Formation of Triads without the Dihydropyridine Receptor α Subunits in Cell Lines from Dysgenic Skeletal Muscle

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Abstract. Muscular dysgenesis (*mdg/mdg*), a mutation of the skeletal muscle dihydropyridine receptor (DHPR) α_1 subunit, has served as a model to study the functions of the DHPR in excitation–contraction coupling and its role in triad formation. We have investigated the question of whether the lack of the DHPR in dysgenic skeletal muscle results in a failure of triad formation, using cell lines (GLT and NLT) derived from dysgenic (*mdg/mdg*) and normal (+/+) muscle, respectively. The lines were generated by transfection of myoblasts with a plasmid encoding a Large T antigen. Both cell lines express muscle-specific proteins and begin organization of sarcomeres as demonstrated by immunocytochemistry. Similar to primary cultures, dysgenic (GLT) myoblasts show a higher incidence of cell fusion than their normal counterparts (NLT). NLT myotubes develop spontaneous contractile activity, and fluores-

cent Ca^{2+} recordings show Ca^{2+} release in response to depolarization. In contrast, GLTs show neither spontaneous nor depolarization-induced Ca^{2+} transients, but do release Ca^{2+} from the sarcoplasmic reticulum (SR) in response to caffeine. Despite normal transverse tubule (T-tubule) formation, GLT myotubes lack the α_1 subunit of the skeletal muscle DHPR, and the α_2 subunit is mistargeted. Nevertheless, the ryanodine receptor (RyR) frequently develops its normal, clustered organization in the absence of both DHPR α subunits in the T-tubules. In EM, these RyR clusters correspond to T-tubule/SR junctions with regularly spaced feet. These findings provide conclusive evidence that interactions between the DHPR and RyR are not involved in the formation of triad junctions or in the normal organization of the RyR in the junctional SR.

XCITATION–CONTRACTION (E-C)[1] coupling in skeletal muscle requires the close interaction of the dihydropyridine receptor (DHPR) in the transverse tubules (T-tubules) and the ryanodine receptor (RyR) in the sarcoplasmic reticulum (SR) (Fleischer and Inui, 1989; Rios et al., 1991). Interactions between DHPRs and RyRs could also play a role in the formation and maintenance of the T-tubule/SR junction called the triad (Yuan et al., 1991; Flucher, 1992). During normal development, both channels are simultaneously expressed and colocalize in clusters (Flucher et al., 1993). These DHPR/RyR clusters correspond to early functional junctions between T-tubules and SR, and they display structural characteristics of mature triads (Flucher et al., 1994). At the EM level, the most

prominent structures of the junctions are the regularly spaced "feet," which correspond to the large cytoplasmic domain of the RyR (Franzini-Armstrong, 1970; Takeshima et al., 1989; Wagenknecht et al., 1989). The link between T-tubules and SR is strong enough to withstand the rigors of triad fractionation procedures (Lau et al., 1977; Kim et al., 1990a); and even after their dissociation, purified T-tubule and heavy SR vesicles maintain the ability to reassociate with one another (Corbett et al., 1985). Thus, it is conceivable that direct or indirect interactions between the DHPR and RyR contribute to the clustering of the channels in the junction and to the association of the two membrane systems during development. While attempts to show direct binding between isolated DHPR and RyR have failed (Brandt et al., 1990), a peptide from the cytoplasmic loop between repeats II and III of the DHPR α_1 subunit functionally interacted with the RyR (Lu et al., 1994). Furthermore, an integral membrane protein from junctional SR, called triadin, binds to both the RyR and the DHPR and is therefore a candidate for mediating interactions between junctional T-tubules and the SR (Kim et al., 1990b; Caswell et al., 1991; see also Knudson et al., 1993).

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1. *Abbreviations used in this paper.* DHPR, dihydropyridine receptor; E-C, excitation–contraction coupling; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; T-tubule, transverse tubule.

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Skeletal muscle of dysgenic mice (*mdg/mdg*) lacks E-C coupling (Powell and Fambrough, 1973; Klaus et al., 1983), the dihydropyridine-sensitive, L-type Ca^{2+} currents (Beam et al., 1986; Rieger et al., 1987), and the component of the gating charge movement associated with voltage sensing (Adams et al., 1990). The primary mutation of muscular dysgenesis is in the α_1 subunit (Chaudhari, 1992; Knudson et al., 1989). During late embryonic development, dysgenic skeletal muscle deteriorates, and homozygous dysgenic mice die upon birth as a result of respiratory failure (Pai, 1965). However, primary cultures of dysgenic muscle can be maintained for several weeks and differentiate well without the DHPR (Flucher, 1992). Structurally, normal triads were generally believed to be missing from dysgenic muscle (Pinçon-Raymond et al., 1990). However, peripheral couplings with regularly spaced feet have been observed in dysgenic myotubes in vitro (Courbin et al., 1989) and in vivo (Franzini-Armstrong et al., 1991). The latter couplings were shown to lack the junctional tetrads, which are characteristically spaced integral membrane particles corresponding to the skeletal muscle DHPR. T-tubule/SR junctions with apparently normal disposition were also found in the best developed regions of the diaphragm of dysgenic embryos at E19 (Franzini-Armstrong et al., 1991). While the α_1 subunit is not expressed in dysgenic cultures, the α_2 subunit is present, but it is not targeted correctly to the T-tubules (Flucher et al., 1991a). Similarly, the integral membrane proteins from junctional SR—the RyR and triadin—are expressed in dysgenic myotubes, but they generally fail to form clusters (Flucher et al., 1993). Nevertheless, in a small number of myotubes, RyR clusters have been observed in the absence of the α_1 subunit of the skeletal muscle DHPR, suggesting that junctional SR can form without the DHPR.

This interpretation, however, was inconclusive: first, because there was only indirect evidence that the RyR clusters in dysgenic myotubes actually represented T-tubule/SR junctions; and secondly, because it could not be ruled out that a DHPR α_1 subunit, other than the skeletal isoform, could substitute for the function in the formation of dysgenic triads that lack the skeletal isoform. The latter possibility was underlined by studies showing that the cardiac DHPR isoform is transiently expressed during early development of skeletal muscle (Chaudhari and Beam, 1993; Varadi et al., 1995) and that dysgenic muscle can express a dihydropyridine-sensitive Ca^{2+} current (I_{Ca-Dys}) with properties similar to that of cardiac muscle (Adams and Beam, 1989).

This problem could now be resolved using an immortalized cell line of dysgenic muscle. The new cell line (GLT) shares all essential characteristics of primary cultures of dysgenic muscle but differentiates normally organized RyR clusters at a high frequency. The analysis of these RyR clusters in dysgenic myotubes showed that (*a*) they correspond to T-tubule/SR junctions with identifiable feet; (*b*) these junctions contain neither of the skeletal muscle DHPR α subunits; and (*c*) they are incapable of skeletal- or cardiac-type E-C coupling. Thus, the molecular differentiation of junctional SR and the formation of T-tubule/SR junctions in developing myotubes is independent of direct or indirect molecular interactions requiring the DHPR α subunits.

Materials and Methods

Generation of the Cell Lines

Primary normal (+/+) and dysgenic (*mdg/mdg*) cultures were prepared as previously described (Klaus et al., 1983; Flucher et al., 1991a), plated at $2.0–2.5 \times 10^5$ per 60-mm culture dish, and transfected using a modification of the Ca^{2+} phosphate method (Graham and Van der Eb, 1973). Briefly, cells were allowed to grow to 50–80% confluency, the medium was changed from plating to growth medium (20% FBS in DME), and the cells were incubated for 24 h in the transfection solution containing 2 µg/ml of the plasmid pRBK (Invitrogen, San Diego, CA) that contains the BK Large T antigen as well as ampicillin- and hygromycin B–resistant sequences. After 34–40 h, the cells were briefly (1 min) exposed to 15% glycerol in Hepes-buffered saline. After further 24-h incubation in growth medium, the cells were split 1:4 or 1:10 and plated in growth medium, containing 100–200 µg/ml hygromycin B (Calbiochem-Novabiochem Corp., La Jolla, CA) for the selection of transfected cells.

