# Changes in Architecture of the Golgi Complex and Other Subcellular Organelles during Myogenesis

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*Abstract.* Myogenesis involves changes in both gene expression and cellular architecture. Little is known of the organization, in muscle in vivo, of the subcellular organelles involved in protein synthesis despite the potential importance of targeted protein synthesis for formation and maintenance of functional domains such as the neuromuscular junction. A panel of antibodies to markers of the ER, the Golgi complex, and the centrosome were used to localize these organelles by immunofluorescence in myoblasts and myotubes of the mouse muscle cell line C2 in vitro, and in intact single muscle fibers from the rat flexor digitorum brevis. Antibodies to the ER stained structures throughout the cytoplasm of both C2 myoblasts and myotubes. In contrast, the spatial relationship between nucleus, centrosome, and Golgi complex was dramatically altered. These changes could also be observed in a low-calcium medium that allowed differentiation while preventing myoblast fusion. Muscle fibers in vivo resembled myotubes except that the ER occupied a smaller volume of cytoplasm and no staining was found for one of the Golgi complex markers, the enzyme α-mannosidase II. Electron microscopy, however, clearly showed the presence of stacks of Golgi cisternae in both junctional and extrajunctional regions of muscle fibers. The perinuclear distribution of the Golgi complex was also observed in live muscle fibers stained with a fluorescent lipid. Thus, the distribution of subcellular organelles of the secretory pathway was found to be similar in myotubes and muscle fibers, and all organelles were found in both junctional and extrajunctional areas of muscle.

MYOGENESIS is a complex process that involves several stages. Each one is characterized by dramatic changes in both gene expression and cellular architecture. During differentiation, mononucleated myoblasts fuse to form multinucleated myotubes; subcellular organelles, such as the Golgi complex, are reorganized (Tassin et al., 1985 *a*,*b*), and new membrane systems, such as the sarcoplasmic reticulum and the T-tubule system, are assembled (Flucher et al., 1991). Then, during further development in vivo, nuclei migrate to the periphery of the cell just beneath the plasma membrane, whereas myofibrils fill the center of the fiber (Kelly and Zacks, 1969). Fully mature muscle fibers are highly organized cells with several segregated structural and functional domains: these include the neuromuscular junction (NMJ),[1] the myotendinous junction, the contractile apparatus, and the subsarcolemmal cytoplasm.

How such domains are formed and maintained is still poorly understood despite careful observations of their development (Kelly and Zacks, 1969; Dennis et al., 1981; Salpeter, 1987*a*). Recent work on localized expression of acetylcholine receptor (AChR) subunit genes in vivo (Merlie and Sanes, 1985; Fontaine et al., 1988; Fontaine and Changeux, 1989; Goldman and Staple, 1989; Brenner et al., 1990) and with hybrid muscle cells in vitro (Pavlath et al., 1989; Ralston and Hall, 1989*a*,*b*; Rotundo and Gomez, 1990) has suggested a mechanism whereby localized gene expression and protein biosynthesis lead to the formation of domains enriched in specific gene products (Hall and Ralston, 1989).

The Golgi complex plays a central role in protein targeting and in the maintenance of domains in various cell types (Mellman and Simons, 1992). Surprisingly little is known, however, of the changes affecting the Golgi complex and other subcellular organelles of the secretory pathway during myogenesis. Tassin et al. (1985*a*,*b*) have shown that both the Golgi complex and the centrosome are reorganized during differentiation of muscle cells in vitro. In myoblasts, the Golgi complex is next to the nucleus to which it forms a cap (this organization of the Golgi complex will be referred to as polar); the centrosome is made of a pair of centrioles surrounded by the pericentriolar material which contains the microtubule-organizing centers. In myotubes, the Golgi

This work was initiated and partly completed in the Department of Physiology at the University of California, San Francisco.

1. *Abbreviations used in this paper*: AChR, acetylcholine receptor; β-COP, β-coat protein; C5-DMB-Cer, N-[5-(5,7-dimethyl BODIPY)-l-pentanoyl]-D-erythro-sphingosine; FDB, flexor digitorum brevis; NMJ, neuromuscular junction; Rho-butx, rhodamine-α-bungarotoxin.

complex is distributed in a narrow band around the nuclei (and will be referred to as perinuclear); the centrosomes are not associated with nuclei on a one-to-one basis anymore and the pericentriolar material is found both in centrosomes and around the nucleus. Tassin et al. (1985*a*,*b*) limited their work to muscle cells in vitro. Later, work on chick muscle fibers led Jasmin et al. (1989) to report that the distribution of the Golgi complex in vivo differed from that in vitro: the Golgi complex seemed to be present near the nuclei associated with the NMJ but absent from the rest of the muscle fiber. The NMJ represents <0.1% of the total surface of a muscle fiber. The authors suggested that this polar distribution would reinforce differences in gene expression between the junctional and extrajunctional part of the muscle.

Because the experimental systems, techniques, and tools used by Tassin et al. (1985*a*,*b*) and Jasmin et al. (1989) differed, it is difficult to compare their results. It is important to ascertain whether there is a difference between myotubes in vitro and muscle fibers in vivo, in regard to the distribution of subcellular organelles, since much of the work in vitro is aimed at understanding events taking place in vivo, and implicitly assumes that myotubes and muscle fibers are at least similarly organized.

The goal of the present work was to describe the distribution of the subcellular organelles of the secretory pathway at different stages of myogenesis, in vitro and in vivo; to compare their organization in myotubes and muscle fibers; and to compare the junctional and extrajunctional regions of muscle fibers.

The distribution of the ER, of centrosomes, and of the Golgi complex was examined in mammalian muscle cells in culture and in freshly dissociated single muscle fibers from rats. A panel of antibodies and a vital lipid stain were used to study the distribution of the organelles by immunofluorescent staining; electron microscopy was used to confirm the identification of the organelles by morphology. The results show that the distribution of subcellular organelles, in muscle fibers in vivo, is similar to that of myotubes in vitro. In particular, the Golgi complex was clearly identified in areas both near and far from the NMJ.

## Materials and Methods

### Antibodies and Reagents

The primary antibodies are described in Table I. Fluorescein-conjugated anti-mouse or anti-rabbit antibodies were obtained from Cappel Laboratories (Malvern, PA). Bisbenzimide (HOECHST) and bungarotoxin were purchased from Sigma Chem. Co. (St. Louis, MO). Rhodamine-α-bungarotoxin (Rho-butx) was prepared according to Ravdin and Axelrod (1977). Collagenase A (Boehringer Mannheim Corp., Indianapolis, IN) was a gift from Mark Lupa.

