelle of the molecule (for review see reference 19). For instance, the distribution of certain proteins targeted for nuclei (39), the Golgi apparatus, or the contractile apparatus (31) is limited to the source nucleus and a few neighboring nuclei, whereas soluble cytoplasmic proteins become widely distributed in the myotube (19, 39). Both situations, limited as well as wide distribution, have been described for plasma membrane proteins (31, 38).

This is the first report of the nuclear domain of a membrane protein localized in the T-tubule system and shows that a normal nucleus incorporated into a diseased muscle cell may be capable of providing its gene products to a region that corresponds to approximately four host nuclei and is finite in length. The distribution of the DHP receptor in its nuclear domain may occur by local insertion of transport vesicles, by lateral diffusion of the polypeptide in the T-tubules that exist during most of development or through the migration of the rescue nucleus itself (11), leaving behind a trail of α_1 mRNA or the polypeptide. Several examples in which the rescue nucleus was found at one end of the nuclear domain rather than in the center support this latter possibility.

In addition to the rescue of α_1 subunit expression per se, the normal distribution patterns of both α subunits were restored by the fusion of dysgenic myotubes with normal cells. This could clearly be seen when the α_1 subunit appeared in its normal clustered distribution in rescued cells. Furthermore, in rescued cultures labeled for α_2, regions of apparently normal α_2 distribution were observed in the midst of distorted labeling patterns. Wherever such "normal" regions were found they coincided with the location of a foreign nucleus. However, more than half of the foreign nuclei in rescued myotubes were not localized in regions of "normal" α_2 distribution. Since two thirds of the foreign nuclei were also not expressing α_1, we believe that the same population of nuclei is responsible for both phenomena. Thus, newly synthesized α_1 subunits in rescued myotubes did not only assume a normal clustered distribution themselves, but apparently caused the aggregation of the α_2 subunits in DHP receptor-rich domains of the T-tubules as well.

The subunit composition of the DHP receptor complex has been proposed on the basis of the co-purification of the α_1, α_2, β, and γ polypeptides from isolated triads (24). However, up to now, there has been relatively little evidence for in situ interactions between the subunits or for possible functions of the subunits other than α_1. In the present study we have demonstrated that: (*a*) the α subunits of the skeletal muscle DHP receptor coexist in DHP receptor-rich domains of the developing T-tubule system; (*b*) the α_2 subunit fails to form clusters in the absence of the α_1 subunit; and (*c*) that reconstitution of α_1 subunit expression restores the normal organization of the α_2 subunit in the T-tubule membranes. Taken together these results provide strong evidence for a direct interaction between the two α subunits of the skeletal muscle DHP receptor and suggest a possible role for these interactions in the specialization of the junctional membrane of the T-tubules.

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