Several hygromycin-resistant cell lines have been characterized. The mutant cell line was called GLT for muscular dys<u>G</u>enic (*mdg/mdg*) <u>L</u>ine transfected with the Large <u>T</u> antigen, and the wild-type cell line was called NLT for <u>N</u>ormal (+/+) <u>L</u>ine transfected with the Large <u>T</u> antigen. Passage numbers refer to the number of passages after the first transfer from a cloned ring culture to a 25-cm² flask. We estimated each passage (once a week) represents ~10 generations of cell division. Testing for optimal differentiation conditions was performed on passages 2 through 5. All characteristics reported here are from cell cultures at passage 7 or higher. Cells were plated and proliferated in growth medium containing 10% FBS and 10% horse serum, and differentiated in fusion (differentiation) medium containing 2% horse serum. For immunofluorescence labeling, cells were plated at 2×10^4 per 35-mm culture dish, 5×10^3 per 13-mm carbon-coated coverslip with 0.1% gelatin coat. For Ca^{2+} recording, cells were plated at 1×10^4 per 25-mm coverslip. Cultures were analyzed 1 to 2 wk after plating.

DNA Pulse-labeling

The cells were incubated with 0.25 methyl nCi/ml [³H]deoxythymidine (Amersham Corp., Arlington Heights, IL) for 30 min 2 d after initial fusion. They were then fixed in buffered 1% formalin, treated for routine autoradiography, and counter-stained with 1% aqueous toluidine blue (Stockdale and Holtzer, 1961).

Analysis of Heterospecific Fusion

To assess the rate of fusion of the primary and cell line myotubes with cells from different species or tissues, rat fibroblasts (JRF) or myoblasts (L6) were added to the mouse cultures at the onset of fusion. Rat fibroblasts were added at 2×10^4 per 35-mm dish to normal (+/+) and dysgenic primary cultures 4–5 d after plating. Both JRF fibroblasts and L6 cells were added to the dysgenic cell lines at the same concentration. After 8 d of coculture for primaries and 4–5 d for the cell lines, cultures were incubated with 10 µg/ml Höechst No. 33342 nuclear stain (Polysciences, Inc., Warrington, PA) for 45 min, and then fixed with 100% methanol at −20°C. Cultures were viewed with epifluorescence optics and analyzed for isolated rat nuclei (homogenous pattern) in myotubes containing predominantly mouse nuclei (punctate pattern). For scoring criteria and representative images, see Flucher et al., 1991a, 1993.

Immunofluorescence Labeling

1- or 2-wk-old cultures grown on coverglasses were fixed and immunostained as previously described (Flucher et al., 1993). Methanol-fixed cultures were incubated with 10% normal goat serum in PBS containing 0.2% BSA (PBS/BSA) for 30 min, 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 h at room temperature and washed again. The coverglasses were then mounted in 90% glycerol, 0.1 M Tris, pH 8.0, with 5 mg/ml *p*-phenylenediamine to retard photobleaching. The working dilutions and the sources of primary and secondary antibodies are listed in Table I. The samples were evaluated on a microscope (Axioskope; Carl Zeiss, Inc., Thornwood, NY) with epifluorescence and phase-contrast optics and documented on 35-mm black-and-white film. All antibodies were either commercially obtained (see Table I) or care-

Table I. Antibodies Used in Present Study

Antibody/Specificity	Type	Dilution	Source
RyR	rabbit, affinity purified	1:5,000	Flucher et al., 1993
DHPR-α	mouse monoclonal	0.1 μM	Morton and Froehner, 1987
DHPR-α_2	mouse monoclonal	0.1 μM	Morton and Froehner, 1989
Ca^{2+}-ATPase, fast isoform	mouse monoclonal	5 μg/ml	Kaprielian and Fambrough, 1987
T-tubules (TT2)	rabbit, affinity purified	1:800	Flucher et al., 1991b
Troponin-T	mouse monoclonal	1:100	Sigma Chemical Co. (T-6277)
α-actinin	rabbit	1:50	Sigma Chemical Co. (A-2543)
Mouse IgG (FITC/TRITC)	goat, affinity purified	1:200–400	Cappel Products
Rabbit IgG (FITC)	goat, affinity purified	1:200–400	Cappel Products
Mouse IgG (Cy3)	donkey, affinity purified	1:4,000	Jackson Immunochemicals

fully characterized for their use in immunofluorescence experiments in previous studies (Flucher et al., 1991a, 1993, 1994). Controls, like the omission of primary antibodies, and incubation with inappropriate antibodies, were routinely performed.

Electron Microscopy

Fixation, embedding, and sectioning of the muscle cell cultures were performed as described previously (Flucher et al., 1994). Briefly, cells were fixed in cacodylate-buffered glutaraldehyde for 15 min at room temperature and then in the same fixative with the addition of 0.15% tannic acid for 15 min at room temperature plus 1 h at 4°C. Cultures were postfixed with osmium tetroxide, en bloc stained with uranyl acetate, dehydrated in ethanol, and then embedded in Epon. Thin sections ($\sim$80 nm) were stained with uranyl acetate and lead citrate and viewed with an electron microscope (JEOL 1200; JEOL USA, Peabody, MA).

Quantitation of the density of junctions between SR and T-tubules was performed as previously described (Flucher et al., 1994). In brief, sections were scanned at a magnification of 10,000, and from each of the 10 myotube profiles, a series of 2–9 micrographs was taken. The cytoplasmic area in each micrograph was measured with NIH-Image software (written by W. Rasband, National Institutes of Mental Health/National Institutes of Health; available from National Information Technology Services, Alexandria, VA), and the number of junctions per 1,000 μm^2 of cytoplasm was calculated. Junctions between SR and T-tubules were defined by a close apposition between SR cisternae and T-tubules, and the presence of electron-dense material within the junction. This characteristic structure was easily recognized even if the junctional feet were not resolved due to the plane of the section. Multiple junctions (consisting of more than one SR and T-tubule profile; e.g., pentads) were counted as one junction as long as the continuity of the structure was recognizable. In immunofluorescence-labeled preparations, such multiple junctions would appear as a single large cluster. The measure of the area density of junctions used here should be taken as an operational measure to allow the comparison with the data from normal rat primary cultures (Flucher et al., 1994). The analysis method does not allow the interpretation of these measurements in terms of the number of junctions per unit of cytoplasmic volume.