### Cell Culture

Details of the culture of the C2C12 subclone of the C2 mouse muscle cell line (Yaffe and Saxel, 1977) can be found elsewhere (Ralston and Hall, 1989*a*). Briefly, myoblasts plated on glass coverslips were kept in growth medium (Dulbecco's minimal essential medium [DMEM], 20% FBS, 0.5% chick embryo extract, 2 mM glutamine, 100 U/ml pen-strep) until 70–80% confluence, and then switched to fusion medium (DMEM, 5% horse serum, 2 mM glutamine), fed daily for 2–3 d, and stained. All media components were purchased from Gibco BRL (Gaithersburg, MD) except FBS obtained from Irvine Scientific (Irvine, CA).

### Preparation of Single Muscle Fibers

Single muscle fibers were prepared from the flexor digitorum brevis (FDB) muscle of 4–6 wk old Munich Wistar rats (Bekoff and Betz, 1977; Lupa and Caldwell, 1991). Briefly, the muscle was dissected in PBS, and transferred to an Eppendorf tube containing 2.5 mg collagenase A (Boehringer Mannheim Corp.) and 1 mg BSA (Sigma Chem. Co.) in 1 ml of DMEM. The tube was placed on a rotating wheel at 37°C for 90 min. The muscle was then transferred to a clean tube containing 1 ml of DMEM supplemented with 2.5% horse serum (Gibco Laboratories, Grand Island, NY). The fibers were dissociated by trituration into Pasteur pipettes with flame-narrowed bores of decreasing size. Medium was added to a final 3 ml per muscle and the fiber suspension was plated onto glass coverslips (0.2 ml fiber suspension per coverslip) pretreated with Matrigel (Collaborative Research Inc., Bedford, MA). The coverslips were placed in a $CO_2$ incubator at 37°C for 15–30 min to allow the fibers to settle and attach.

### Immunofluorescent Staining

For staining of the NMJ of FDB fibers, Rho-butx was added, to a final concentration of ~10 nM, to the fiber suspension before plating. The staining could be blocked by adding unlabeled bungarotoxin (Sigma Chem. Co.) to a final concentration of 2.5 μM.

For antibody staining, coverslips with C2 cells or FDB fibers were removed from the incubator and placed on ice for 10 min. The supernatant was then removed by gentle aspiration and replaced by 1% cold paraformaldehyde in PBS. The cells were fixed for 20 min on ice. All subsequent steps were performed at room temperature. The cells were permeabilized with 1% Triton X-100 in PBS (except when staining with the anti-β coat protein [β-COP] antibody, where 0.1% Triton X-100–0.05% SDS was used), blocked for 1 h in PBS containing Triton X-100 (0.1%), BSA (1%), and FBS (5%), and incubated for 2 h with the primary antibody in the same solution. After three washes in PBS, coverslips were incubated for 1 h with the appropriate fluorescein-conjugated secondary antibody (Organon Teknika, Malvern, PA), rinsed, stained with bisbenzimide (HOECHST 33258, 0.25 μg/ml, from Sigma Chem. Co.) for a few minutes, and mounted in glycerol supplemented with paraphenylenediamine (Platt and Michael, 1983).

The vital Golgi dye *N*-[5-(5,7-dimethyl BODIPY)-1-pentanoyl]-D-*erythro*-sphingosine (C5-DMB-Cer) (Pagano et al., 1991) was purchased from Molecular Probes Inc. (Eugene, OR), prepared according to Pagano and Martin (1988), and used as recommended (Pagano et al., 1991). A coverslip with live cells was mounted on a slide fitted with a silicon rubber chamber ("Ronsil," North American Reiss, Bellemead, NJ). Images were collected on a laser scanning confocal microscope equipped with a krypton/argon mixed gas laser (model MRC-600; Bio-Rad Laboratories, Richmond, CA).

### Electron Microscopy

After dissection of the FDB in PBS, the muscle was fixed at room tempera-

*Table I. Antibodies Used in This Work*

| Organelle | Antigen | Type/Name | Reference |
|---|---|---|---|
| ER | Membrane fraction | pc | Louvard et al., 1982 |
| Centrosome | Pericentrin | pc | Doxsey and Kirschner, 1992 |
| Golgi | α-mannosidase II | pc | Moremen and Touster, 1985 |
| Golgi | Clathrin heavy chain | mc, X32 | Blank and Brodsky, 1986 |
| Golgi | β-COP | mc, M3A5 | Allan and Kreis, 1986 |

*pc*, rabbit polyclonal; *mc*, mouse monoclonal.

