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# The Expression of Dystrophin-associated Glycoproteins During Skeletal Muscle Degeneration and Regeneration. An Immunofluorescence Study

RUTH VATER, PH.D., JOHN B. HARRIS, PH.D., LOUISE V. B. ANDERSON, PH.D.,
STEVEN L. ROBERDS, PH.D., KEVIN P. CAMPBELL, PH.D.,
AND MICHAEL J. CULLEN, D.PHIL.

**Abstract.** The distribution and expression of dystrophin and three of the dystrophin-associated glycoproteins (DAG), α-dystroglycan (156 kDa DAG), β-dystroglycan (43 kDa DAG) and adhalin (50 kDa DAG) in rat skeletal muscle were studied during a controlled cycle of degeneration and regeneration induced by the injection of a snake venom. Cryosections of muscle at various stages of degeneration and regeneration were labeled using monoclonal antibodies to the three glycoproteins and examined at fixed time points after venom injection. Adhalin and α-dystroglycan remained present at the sarcolemma throughout the entire cycle of degeneration and regeneration. β-Dystroglycan, on the other hand, was lost from the sarcolemma by 12 hours and reappeared 2 days after venom injection when new muscle fibers were being formed. Dystrophin was not lost from the sarcolemma until 24 hours after venom injection and did not reappear at the membrane until 4 days. It is suggested that dystrophin and the glycoprotein complex are synthesized separately, both temporally and spatially, and only become associated at the plasma membrane during the later stages of regeneration. The expression of β-dystroglycan in the regenerating muscle fibers was first seen at sites of newly forming plasma membrane that were closely associated with the old basal lamina tube. The basal lamina may therefore have a regulatory or modulatory role in the expression of the DAG.

**Key Words:** Adhalin; α-Dystroglycan; β-Dystroglycan; Dystrophin; Muscle degeneration; Muscle regeneration; Plasma membrane.

## INTRODUCTION

The degenerative myopathy, Duchenne muscular dystrophy (DMD), is the most common X-linked disorder in humans (1) and is associated with the absence of or a defect in dystrophin, the protein product of the gene located at position p21 on the short arm of the X-chromosome (2, 3). In previous studies, dystrophin has been shown to be localized to the plasma membrane of healthy muscle fibers (4–8). Ultrastructural studies using gold-labeled monoclonal antibodies raised against defined epitopes of dystrophin showed that the dystrophin molecules label at intervals of approximately 165 nm along the internal face of the plasma membrane in both longitudinal and transverse orientation on sections of rat skeletal muscle. This labeling interval was in close agreement with measurements of the length of the putative dystrophin molecule isolated from chicken gizzard made using the rotary-shadowing method (9), although slightly different from the measurements made from rabbit muscle (10), and led to the suggestion that dystrophin molecules formed a regular lattice (11–14).

In 1989, Campbell's group showed that the linkage between dystrophin and the plasma membrane in skeletal muscle was mediated by a complex of glycoproteins (15). This complex was shown to contain an intracellular 59 kDa protein (syntrophin); four transmembrane proteins of 50 kDa (adhalin), 43 kDa (β-dystroglycan), 35 kDa and 25 kDa; and an extracellular protein of 156 kDa (α-dystroglycan) (16–18). Further work showed that the dystrophin molecule associated with syntrophin and β-dystroglycan (19), while α-dystroglycan bound to laminin in the basal lamina (20). It was later found that when dystrophin was absent in the muscles of patients with DMD, and of mice with X-linked muscular dystrophy (*mdx*), the dystrophin-associated glycoproteins (DAG) were also drastically reduced or absent (16, 20–23). Several other pieces of evidence imply that one or all of the constituents of the complex of glycoproteins play a major role in the normal physiology of the muscle cell: (i) a deficiency of adhalin is linked with severe childhood autosomal recessive muscular dystrophy (24, 25); (ii) a deficiency of adhalin is associated with hereditary cardiomyopathy in the hamster (26); and (iii) a number of severely affected DMD patients have been described who possess a truncated form of dystrophin specifically lacking the entire C-terminal region of the protein (see, for example, 27). It has been speculated that the severity of the phenotype of these patients could be due either to the loss of the C-

From the Muscular Dystrophy Group Research Laboratories, Regional Neurosciences Centre, Newcastle General Hospital, Newcastle upon Tyne, United Kingdom (RV, JBH, LVBA, MJC), and the Howard Hughes Medical Institute and Department of Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa (SLR, KPC).