Ca^{2+} Recording

Recordings of intracellular free Ca^{2+} concentrations were performed as described in Flucher et al. (1993) with a dual-emission photometry system. Cultures plated on 25-mm round coverslips were incubated in the fluorescent Ca^{2+} indicator fluo-3 AM (5 μM) and SNARF-1 AM (0.5 μM) plus 0.1% Pluronic F-127 (all from Molecular Probes, Eugene, OR) for 30 min at room temperature. Ca^{2+} measurements were carried out on an inverted microscope (Axiovert; Carl Zeiss, Inc.) with epifluorescence using a 488-nm band-pass filter for the simultaneous excitation of fluo-3 and SNARF-1. The illumination was attenuated so that the fluorescence signals emitted during a Ca^{2+} transient could just be seen with the naked eye through the eyepieces. Fluorescent measurements from a field of 30 × 30 μm were performed with a dual photometer system (Photon Technology Intl., South Brunswick, NJ) using a 590-nm dichroic mirror with a 530-nm barrier filter for fluo-3 and a 610-nm barrier filter for SNARF-1 (Omega Optical, Inc., Brattleboro, VT). The two channels were recorded at a sampling rate of 200 Hz, and the ratio of the two emission wavelengths was calculated using PTI Oscar software (Photon Technology Intl.). Electrical
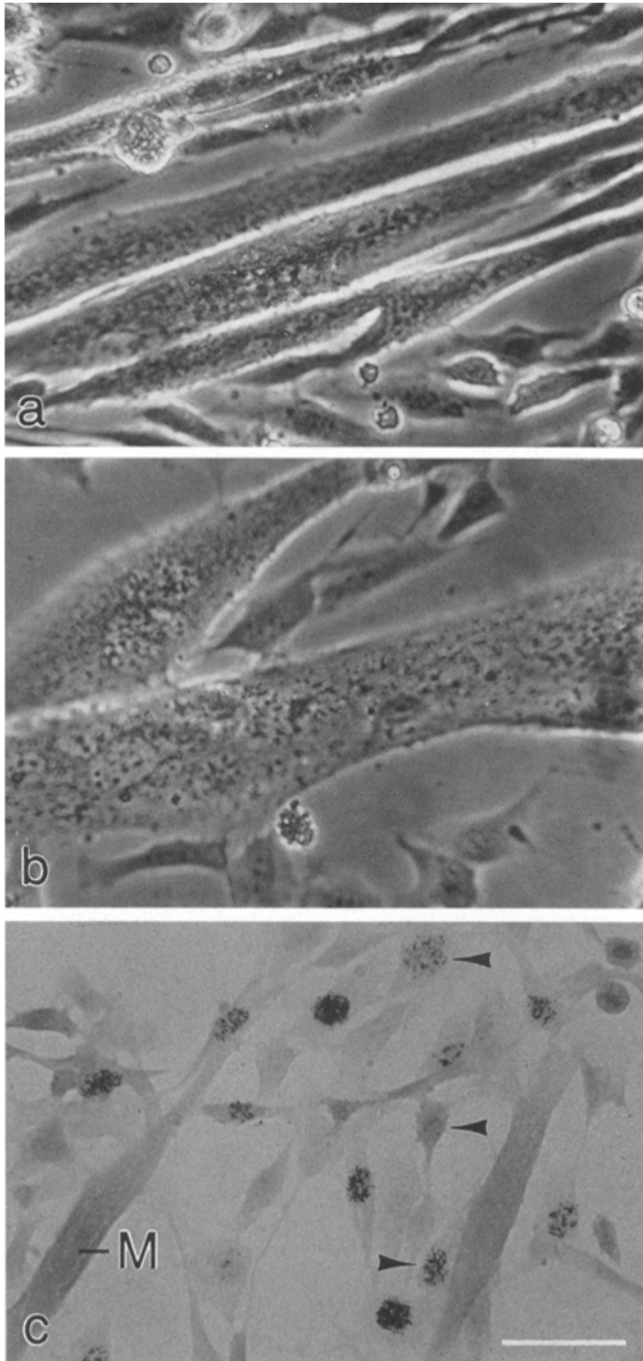
field stimulation was performed with a 1-ms pulse of 15–20 V passed across the 19-mm-diam well of the incubation chamber. Passive depolarization and caffeine-induced Ca^{2+} release were stimulated by rapidly replacing half of the incubation medium with twofold concentrated KCl or caffeine solution, respectively.

Results

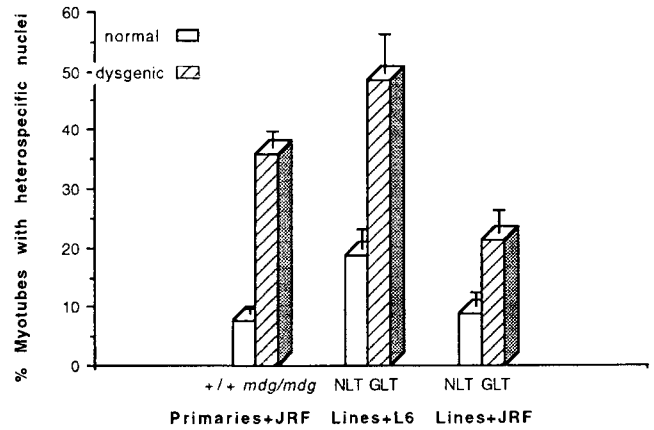### Characteristics of the Cell Lines GLT and NLT

General Features. Dissociated myoblasts from normal and dysgenic mice were transfected with a plasmid carrying the BK Large T antigen. Cells were selected based on their ability to continuously proliferate in serum-rich growth medium and their ability to leave the cell cycle, fuse, and differentiate after partial serum withdrawal (see Materials and Methods). The onset of myoblast fusion occurred 2 d after changing to differentiation medium and continued for up to 1 wk. Eventually all myoblasts fused and formed short (two to six nuclei) to medium-long (20–40 nuclei) myotubes with a low degree of interconnection (Fig. 1, *a* and *b*). Some large myotubes were up to 800 μm long. The myofiber stage with extensive cross-striations seen in phase-contrast and peripherally located nuclei has not been observed. Both cell lines could be passed up to 20 times before a decreased ability to develop myotubes with more than two to four nuclei occurred. [^{3}H]deoxythymidine pulse-labeling at the beginning of fusion showed that all multinucleated myotubes and some mononucleated cells have stopped synthesizing DNA, while a large portion of the mononucleated cells were still mitotically active (i.e., incorporating [^{3}H]deoxythymidine into their nuclei) (Fig. 1 *c*). This indicates that the Large T antigen is active in serum-rich medium but becomes downregulated after serum withdrawal. Therefore, it is unlikely to interfere with normal muscle differentiation or with experiments performed on differentiated myotubes. In the description below, we do not attempt a developmental comparison; all the data reported are taken from cultures at the height of maturity, i.e., at $\sim$5–7 d after fusion.

Heterologous Fusion. Primary cultures of dysgenic muscle are known to fuse at an increased rate with cells from other species and tissues (Powell, 1990; Chaudhari and Beam, 1990; Flucher et al., 1991a; Flucher et al., 1993). Such increased heterologous fusion has also been observed in the GLT cell line (Fig. 2). Mouse and rat nuclei can be distinguished by their characteristic labeling pattern with the dye Höechst No. 33342 (see Flucher et al.,

Figure 2. Comparison of heterospecific cell fusion of normal and dysgenic primary cultures (+/+; mdg/mdg) and cell lines (NLT; GLT). At the onset of cell fusion, 2 × 10⁴ JRF fibroblasts or L6 myoblasts were added to each 35-mm dish of the muscle cultures. After fusion the cells were labeled with the nuclear dye Höechst No. 33342 and screened for the presence of (heterospecific) rat nuclei in the mouse myotubes. Both GLT myotubes and primary cultures of dysgenic muscle exhibit a more than twofold enhanced incorporation of rat nuclei. Heterospecific fusion with nonmuscle cells (the rat fibroblast line JRF) is about half of that observed with a rat muscle cell line (L6). This data is derived from a study of at least three different individually plated cultures for each of the six conditions. The number of myotubes counted from each group are: mdg/mdg +JRF (175); +/+ +JRF (205); GLT+ L6 (639); NLT + L6 (670); GLT + JRF (649); NLT + JRF (834).

Figure 1. Differentiation of normal (NLT) and dysgenic (GLT) muscle cell lines. Phase-contrast micrographs of NLT (+/+) (a) and GLT (mdg/mdg) (b) myotubes 4 d after addition of differentiation medium. (c) Pulse-labeling cultures with [³H]thymidine for 30 min 2 d after the addition of differentiation medium results in silver grains over a large portion of the mononucleated cells (arrowheads), while nuclei of multinucleated myotubes (M) show no indication of DNA synthesis. Bar, 50 μm.

1991a, 1993). In cocultures of GLTs with JRF, about two times as many (21.3 ± 3.9% [mean ± SEM]) rat nuclei were found in the mouse myotubes compared to NLT/JRF cocultures (8.9 ± 2.3%). The overall rate of fusion was higher when using the rat muscle cell line L6 instead of fi-

broblasts, but here, too, the dysgenic line showed twice as much fusion as the normal cell line (GLT/L6, 48.7 ± 6.5%; NLT/L6, 18.7 ± 3.9%). Thus, GLT myotubes share with primary dysgenic myotubes the characteristics of elevated (heterologous) fusion.

Expression of Myofibrillar Proteins. Immunofluorescence labeling of the GLT and NLT cell lines showed that, like normal and dysgenic primary cultures, the cell lines expressed skeletal muscle-specific proteins upon differentiation of myotubes. Troponin T, α-actinin (Fig. 3), and myosin (not shown) were localized in GLT and NLT myotubes. While cross-striated cells were found in the cell lines, the proportion of cross-striated myotubes, as well as the degree of cross-striated organization of the myofibrils within individual myotubes, was lower than in primary cultures. Thus, GLT and NLT cells showed the features of early myotube differentiation in primary culture; however, the cell lines fell behind primary cultures in the later stages of differentiation. This tendency has also been observed in the myogenic cell lines C₂C₁₂ and L6 (Flucher, B.E., and J.A. Powell, unpublished results).