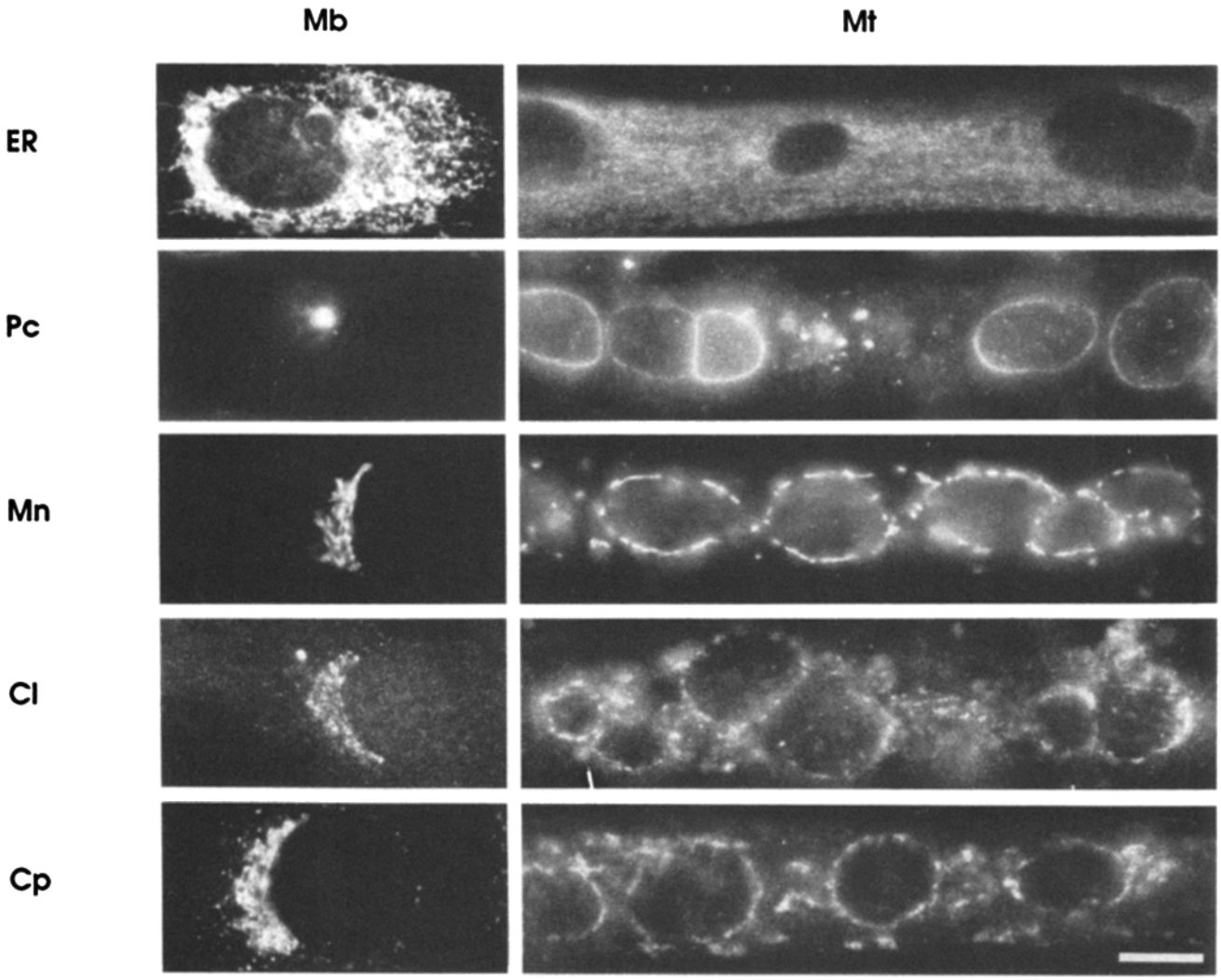


*Figure 1.* Redistribution of subcellular organelles during differentiation of muscle cells in culture. Myoblasts (*Mb*) and myotubes (*Mt*) of the mouse muscle cell line C2 were stained, as described in Materials and Methods, with antibodies recognizing, respectively, the ER, pericentrin (*Pc*), α-mannosidase II (*Mn*), clathrin (*Cl*), and β-COP (*Cp*) followed by a fluorescein-conjugated second antibody. Bar, 10 μm.

ture for 90 min with 2.5% glutaraldehyde in 0.1 N sodium cacodylate buffer (pH 7.4). All subsequent steps were performed by staff of the NINDS Electron Microscopy Facility. The muscle was trimmed, osmicated, treated with uranyl acetate, and embedded in Epon following standard procedures.

## Results

### Subcellular Organelles Are Reorganized during Muscle Cell Differentiation In Vitro

A panel of antibodies (described in Materials and Methods) was chosen to follow the changes in subcellular organelle distribution that take place during myogenesis. Antibodies were selected that would recognize the ER and the Golgi complex, organelles involved in protein biosynthesis. The markers of the Golgi complex were an antibody against α-mannosidase II, a resident Golgi enzyme, and antibodies against clathrin heavy chain and β-COP, the coat protein of nonclathrin-coated vesicles (Duden et al., 1991; Serafini et al., 1991). Because of the close relationship between microtubule-organizing material and the Golgi complex (Tassin et al., 1985*a*,*b*), an antibody against pericentrin, a protein of the pericentriolar material of centrosomes (Doxsey and Kirschner, 1992), was also included.

The antibodies were first used to characterize the changes in subcellular organelle distribution that accompany muscle differentiation in vitro. The changes in staining pattern observed when myoblasts of the mouse muscle cell line C2 fuse to form myotubes are illustrated in Fig. 1. ER staining was seen throughout the cell in both myoblasts and myotubes but whereas myoblasts showed a reticular ER similar to that observed in other mononucleated cell types (Terasaki and Reese, 1992), the stained elements appeared thinner in myotubes and generally aligned along the myotube axis. At this stage of development, myofibrils are not well organized in the cytoplasm of the myotubes, very few of which show Z bands (not shown); T-tubules as well are aligned along the myotube axis (Flucher, et al., 1991). For the other organelles examined here, more dramatic changes were easily seen. Changes in distribution of the Golgi complex, from polar to perinuclear, could be observed with each marker that was used. In parallel, the centrosome seemed to be reduced, in myotubes, to a smaller organelle, whose association with in-

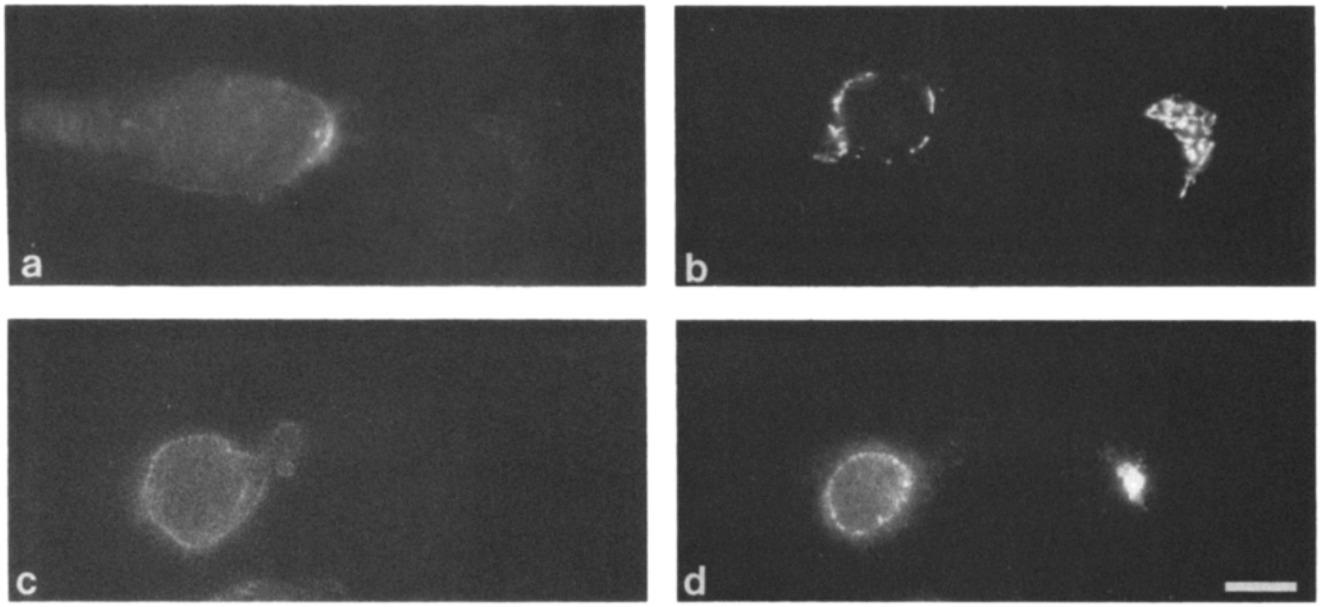


*Figure 2.* Redistribution of the Golgi complex and of the centrosomes takes place during differentiation without cell fusion. Cultures of C2 myoblasts were grown for 2 d in fusion medium supplemented with 1.5 mM EGTA; they were then stained with Rho-Butx (*a* and *c*) and with anti-α-mannosidase II (*b*) or antipericentrin (*d*) followed by fluorescein-conjugated second antibody, and observed by fluorescence with the appropriate filter. In each field two unfused cells are shown, one of which has differentiated and is stained with Rho-butx. Bar, 10 μm.

dividual nuclei was not clear. Several of these can be seen in the cytoplasmic gap between two nuclei on Fig. 1. In addition, perinuclear staining, undetectable in myoblasts, became prominent in myotubes. These changes are similar to those observed by Tassin et al. (1985*a*,*b*) in cultures of human myotubes. Similar staining patterns were observed when rat primary muscle cell cultures were stained instead of the C2 (not shown) and a similar distribution of the ER and of the Golgi complex has been shown by electron microscopy in myotubes of the rat muscle cell line L5 (Horovitz et al., 1989). No staining was seen when primary antibodies were omitted (not shown). Thus, differentiation in vitro is accompanied by profound changes in subcellular organization.