Expression of Muscle Membrane Proteins. Despite the lack of the α₁ subunit of the DHPR, T-tubules and the SR developed normally in primary cultures of dysgenic muscle (Flucher et al., 1991a; Flucher, 1992). Immunolabeling of GLT (Fig. 4) and NLT myotubes (not shown) with specific markers for these membrane systems, the SR Ca²⁺-ATPase and the T-tubule antibody TT2, showed the expression of both membrane systems in the cell lines. As ex-
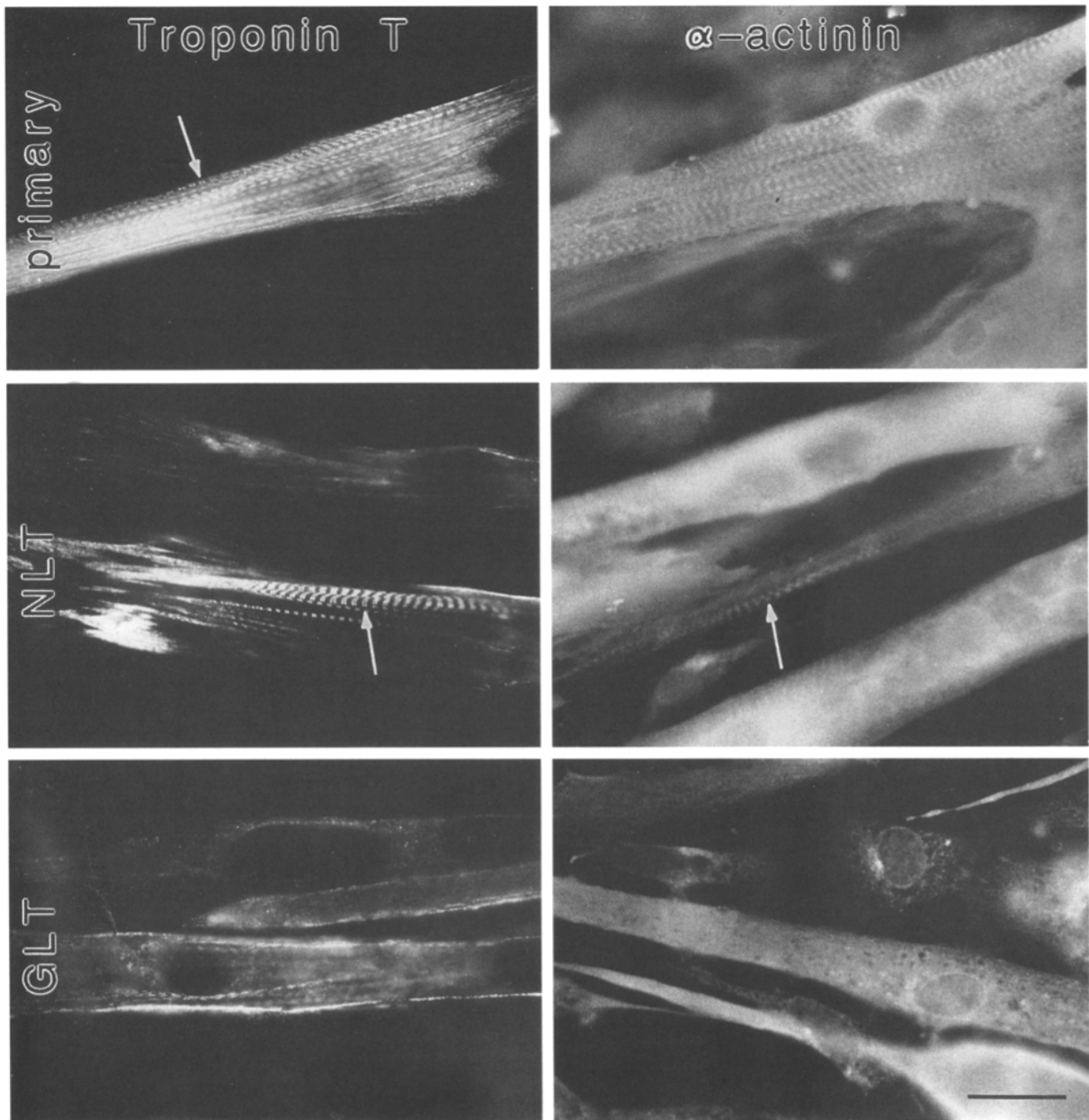
Figure 3. Expression of myofibrillar proteins in cultures of normal and dysgenic muscle. Primary cultures (*mdg/mdg, top left; +/mdg?, top right*) and cell lines (*NLT, center; GLT, bottom*) were immunolabeled with anti-troponin T (*left*) and anti-α-actinin (*right*). Troponin T is only expressed in fused myotubes of all culture types. α-Actinin is expressed in myoblasts and in myotubes. A cross-striated organization of the myofibrils is achieved less frequently in the cell lines than in the primary cultures. Bar, 25 μm.

pected, the α_1 subunit of the skeletal muscle DHPR was lacking in GLT myotubes. The degree of SR and T-tubule differentiation in these cell lines was that typically observed in young primary culture myotubes (Flucher, 1992; Flucher et al., 1994). Beginning cross-striation of the Ca^{2+}-ATPase in the periphery of the GLT myotubes indicated the early accumulation of SR at the I-band, while T-tubules remained mostly randomly oriented.

Organization of Triad Proteins in GLT and NLT Myotubes

The Ryanodine Receptor (RyR). In the normal muscle cell line (NLT), there was an exact point-to-point colocalization of the RyR and the α_1 subunit of the DHPR (Fig. 5). This colocalization represented the juxtaposition of the DHPR in the T-tubules across from the RyR in the termi-
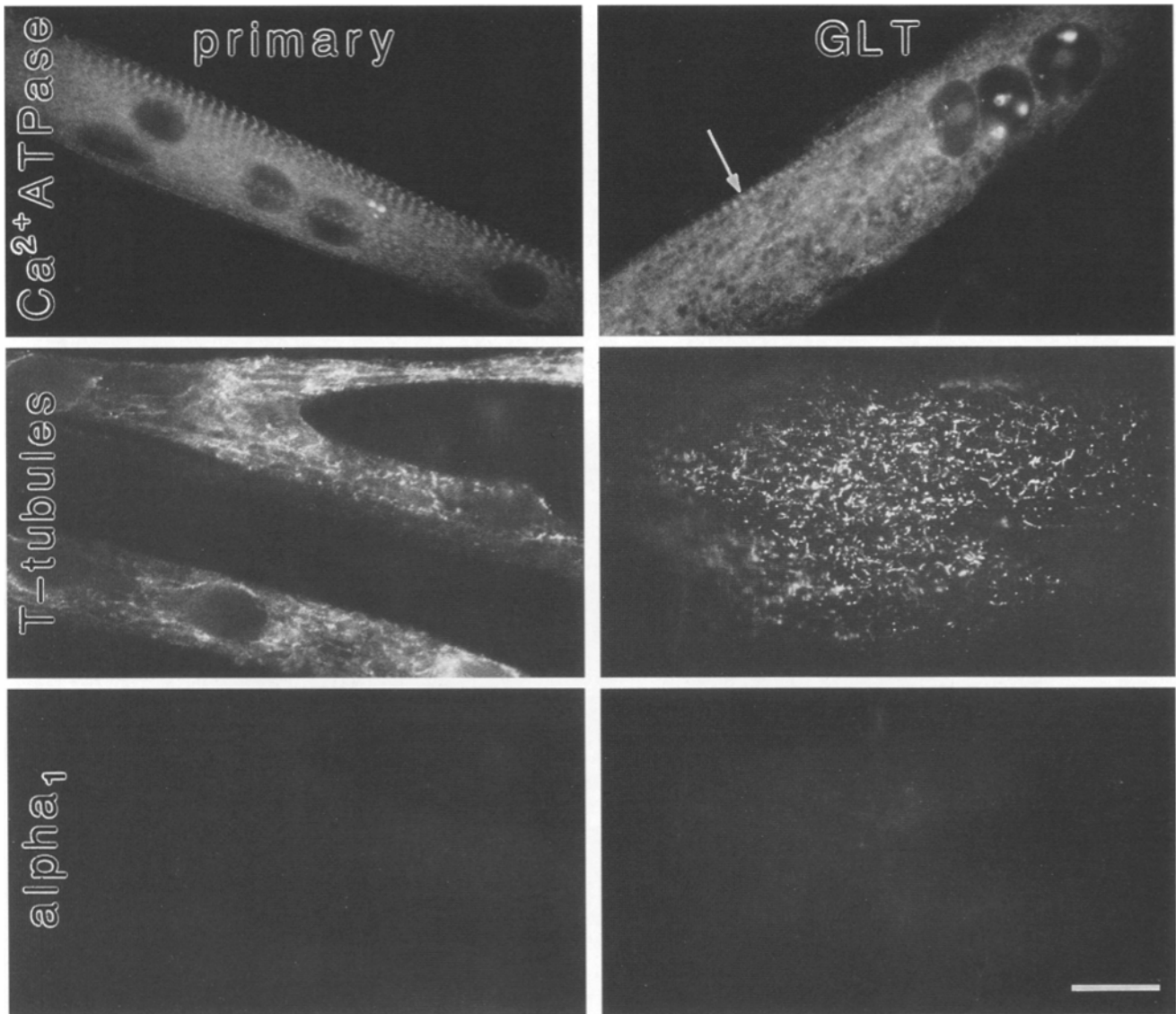
Figure 4. Expression of triad proteins in primary cultures and cell lines of dysgenic muscle. Primary *mdg/mdg* myotubes (*left*) and GLT myotubes (*right*) were immunolabeled with antibodies against the SR Ca^{2+}-ATPase (*top*), T-tubule antigens (*center*), and the α_1 subunit of the skeletal muscle DHPR (*bottom*). The SR and the T-tubule system differentiate normally in primary dysgenic and GLT cells. The DHPR α_1 subunit is lacking in both dysgenic culture systems. (*Arrow*) SR accumulation at the I band. Bars: (*upper panels*) 30 μm; (*center and bottom panels*) 20 μm.

nal cisternae of the SR. In the dysgenic cell line (GLT), two expression patterns of the RyR could be observed: either the RyR was distributed diffusely throughout the SR (Figs. 5 and 6, *center left*), or it was clustered in distinct RyR-rich domains in the SR (Figs. 5 and 6, *bottom left*) (see Flucher, 1992; Flucher et al., 1993, for comparison with SR ATPase labeling pattern). The two types of RyR distribution were not mutually exclusive but could coexist in one myotube (Fig. 5, *center left*). The clustered distribution was indistinguishable from the normal distribution pattern of the RyR in the presence of the DHPR α_1 subunit. However, in GLT myotubes the α_1 subunit was always absent. The extent of RyR clustering was not correlated with the overall differentiation of the myotube. Even poorly differentiated myotubes could form RyR clusters, while some cross-striated myotubes with diffuse RyR dis-

tribution were seen (compare Fig. 5, *center*, with Fig. 6, *bottom*). This lack of correlation between RyR clustering and general differentiation also became apparent in the comparison of GLT myotubes with primary cultures. In the latter, only a few myotubes with clustered RyRs were observed (Flucher et al., 1993), while the clustered distribution pattern was prevalent in GLT cultures that matured to a lesser degree than dysgenic primary cultures. The diffuse type of RyR distribution sometimes, but not always, revealed the tubular structure of the SR. Nevertheless, the diffuse RyR distribution pattern differed from that of the Ca^{2+}-ATPase (compare Figs. 5 and 6, *center left*, with Fig. 4, *top*), suggesting that even the diffusely distributed RyR does not penetrate all regions of the SR.

The DHPR α_2 Subunit. In NLT myotubes, the α_2 subunit of the DHPR also displayed a clustered distribution pat-
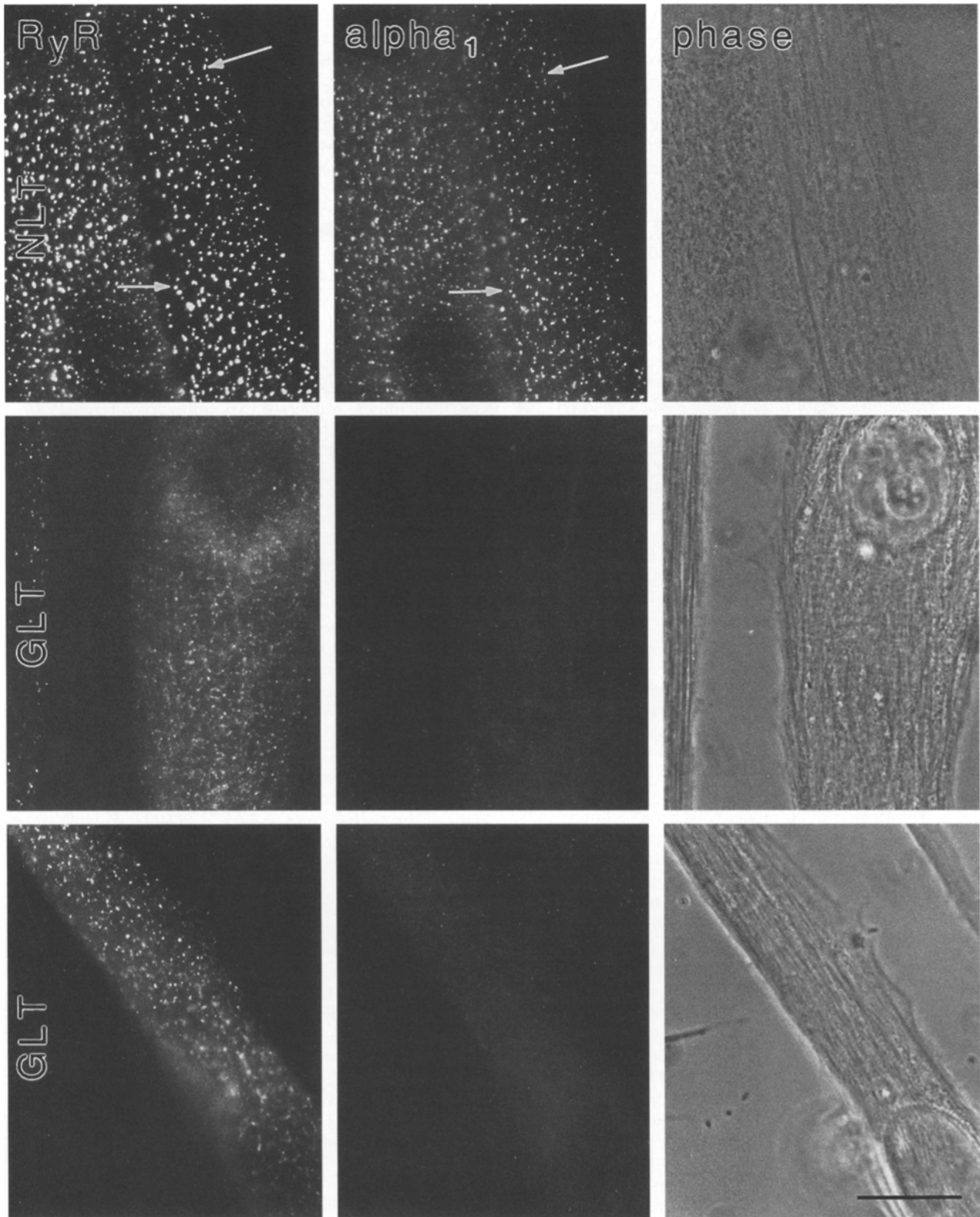
Figure 5. Localization of ryanodine receptors (*RyR*) and DHPR α_1 subunits (*alpha$_1$*) in normal, NLT, and dysgenic, GLT, cell lines. In NLT myotubes, RyR and the α_1 subunit are clustered and colocalized (*arrows*). This colocalization is indicative of T-tubule/SR junctions. The DHPR α_1 subunit is absent from GLT myotubes but the RyR is expressed. RyRs in GLT myotubes exist in two distinct expression patterns: diffuse distribution in the SR (*middle panels*) and clustered RyR distribution (*bottom panels*) (for further examples, see also Fig. 6). Both distribution patterns of the RyR are found at approximately equal frequencies. There is no correlation between RyR clustering and the overall differentiation of myotubes. Note the cross-striations seen in the phase-contrast image of the myotube shown in the middle panels. Bar, 20 μm.