### *Differentiation, Not Fusion, Causes the Changes in Distribution of the Golgi Complex and of the Centrosome In Vitro*

The changes in cellular organization observed during differentiation could be induced by the changes in cellular geometry that take place when cells fuse. Alternately, they could be directly linked to the execution of the myogenic program and take place independently of cell fusion. To find out which of these alternatives was correct, C2 cells were grown in fusion medium supplemented with 1.5 mM EDTA. In these conditions, fusion is prevented but all other aspects of differentiation proceed normally (Fambrough and Rash, 1971; Prives and Paterson, 1974; Hu and Olson, 1990). After 2 d in EGTA, cultures were stained with Rho-butx to detect surface AChR and with the antimannosidase antibody to stain the Golgi complex or with the antipericentrin antibody to stain centrosomes. A fraction of the cells differentiated and were stained with the toxin. All cells that appeared positive for AChR staining presented a perinuclear, myotube-like Golgi complex (Fig. 2, *a* and *b*). Cells that did not express the AChR had a polar, myoblast-like Golgi complex (same figure). Similarly, all AChR-expressing cells presented the perinuclear pericentrin distribution characteristic of myotubes, whereas AChR-negative cells showed a myoblast-like single centrosome (Fig. 2, *c* and *d*). Similar results were obtained with the nonfusing muscle cell line BC3H1 (not shown).

These experiments, then, indicate that the changes in centrosome and Golgi complex pattern during muscle cell differentiation result from the activation of the myogenic program and occur independently from myoblast fusion.

### *Staining with Markers of the ER, the Centrosome, and the Golgi Complex Is Observed Extrajunctionally in Muscle Fibers In Vivo*

Single muscle fibers from young mature rats were isolated, stained with the antibodies, and observed as whole mounts. Staining was observed in both junctional and extrajunctional areas (the term junctional refers to the very small area at and very near the site of the NMJ; the term extrajunctional to the rest of the fiber). Examples of staining in extrajunctional areas are presented in Fig. 3. The staining with the antibody to the ER, which in myotubes extended throughout the cell, was seen in the myofibers to be restricted to a small area around and between nuclei. This change in distribution presumably results from exclusion of the ER from the central part of the fiber occupied by myofibrils and must follow the migration of the nuclei to the edge of the muscle fibers. In addition, a weaker striated staining was also seen that could reflect common elements between ER and sarcoplasmic reticulum. Pericentrin staining was generally similar to that observed in myotubes in that it showed small organelles and perinuclear staining. In myofibers, however, each residual centrosome (Fig. 3, *arrowheads*) appeared to be associated with one nucleus.

When markers of the Golgi complex were used, both

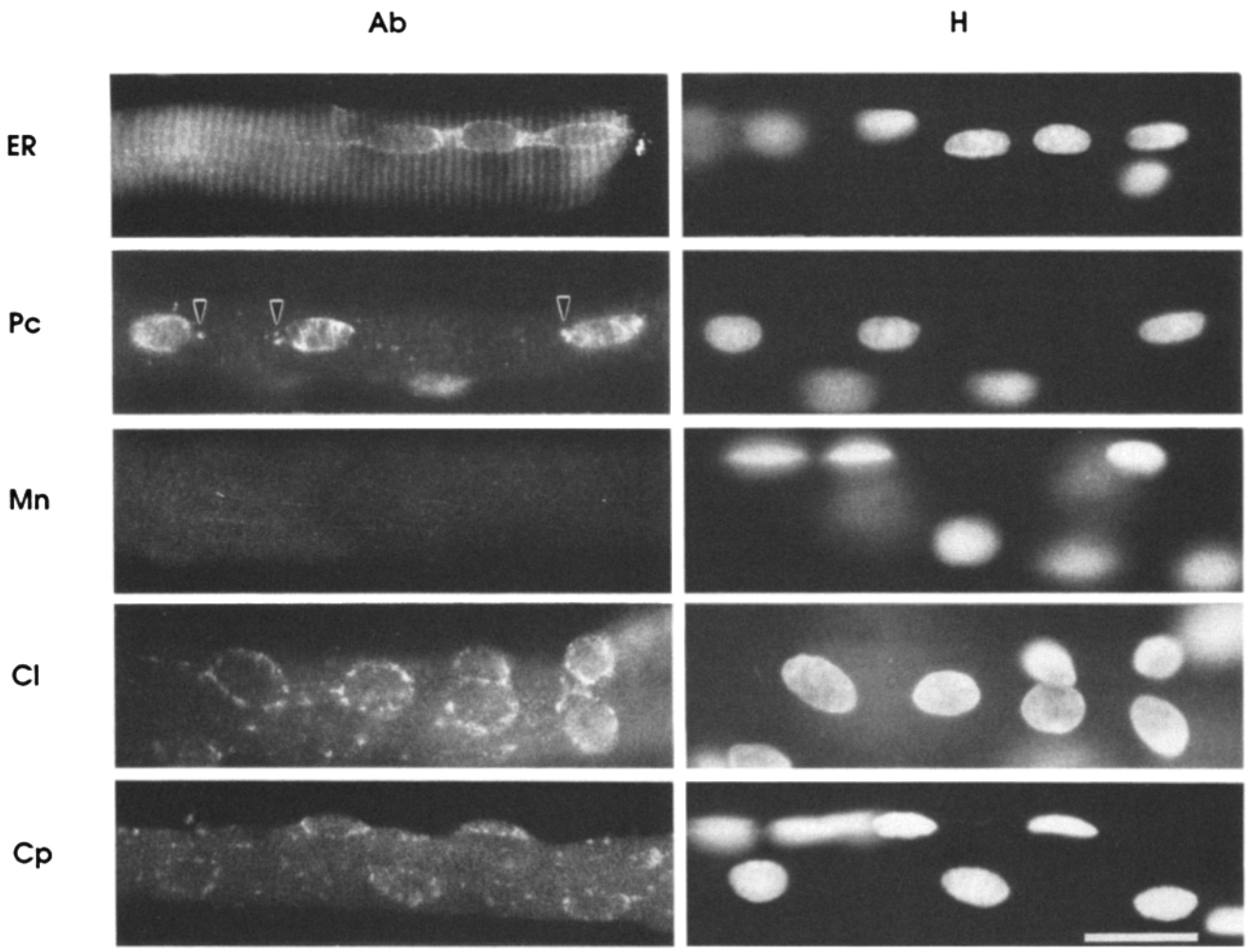


*Figure 3.* Distribution of subcellular organelles in extrajunctional areas of single muscle fibers. Muscle fibers from rat FDB dissociated by collagenase treatment (see Materials and Methods) were stained with antibodies (*Ab*) against the ER, pericentrin (*Pc*), α-mannosidase II (*Mn*), clathrin (*Cl*), and β-COP (*Cp*) followed by fluorescein-conjugated second antibody, and with HOECHST (H). The arrowheads in the pericentrin figure point to the centrosomes, each one close to a nucleus. Bar, 20 μm.

clathrin and β-COP antibodies produced a discontinuous perinuclear staining similar to that observed throughout the myotubes. Curiously, in myofibers, no staining was detected with anti-α-mannosidase II.