tern in point-to-point colocalization with the RyR (examples indicated by *arrows* in Fig. 6). This distribution was consistent with the localization of the α_2 subunit in the T-tubule/SR junction. However, in the dysgenic line (GLT), the α_2 subunit was never found in discrete clusters, which would have indicated a junctional localization. To the contrary, the α_2 subunit in GLT myotubes was clearly mistargeted (Fig. 6, *center*). As previously observed in dysgenic primary cultures, it was found in extensive cytoplasmic compartments that differed in shape from T-tubules, SR, or other known organelles. Furthermore, in GLTs, the α_2 subunit was often localized diffusely in the plasma membrane of the dysgenic cell line. In none of these distribution patterns was the DHPR α_2 subunit codistributed with the RyR. The distribution of the α_2 subunit in GLT myotubes was independent of the type of RyR localization, regardless of whether the RyR was clustered or diffusely distributed.

Ultrastructural Organization of the RyR. The RyR has been identified as the foot protein that periodically spans the T-tubule/SR or sarcolemma/SR junctions. The clustered distribution of the RyR in many GLT myotubes is indicative of a junctional location. However, it was necessary to determine whether the RyR clusters in dysgenic myotubes corresponded to a junctional localization or whether they represented RyR-rich domains of free SR without association with T-tubules. EM preparations of parallel cultures to those used for the immunofluorescence studies were screened for the existence of T-tubule/SR junctions. Indeed, in well-differentiated GLT cultures, such junctions were found at a density of 61 (± 9.4) per 1,000 μm^2 cytoplasm ($n = 44$ micrographs from 10 myotube profiles). This is similar to the density reported earlier for 7–10-d-old normal rat myotubes (Flucher et al., 1994), which was to be expected based on the apparent density of clusters seen in immunofluorescence-labeled preparations from the parallel GLT cultures (Figs. 5 and 6). The junctions were of heterogeneous composition (dyads, triads, inverted triads, and multiple junctions) that is typical for young myotubes. Most importantly, however, well-preserved junctions clearly showed evenly spaced feet spanning the gap between T-tubules and SR (Fig. 7). Thus, T-tubule/SR junctions develop in the dysgenic cell line GLT, and RyRs can assume their normal organization in these junctions even though the α subunits of the DHPR are not present in the T-tubules.

Functional Properties of GLT and NLT Myotubes. The lack of the DHPR α_1 subunit in dysgenic skeletal muscle causes the failure of E-C coupling. The high degree of junction formation and the apparently normal organization of the SR Ca^{2+} release apparatus in the GLT cell line made it necessary to reevaluate their Ca^{2+} release properties. Consistent with the expected behavior of dysgenic myotubes, GLT cells did not show spontaneous contractile behavior, whereas spontaneous twitches were occasionally observed in NLT cells. GLT and NLT cultures were loaded with the fluorescent Ca^{2+} indicator fluo-3 plus a reference dye and were analyzed with microfluorometry (Fig. 8). In NLT myotubes, the stimulation of action potentials resulted in normal Ca^{2+} transients (compare with Flucher et al., 1994). Passive depolarization of the myotubes with 80 mM KCl caused a long-lasting, slowly inactivating Ca^{2+} release.

Both Ca^{2+} transients, induced by active and passive depolarization, were independent of extracellular Ca^{2+}. Thus, NLT myotubes showed normal, skeletal muscle-type E-C coupling. In contrast, GLT myotubes were incapable of depolarization-induced Ca^{2+} release. Neither electrical stimulation nor KCl depolarization triggered Ca^{2+} release. However, GLT myotubes responded to the application of 8 mM caffeine with a strong Ca^{2+} transient, indicating that the Ca^{2+} storage and release sites were functional. Thus, the dysgenic cell line GLT is deficient in E-C coupling despite the relatively high degree of junction formation between T-tubules and the SR.

Discussion

The Dysgenic Cell Line GLT

In the present study, we have generated an immortalized dysgenic muscle cell line with a normal counterpart ($+/+$) for the use in the study of E-C coupling. Another dysgenic line has previously been produced without a known ($+/+$) equivalent (Pinçon-Raymond et al., 1991). Myotubes of our present dysgenic cell line show all general characteristics of the well-described *mdg/mdg* primary cultures (Flucher et al., 1991a; Flucher, 1992), and they develop an exceptionally high degree of differentiation of the E-C coupling apparatus, as seen in abundant RyR clusters and T-tubule/SR junctions with regularly spaced feet. These features allowed us to demonstrate that junction formation as well as the organization of the RyR in these junctions is independent of the α subunits of the DHPR. Table II summarizes the properties of the new cell lines and compares them with the properties of primary cultures from *mdg/mdg* and $+/+$ mice. Our immortalized dysgenic cell line resembles its primary counterparts (*a*) in function, in that it is deficient in E-C coupling; (*b*) in the molecular expression patterns of the triad proteins, in that the α_1 subunit of the skeletal muscle DHPR is lacking, while the α_2 subunit and the RyR are expressed; and (*c*) in general features of myogenic differentiation, such as myoblast fusion and expression of myofibrillar proteins. The morphological characteristics of the GLT myotubes are typical for the early myotube stage. This arrest in the early stage of myotube differentiation is, however, also found in other myogenic cell lines like the C_2C_{12} and L6 cells. Most important for the study of E-C coupling in this cell line is its ability to form a structurally normal E-C coupling apparatus that is capable of releasing Ca^{2+} in response to caffeine. Only when the junctions between T-tubules and the SR are established and the Ca^{2+} release apparatus is functional is it possible to draw conclusions about the role of an individual missing component, the DHPR α_1 subunit, in this system.