The same results were obtained with mouse muscle fibers and with rat muscle fibers that were dissociated by dissection rather than by collagenase digestion (not shown).

### *Staining of the Golgi Complex of Live Muscle Fibers with a Fluorescent Ceramide Is Observed throughout the Fiber*

Fluorescent lipids have been used as vital stains for the Golgi complex where they accumulate, presumably because of the role of this organelle in lipid biosynthesis and trafficking. To localize this function in muscle fibers, C5-DMB-Cer, a ceramide analogue labeled with the fluorophore BODIPY (Pagano et al., 1991), was added to the cells as a complex with BSA. C5-DMB-Cer labels the Golgi complex in many cell types, including myotubes (Pagano et al., 1991). Single FDB fibers were incubated with C5-DMB-Cer at 2°C, warmed up to 37°C, and observed live with a confocal microscope. A punctate staining resembling strings of pearls was seen, both as rings around the nuclei and extending along the fiber just beneath the plasma membrane (Fig. 4). An additional weaker cross-striated staining varied in intensity between experiments and is unexplained. The punctate staining was rather regular; the separation between the pearls appeared to be a multiple of the sarcomeric length.

The perinuclear staining appeared rather two-dimensional, forming a ring just below and parallel to the plasma membrane around the slightly bulging nuclei. Thus, for nuclei lying "flat" on the top or bottom side of the fiber, the whole ring could be seen in one single optical section. Three nuclei show this ring in Fig. 4, whereas one nucleus only shows a strong bipolar staining.

The staining with C5-DMB-Cer was observed in all parts of the muscle fiber. Because of the specific excitation and emission filter requirements to observe the Golgi complex at the exclusion of other subcellular structures in the cell (Pagano et al., 1991), double staining was difficult and identification of the junctional area was not attempted.

### *Organelle Distribution at the NMJ of Muscle Fibers*

It has long been known that several clustered nuclei, the

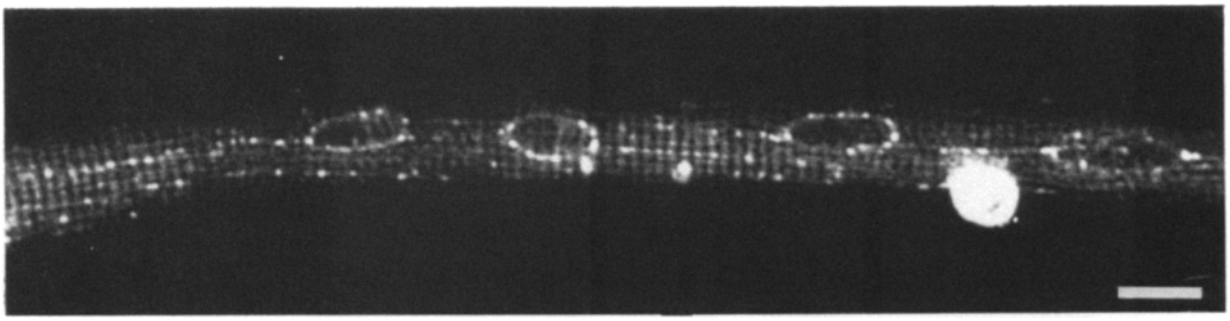

*Figure 4.* Live muscle fiber stained with C5-DMB-Cer. Muscle fibers were stained as recommended (Pagano et al., 1991) and observed live with a confocal microscope. The brightly stained object is a mononucleated cell stuck to the fiber. Bar, 10 μm.

"fundamental nuclei," underlie the NMJ (for review see Couteaux, 1960). To determine whether subcellular organelles associated with these nuclei were organized differently from those found extrajunctionally, the muscle fibers were triple stained with Rho-butx (an AChR ligand) to localize the NMJ, with HOECHST dye to label the nuclei, and with each of the antibodies (Fig. 5). Not only was the perinuclear staining observed, but also a pattern that was similar to that of α-bungarotoxin. It was especially noticeable with antibodies to the ER, to pericentrin, and to clathrin, less so with antibodies to β-COP. Again, no staining at all could be detected with anti-α-mannosidase II antibodies.

### *Intracellular Staining with Rho-butx Provides Visualization of the Organelles Involved in AChR Biosynthesis, In Vivo*

Gu et al. (1989) have shown that staining of permeabilized myotubes of the muscle cell line C2 with Rho-butx labeled intracellular AChR in the ER and Golgi region throughout the cell. Presumably, the labeled AChR molecules were at different stages of the secretory pathway and the staining thus provided a functional assay for the detection of the organelles involved in protein biosynthesis. Muscle fibers were incubated with an excess of unlabeled bungarotoxin to saturate surface AChR, and then permeabilized and stained with Rho-butx. The staining was limited to the junctional nuclei (Fig. 6); it was weak in intensity and perinuclear. No additional pattern similar to external endplate staining was seen.

### *Analysis at the Electron Microscope Level Shows Small Golgi Stacks throughout Muscle Fibers*

To resolve the apparent conflict between the results obtained with anti-α-mannosidase II, which did not label muscle fibers, and those obtained with other markers of the Golgi complex, muscle fibers were analyzed by electron microscopy. Sections were prepared from the whole muscle rather than from dissociated fibers so that identification of the NMJ would be facilitated by the presence of presynaptic elements. Typical Golgi stacks were easily identified in both junctional and extrajunctional areas of the sections. Examples of Golgi stacks in extrajunctional areas are shown in Fig. 7. In one example, (Fig. 7 *a*) the stacks are just next to a nucleus; in the other example (Fig. 7 *b*), two stacks are next to one another in a band of cytoplasm between nuclei, just beneath the plasma membrane. The stacks were generally small (< 5 μm in the largest dimension) and included three to four cisternae. Clathrin-coated vesicles (Fig. 7, *black arrowhead*) and nonclathrin-coated vesicles (*white arrowheads*) were associated with the cisternae. Of 77 nuclei that were examined in 14 sections, 40 (52%) showed well-formed Golgi stacks similar to those shown in Fig. 7; 28 of these nuclei (36%) presented a single stack whereas 12 (16%) presented two stacks. Similar results were obtained when sections were prepared from dissociated muscle fibers (not shown). When longitudinal sections of cultures of C2 myotubes were treated and examined in the same way, they showed longer and more numerous stacks of Golgi cisternae (not shown).