Triad Formation

In an earlier study using the primary *mdg/mdg* cultures, we observed isolated cases of clustered RyR without the DHPR α_1 subunit (Flucher et al., 1993). These observations were in agreement with reports from EM studies showing apparently normal junctions in dysgenic muscle (Franzini-Armstrong et al., 1991). Together, these findings
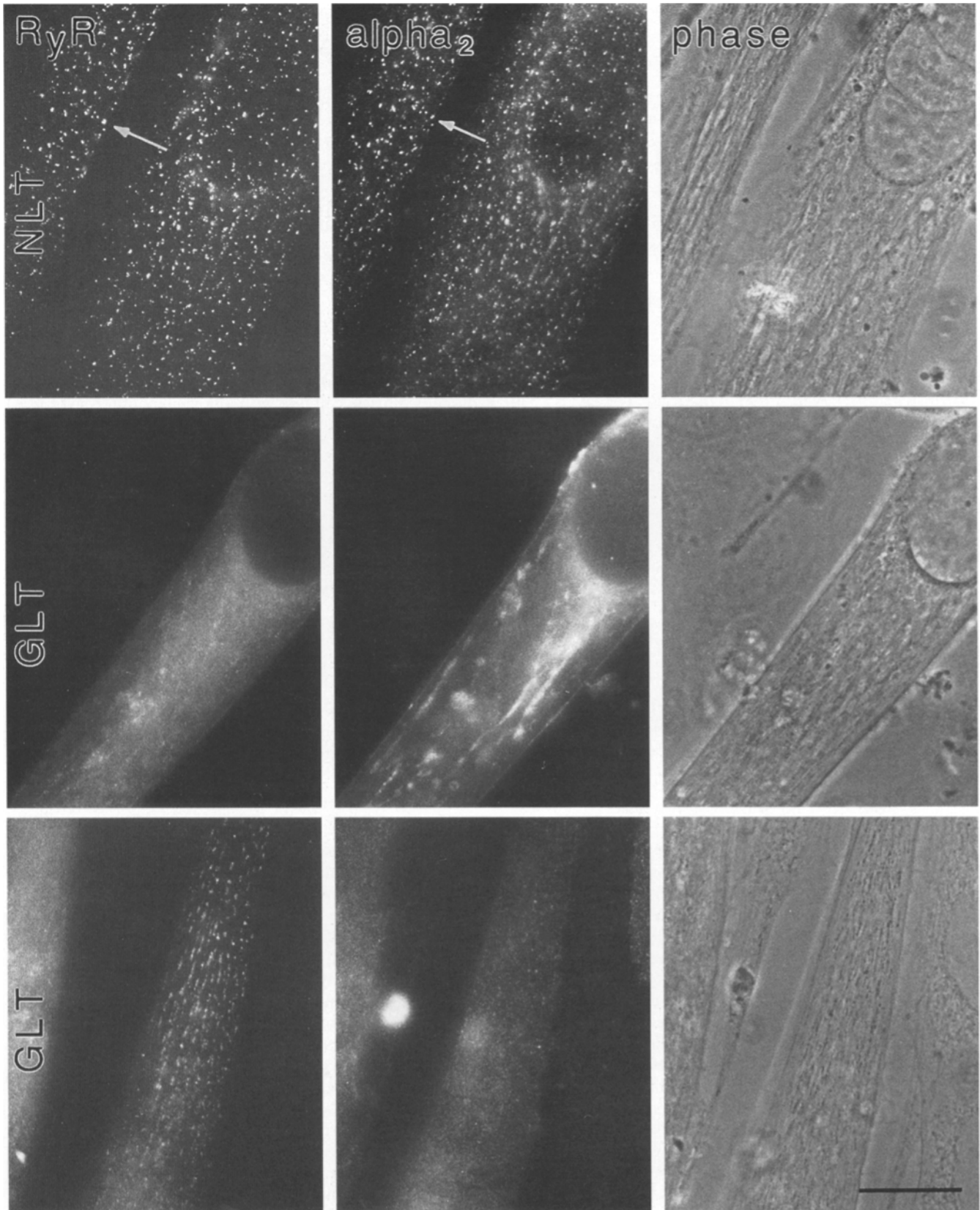
Figure 6. Localization of ryanodine receptors (*RyR*) and DHPR α_2 subunits (*alpha$_2$*) in normal, NLT, and dysgenic, GLT, cell lines. In NLT myotubes α_2 is clustered and colocalized with the RyR (examples indicated by *arrows*). In GLT myotubes, where the α_1 subunit is absent, the α_2 subunit is either mistargeted to the plasma membrane and cytoplasmic compartments (*middle*) or found in a diffuse distribution (*bottom*). In no case is the α_2 subunit clustered or colocalized with the RyR. This is especially apparent in the second example (*bottom*) where the RyR is localized in distinct clusters. Bar, 20 μm.
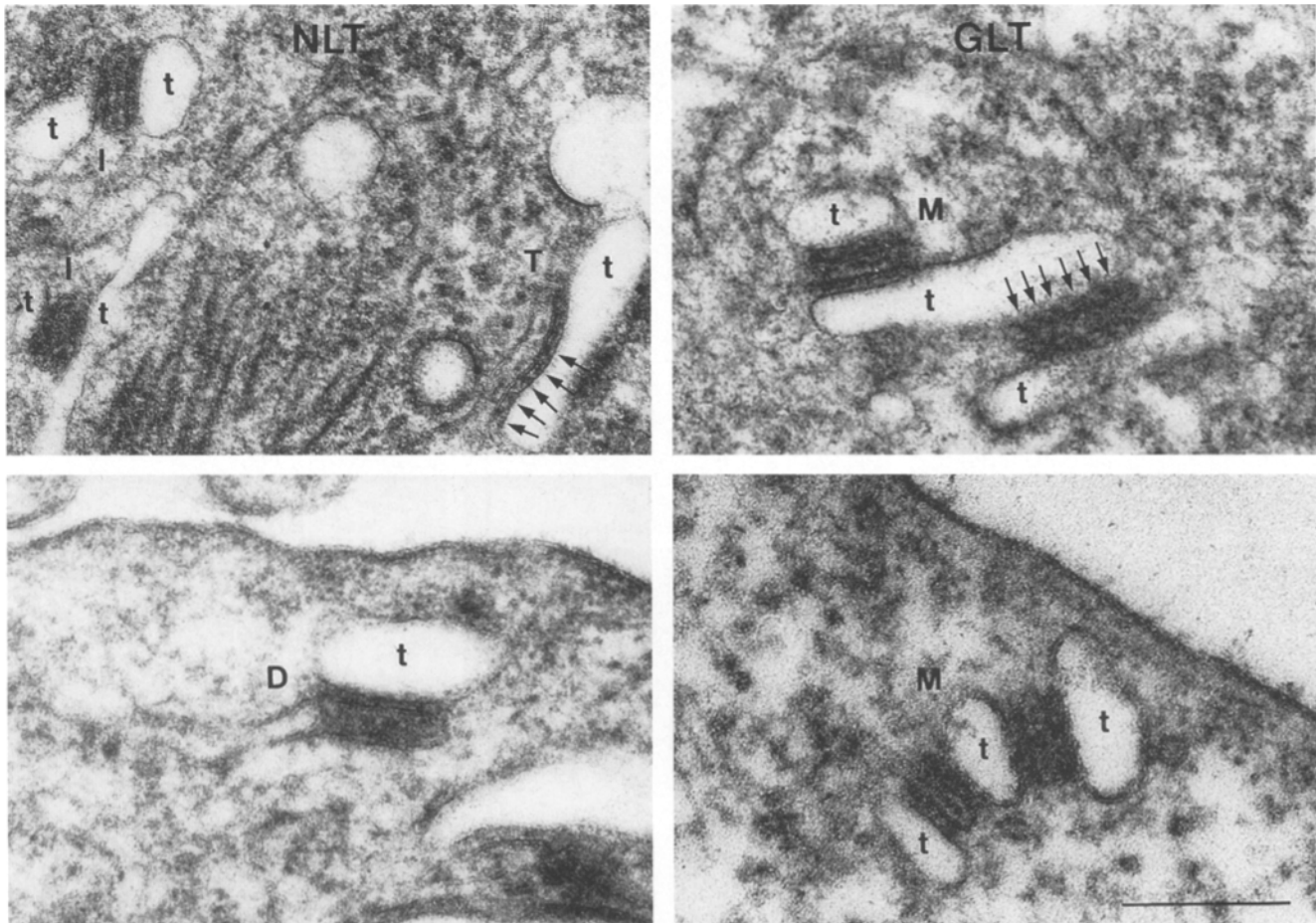
Figure 7. Electron micrographs of T-tubule/SR junctions in normal, NLT (*left*), and dysgenic, GLT (*right*), myotubes. The heterogeneous composition of the junctions (dyads [*D*], triads [*T*], inverted triads [*I*], and multiple junctions [*M*]) as well as the large T-tubule profiles (*t*) are typical for the early stages of triad formation. Note the periodic densities (feet; *arrows*) corresponding to RyR in the junctions and the electron-dense content of the SR, in both the normal and dysgenic junctions. Bar, 0.2 μm.

suggested that triad formation can occur without the skeletal muscle DHPR α_1 subunit. However, the majority of studies on dysgenic muscle reported that junctions with regularly spaced feet are missing (Platzer, 1979; Platzer and Gluecksohn-Waelsch, 1972; Banker, 1977). Our initial observations on primary dysgenic cultures added two important pieces of evidence to support the notion that DHPR–RyR interactions are not involved in triad formation: first, double immunofluorescence experiments showing RyR clusters without the corresponding DHPR α_1 subunit demonstrated that residual, low-level expression of the skeletal α_1 subunit could not account for the formation of a small number of normal junctions; secondly, functional analysis failed to detect any depolarization-induced Ca^{2+} release events, which would be expected if the observed RyR clusters actually resembled complete junctions. Nevertheless, the demonstration that triad formation can occur without the DHPR α_1 subunit fell short of being conclusive because it had not been shown that the RyR clusters actually corresponded to junctions with feet. Furthermore, the possibility remained that another α_1 subunit, which would not be detected with the skeletal-specific antibody, could substitute for the mutated skeletal α_1

subunit. These open questions, which could not be addressed in the primary cultures due to the low incidence of triad formation, have now been resolved using the cell line of dysgenic muscle.