In the 14 sections that were screened, three neuromuscular junctions were found. The area under the junctional folds (Fig. 8) appeared rich in ribosomes and in coated and uncoated vesicles. Golgi stacks could be identified next to three out of four nuclei; they did not appear different from those in other areas of the same fiber. It is then likely that the additional staining pattern observed by immunofluorescence at the NMJ with Golgi complex markers results from an accumulation of secretory and/or endocytotic vesicles.

## *Discussion*

The goal of this work was to study the distribution of the ER, the Golgi complex, and the centrosome at different stages of myogenesis: in particular, to find out whether their organization, in mature muscle fibers, is similar to that in myotubes in culture; whether there are differences between junctional and extrajunctional regions of muscle fibers; and investigate the reason for the profound changes in organelle architecture observed during differentiation.

The most important result is that the Golgi complex, in muscle fibers in vivo, is distributed throughout the muscle fiber and is organized like the myotube Golgi complex. This conclusion was reached on the basis of the staining obtained with anticlathrin, anti-β-COP, and a fluorescent ceramide, and confirmed by the observation in electron microscopy of characteristic stacks of Golgi cisternae, next to nuclei and between nuclei, just beneath the plasma membrane.

This conclusion stands in sharp contrast to that of Jasmin et al. (1989). Failing to detect extrajunctional staining in chick muscle sections labeled with an antibody against a Golgi complex antigen, these authors suggested that there was no extrajunctional Golgi complex in mature muscle. In the present work, an antibody against α-mannosidase II (Moremen and Touster, 1985), often used to localize the Golgi complex in cultured cells, also failed to stain mature muscle fibers. α-Mannosidase II is a resident Golgi enzyme

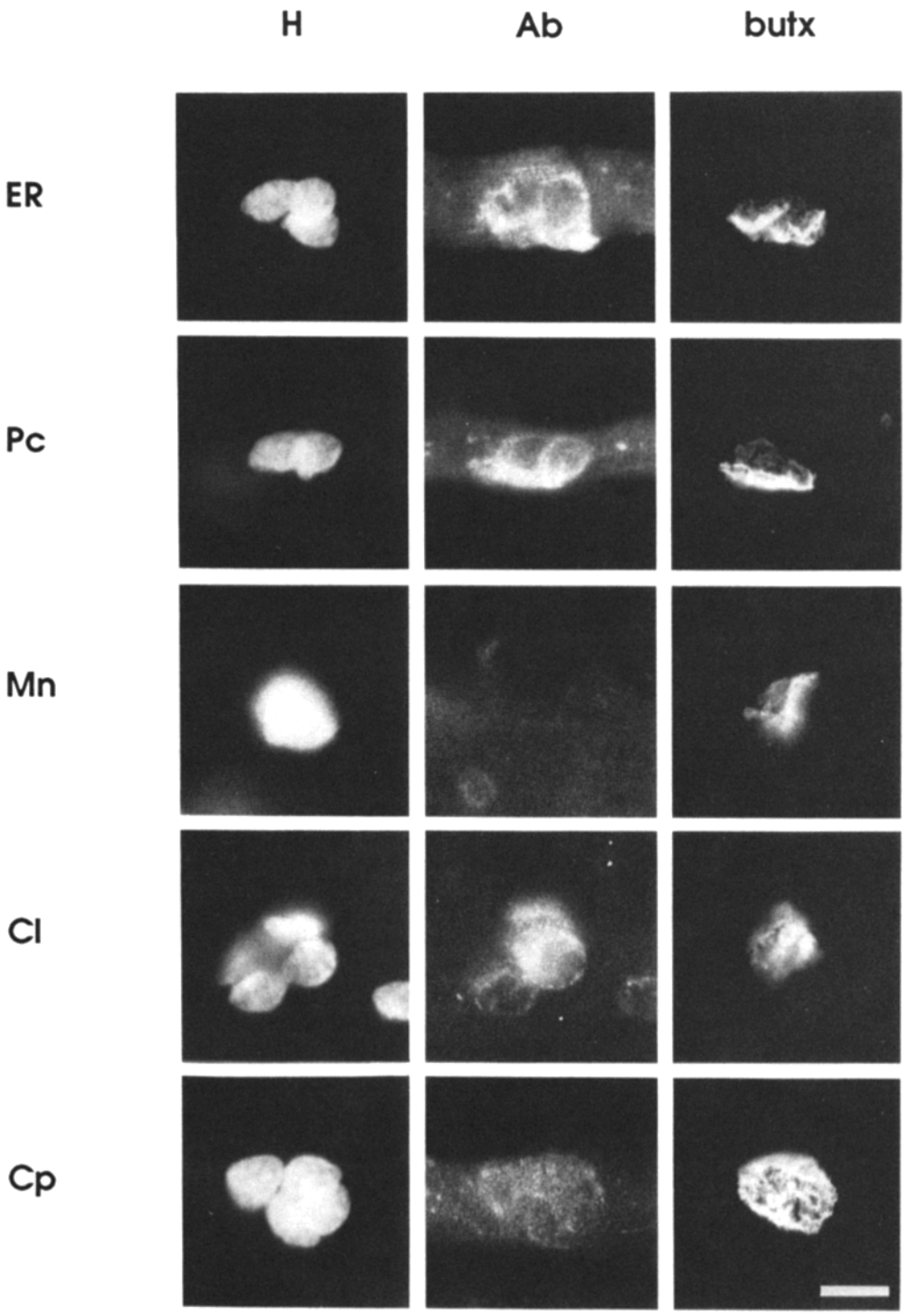


*Figure 5.* Staining at the NMJ of single muscle fibers. To localize the NMJ, fibers were stained before permeabilization with Rho-butx (*butx*). They were then stained, as previously, with antibodies (*Ab*) against the ER, pericentrin (*Pc*), α-mannosidase II (*Mn*), clathrin (*Cl*), and β-COP (*Cp*). Nuclei were stained with HOECHST (*H*). Bar, 10 μm.

involved in the processing of complex carbohydrate chains. The level of α-mannosidase II appeared to decrease below detection level during muscle maturation. The enzyme may be replaced by a different isoform (Fukuda et al., 1990) as happens with muscle-specific proteins such as myosin heavy chain (Whalen et al., 1981) and AChR subunits (Gu and Hall, 1988). Alternately, the level of α-mannosidase II activity required in mature muscle may be very low. Results with other markers suggested that the Golgi complex was still present but its contents changed.

As new antibodies to proteins transiently associated with the Golgi complex are characterized (Willison et al., 1989; Goud et al., 1990; Stearns et al., 1990), more markers become available for the detection of this organelle. With all such markers, one potential problem, underlined by the results reported here, is that their expression and localization may vary between cell types or according to developmental or physiological conditions. Because clathrin and β-COP play an important role in protein trafficking in eukaryotes, their presence may be more consistent. However, clathrin and β-COP are also associated with coated vesicles in the cytoplasm and clathrin forms coated pits on cell membranes.

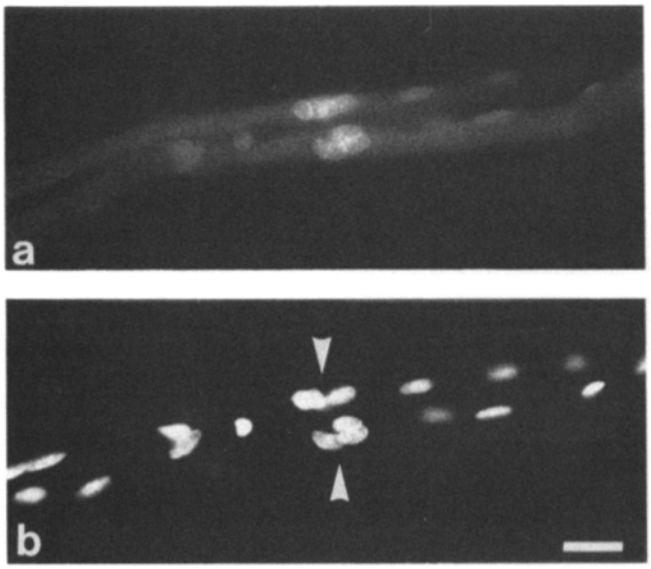


*Figure 6.* Staining of intracellular AChR in FDB muscle fibers. Staining of surface AChR was blocked by an excess of unlabeled bungarotoxin. Fibers were then washed, permeabilized, and stained with Rho-butx (*a*) and with HOECHST (*b*). Two fibers lie together in the figure; the clusters of junctional nuclei are indicated by arrowheads. Bar, 20 μm.

Thus, additional markers, such as a fluorescent lipid, and morphological analysis by electron microscopy were necessary to confirm that the staining observed corresponded to the Golgi complex.

Staining of the Golgi complex observed with the fluorescent ceramide differed from that obtained with protein markers of the Golgi complex in that it was more punctate and regular in appearance. Interestingly, the same punctate and bipolar staining has been observed when fixed FDB fibers were stained with an antibody to the muscle-specific GLUT4 glucose transporter (Ploug, T., and E. Ralston, unpublished observations). Thus, these fine differences in staining pattern do not result from changes during muscle fiber fixation but could, instead, result from the association of the marker with different compartments of the Golgi complex.

Another finding of this work is that the organization of the Golgi complex at the NMJ is similar to its organization elsewhere along the muscle. When the NMJ was identified by labeling with Rho-butx and the staining with markers of the ER, of the Golgi complex, and of centrosomes around the junctional nuclei examined, it appeared stronger than elsewhere in the fiber and formed a pattern similar to that of Rho-Butx (Fig. 5). The electron micrographs, however (Fig. 8; also see Salpeter, 1987*a*), showed that the number of stacks or the number of cisternae per stack was not different from that found in other areas of the muscle. Several nuclei are clustered at the NMJ and the layer of cytoplasm that separates them from the plasmalemma is severalfold thicker than elsewhere because of the postsynaptic membrane folds (Salpeter, 1987*b*). Thus, nearly any cytoplasmic component would appear enriched at the junction by conventional fluorescence microscopy. In addition, the junctional cytoplasm appeared rich in various types of coated vesicles, presumably accounting for the increased clathrin and β-COP staining. On the other hand, numerous molecules have been shown to be specifically enriched at AChR clusters in vitro (for review see Bloch and Pumplin, 1988) or in vivo (Froehner et al., 1987; Sealock et al., 1989; Jasmin et al., 1990) and this could be the case for pericentrin.

Mannosidase staining was not detected at the NMJ either, indicating that the down regulation of the enzyme happens throughout the muscle. The staining reported by Jasmin et al. (1989), at the junction only and polar rather than perinuclear, suggests that regulation of their marker could be different. Junctional staining, both with antibodies against markers of the Golgi complex and with Rho-butx to label intracellular AChR (Fig. 6), was perinuclear. Similarly, Simon et al. (1992) observed a perinuclear pattern around the synaptic nuclei in muscle fibers of transgenic mice expressing human growth hormone. Thus, it is concluded that the organization of the Golgi complex is the same throughout the mature muscle fiber as it is throughout the myotube, and that mRNA localization, rather than differential subcellular organelle distribution, is responsible for the maintenance of domains such as the NMJ.

Although the changes in the Golgi complex are very dramatic at the light microscopy level, they only affect the size of the stacks and their spatial arrangement. They do not appear to modify the membrane cycling between the Golgi complex and the ER: treatment with Brefeldin A (Lippincott-Schwartz et al., 1989) resulted in rapid redistribution of Golgi proteins to the ER in both myoblasts and myotubes (data not shown). The mechanism of the changes in subcellular organelle distribution during myogenesis is not known. Nor is it known whether the elements of the Golgi complex in a myotube or muscle fiber are connected, as suggested in other cell types (Cooper et al., 1990; Rambourg and Clermont, 1990).

The observation that microtubules, in cultured myotubes, were nucleated by the perinuclear microtubule-organizing centers and that centrosomes were not associated with nuclei on a one-to-one basis (Tassin et al., 1985*a*,*b*; and this work) suggested that the centrosome is not essential in differentiated muscle cells. It was shown here (Fig. 3) that in muscle fibers, however, each centrosome was associated with a nucleus in a rather precise arrangement. In lymphocytes, the position of the centrosome defines the cell axis (Geiger et al., 1982; Kupfer et al., 1983). The centrosomes could also play a role in the migration of the nuclei towards the plasmalemma during muscle maturation: normal nuclear migration towards the cell cortex is prevented when association between centrosomes and nuclei is disrupted in Drosophila oocytes (Raff and Glover, 1989).

The finding that the changes in subcellular organelle distribution are activated by differentiation, independently of cell fusion, suggests that tissue-specific transcriptional factors regulate components of the centrosomes, of the cytoskeleton, or of the Golgi complex. Remarkably, similar changes in the organization of the Golgi complex take place during the development of hippocampal neurons in culture (Goslin et al., 1990). Since both muscle and nerve cells are differentiated and postmitotic, it is possible that different tissue-specific factors or cell cycle control proteins interact with common structural proteins to regulate the cell architecture.