First of all, finding a large number of myotubes with normally clustered RyR but without the skeletal muscle DHPR α_1 subunit strengthened the hypothesis that the molecular differentiation of the terminal SR cisternae does not require interactions with the DHPR in the T-system. Consequently, the lack of this differentiation, i.e., myotubes with diffusely distributed RyR, is a secondary effect of the mutation. Secondly, our EM observations of T-tubule/SR junctions with evenly spaced feet show that the clustered distribution of the RyR corresponds to the normal organization of this protein in the terminal cisternae and that this normal organization of RyRs develops in T-tubule/SR junctions rather than in free SR. Thus, not only is the differentiation of the Ca^{2+} release compartment in the SR independent of the DHPR α_1 subunit, but of the formation of the T-tubule/SR junctions as well. In normal cardiac muscle, isolated SR Ca^{2+} release domains with evenly spaced feet have been described (Jorgensen et al., 1993; Junker et al., 1994). However, in mammalian

NLT

GLT
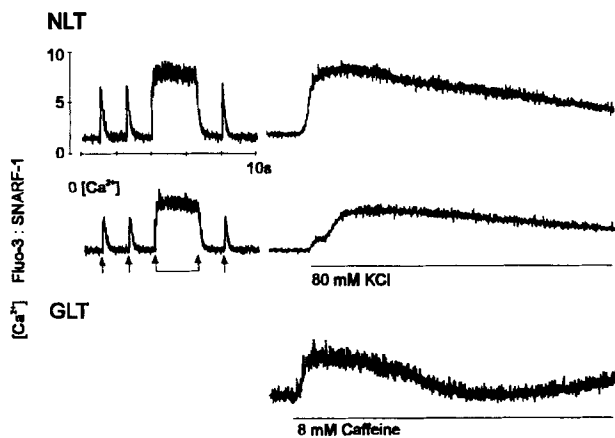
Figure 8. Ca^{2+} transients in cell lines of normal (NLT) and dysgenic (GLT) muscle. Myotubes were simultaneously loaded with the fluorescent Ca^{2+} indicator, fluo-3, and SNARF-1 (used as a reference dye), and analyzed with a dual channel microfluorimeter. Normal cells show release of Ca^{2+} from internal stores upon single, extracellular stimulation (1-ms pulse of 20 V; arrows) or a train of stimuli (10 Hz for 2.5 s; bracket between arrows) and upon high potassium (80 mM KCl; solid line) depolarization. This response is skeletal specific since it is not dependent upon external Ca^{2+}. The traces from NLTs are from the same myotube with (top) and without Ca^{2+} (middle). Dysgenic cells (GLT) failed to respond to electrical or KCl depolarization, but they responded with Ca^{2+} release to the addition of 8 mM caffeine (lower trace; solid line). (Left) 10-s traces; (right) 20-s traces; (abscissa) arbitrary ratio units, proportional to free cytoplasmic $[Ca^{2+}]$, apply to all traces.

skeletal muscle, it seems that the molecular differentiation of the SR Ca^{2+} release sites is restricted to T-tubule/SR and sarcolemma/SR junctions (Sun et al., 1995). During skeletal muscle development, the organization of the feet (RyR) in the SR and the DHPR in the sarcolemma and T-tubules occurs simultaneously, upon or shortly after the initial docking of the two membrane systems (Flucher et al., 1994; Takekura et al., 1994). This is consistent with the situation in dysgenic muscle where the formation of junctions without feet has been described frequently, but the clustering of the RyR occurs only under advantageous circumstances (Garcia et al., 1990; Franzini-Armstrong et al., 1991; present study).

Can other DHPR α_1 subunit isoforms be responsible for the observed clustering of RyR in the T-tubule/SR junctions? A primary candidate for such a substitution would be the cardiac isoform. It is transiently expressed during skeletal muscle development (Chaudari and Beam, 1993; Varadi et al., 1995) and has been shown to functionally interact with the skeletal muscle RyR (Tanabe et al., 1991). Furthermore, the DHP-sensitive, Ca^{2+} current called I_{Ca-Dys} with cardiac activation kinetics has been described in dysgenic myotubes (Adams and Beam, 1989). However, RyR clusters in GLT myotubes not only lack the skeletal α_1 subunit but also the α_2 subunit. The α_2 subunit is expressed, but it is mistargeted to the plasma membrane and cytoplasmic compartments distinct from T-tubules. If a heterologous α_1 subunit isoform were to substitute for the mutated skeletal isoform and cause the differentiation of junctions, we would expect the α_2 subunit to associate with that α_1 subunit (for review see Isom et al., 1994). Thus, the absence of the DHPR α_2 subunit from the RyR clusters shows not only that this subunit is not necessary for the structural and molecular differentiation of the triad, but also provides indirect evidence for the lack of any α_1 subunit isoform from T-tubule/SR junctions in dysgenic muscle. This is also supported by the failure to label RyR clusters in GLT myotubes with antibodies recognizing the cardiac α_1 isoform (Flucher, B.E., unpublished results).

Reconstitution of dysgenic myotubes with the cardiac isoform of the α_1 subunit resulted in the restoration of E-C coupling with cardiac characteristics (Tanabe et al., 1991). GLT myotubes are capable of caffeine-induced Ca^{2+} release, but they fail to respond to depolarization with Ca^{2+} release from the SR. Again, if the observed junctions in the majority of GLT myotubes would contain the cardiac α_1 subunit isoform, a restoration of depolarization-induced Ca^{2+} release would have been expected. The failure of E-C coupling is further evidence for the absence of a DHPR α_1 subunit from T-tubule/SR junctions in dysgenic muscle, and thus indicates that the α_1 subunit plays no role in the formation of junction and the organization of the RyR within it. Conversely, the failure of depolarization-induced Ca^{2+} release in myotubes with an otherwise intact and functional T-tubule/SR junction confirms the importance of the DHPR α_1 subunit in skeletal muscle E-C coupling.

Consistent with our present data are recent results from another mutation of the E-C coupling apparatus. In skele-

Table II. Comparison of New Cell Lines with Primary Cultures

	Primary cultures		Cell lines	
	+/+ (normal)	mdg/mdg (dysgenic)	NLT (normal)	GLT (dysgenic)
Spontaneous contractions	+++	—	+	—
Depolarization-induced Ca^{2+} transients	+++	—	+++	—
Caffeine-induced Ca^{2+} transients	+++	+++	+++	+++
DHPR α_1 subunit	clustered	—	clustered	—
DHPR α_2 subunit	clustered	mistargeted	clustered	mistargeted
Ryr	clustered	diffuse* (clustered)	clustered	clustered/diffuse*
Cross-striations	+++	++	+	+
Heterologous fusion	normal	enhanced (4×)	normal	enhanced (2.5×)

*Not colocalized with the DHPR α_2 subunit.

tal muscle of the dyspedic mouse, with a targeted deletion of the RyR gene, triad junctions have been observed that lack the feet (Takeshima et al., 1994; Takekura et al., 1995). Thus, T-tubule/SR junctions can form not only without the DHPR but also without the RyR. Therefore, the idea that interactions between these two triad proteins may be involved in the association of the two membrane compartments must be dismissed. Whereas RyRs can assume their normal disposition in dysgenic myotubes, in muscle of the RyR knockout mouse, DHPR tetrads seem to be missing. This suggests that the RyR exerts an organizing influence on the DHPR, but not vice versa. Taking all this into consideration, a picture of the sequential formation of the triad evolves, beginning with the association of undifferentiated T-tubules and SR, aided by the binding of two currently unidentified constituents of both membrane systems. The contact with the T-tubules then induces the molecular differentiation of the terminal SR cisternae with the regular arrangement of the RyR. This, in turn, may lead to the tetradic organization of the DHPR in the junctional T-tubule membrane, and thus results in the close association of the voltage sensor and the Ca^{2+} release channel that is the prerequisite of their physical interactions in skeletal muscle E-C coupling.

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