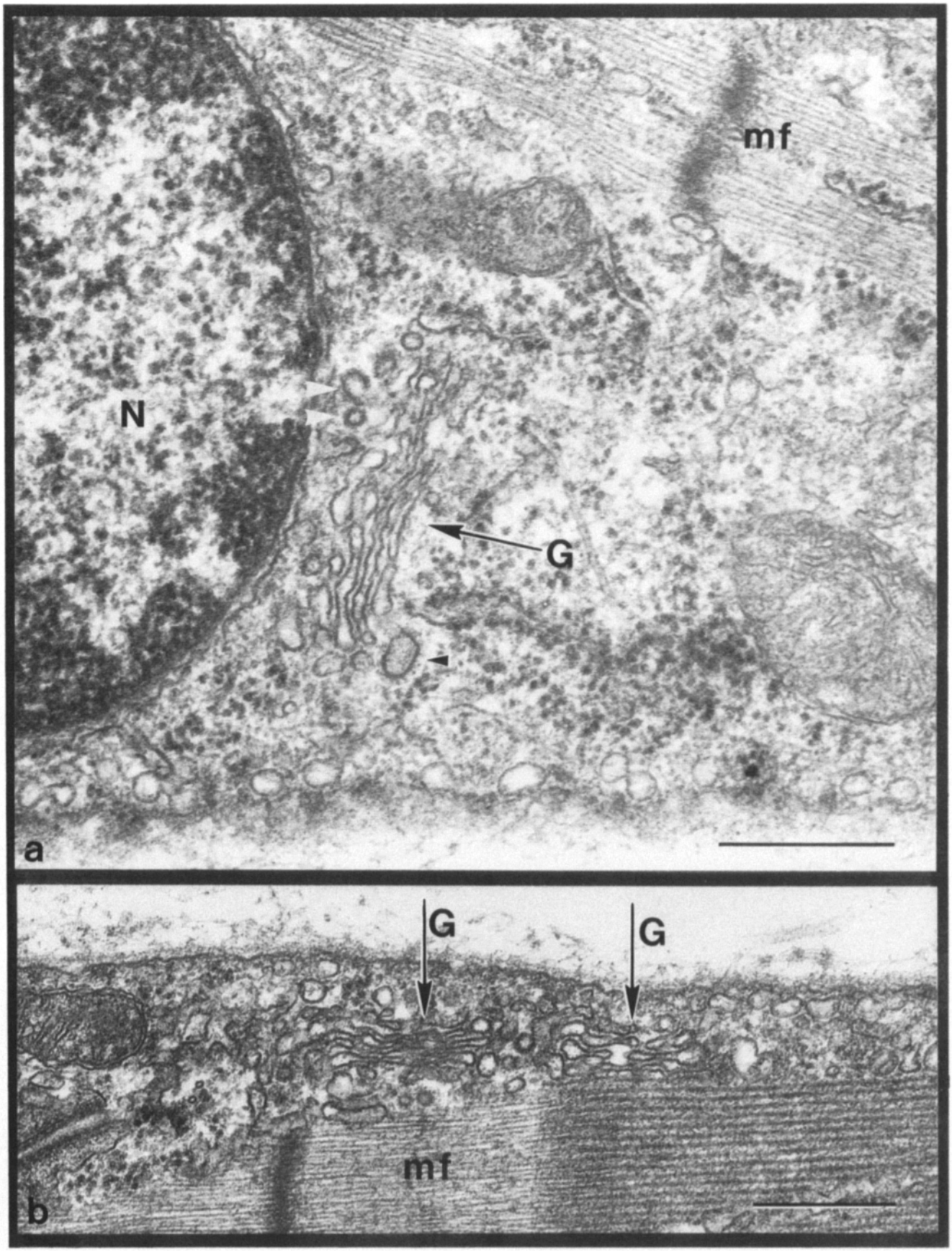


*Figure 7.* Morphological identification of Golgi complex in extrajunctional areas of muscle. Rat FDB was dissected and treated for electron microscopy as described in Materials and Methods. Stacks of Golgi cisternae were observed in the perinuclear area (*a*) or between nuclei (*b*). The Golgi complex is surrounded with clathrin-coated (*black arrowhead*) and nonclathrin-coated (*white arrowheads*) vesicles. *G*, Golgi cisternae; *mf*, myofibrils; *N*, nucleus. Bar, 1 μm.

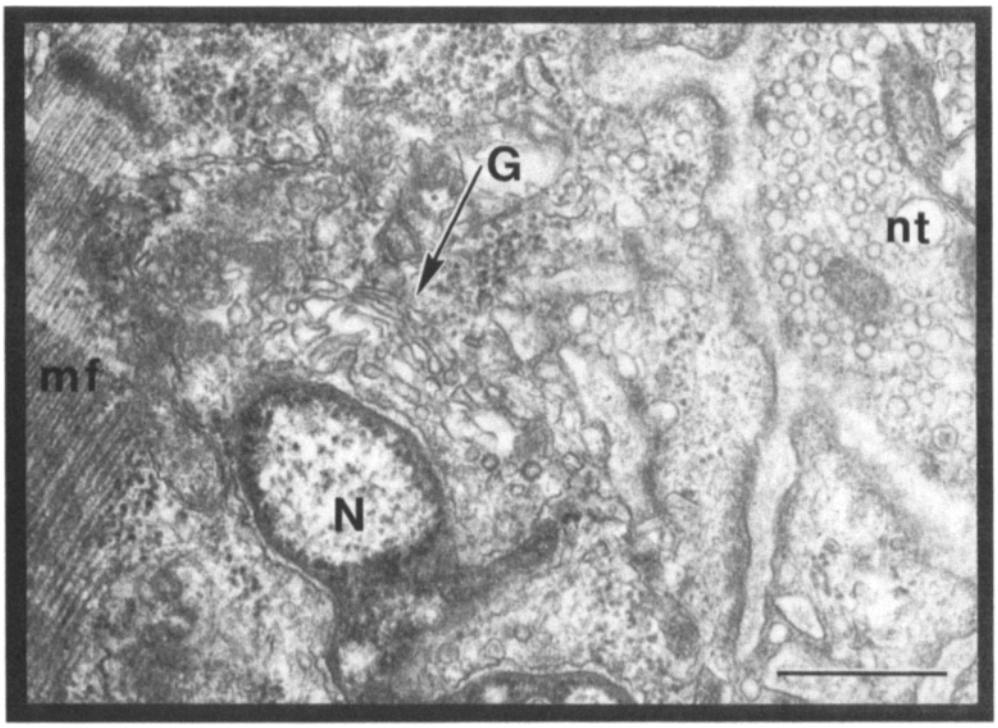


*Figure 8.* Electron microscopic observation of Golgi cisternae at the NMJ. Neuromuscular junctions were identified by the presence of nerve terminals (*nt*) filled with synaptic vesicles and by the folds of the muscle membrane. *G*, Golgi cisternae; *mf*, myofibrils; *N*, nucleus (this nucleus was highly invaginated and only part of it shows in this photograph). Bar, 1 μm.

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