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Correspondence to: Ruth Vater, Ph.D., Muscular Dystrophy Group Research Laboratories, Regional Neurosciences Centre, Newcastle General Hospital, Westgate Road, Newcastle upon Tyne, NE4 6BE, United Kingdom.

terminal domains of dystrophin, the loss of one or more of the DAG, or both (16, 28–31). That the reduction or lack of glycoproteins may be involved, directly or indirectly, in the pathology of muscle of patients with neuromuscular disorders suggests the need for further investigation into the localization, distribution and function of the DAG in muscle fibers.

In an earlier report, Vater et al (8) described a study of the expression and localization of dystrophin in degenerating and regenerating rat skeletal muscle. It was found that dystrophin was expressed and localized to the plasma membrane relatively late after the regenerative process had begun. We hypothesized that the DAG would be expressed and localized earlier than dystrophin in the regenerating muscle fibers, and that the DAG may act as an anchor (or template) for the insertion of dystrophin into the membrane. The experiments described below were devised to explore this hypothesis and to aid our understanding of the relationship between dystrophin and its associated glycoproteins.

## MATERIALS AND METHODS

### Source of Tissue

Female Wistar rats weighing 90–120 g were anesthetized using a combination of 2.5% Halothane, 500 l/min $N_2O$ and 300 l/min $O_2$. A single subcutaneous injection of 15 μg of whole venom (0.2 ml of 75 μg/ml in 0.9% w/v NaCl) from the Australian tiger snake, *Notechis scutatus scutatus* (Sigma, code V-0251), was made into the dorsolateral aspect of the left hind limb at the line of demarcation between the gastrocnemius and tibialis anterior muscles (32). The injection was made thus so that the venom was introduced into the immediate vicinity of, rather than directly into, the underlying soleus muscle, thereby avoiding physical damage of the soleus muscle by the needle, and preventing necrosis incurred by such damage. Contralateral muscles served as controls. Both soleus muscles were removed at 1 hour (h), 3 h, 6 h, 12 h, 1 day (d), 2 d, 3 d, 4 d, 7 d and 21 d post-injection for examination. Muscles from six animals were used at each time point. The data illustrated in Figures 2 to 9 are typical examples of the observations made. At all times the animals were treated in accordance with the requirements of the Animals (Scientific Procedures) Act, 1986.

### Preparation of Muscles for Immunocytochemistry

The muscles were pinned to dental wax under slight tension and lightly fixed for 1 h in 2% w/v paraformaldehyde plus 0.001% w/v glutaraldehyde in 0.1 M phosphate buffer (PB, composition 0.02 M $NaH_2PO_4.2H_2O$; 0.08 M $Na_2HPO_4$; pH 7.35) at 4°C. This procedure allowed the preservation of antigenicity and, at the same time, the ultrastructural architecture of the muscle, which was to be studied ultimately both at the light and electron microscopic levels. After fixation, the muscles were washed in phosphate-buffered saline (PBS, composition 0.1 M PB; 0.15 M NaCl; pH 7.2), trimmed, and their mid-belly portions cut into small blocks of approximately 1.0 mm × 0.5 mm × 0.5 mm. The small tissue blocks were cryoprotected overnight in 2.3 M sucrose in 0.1 M PB at 4°C. The blocks were then mounted on aluminium rivets, plunge-frozen in liquid nitrogen, and stored in liquid nitrogen until use.

Longitudinal and transverse sections were cut using a Reichert FC4D cryoultramicrotome (knife temperature −95°C, block temperature −75°C) and transferred onto gelatin-coated slides on a drop of 2.3 M sucrose. The slides were then immersed in PBS, washed in 50 mM ammonium chloride in PBS, and excess moisture removed by gentle blotting. The sections were incubated for 1 h at room temperature in 10 μl of the primary antibody (monoclonal anti-dystrophin antibodies against the rod domain, Dy4/6D3 [IgG subclass 2a], and the C-terminal domain, Dy8/6C5 [IgG subclass 2a, see 33, 34], diluted 1:10 with PBS containing 0.5% w/v bovine serum albumin [BSA] or rabbit serum albumin [RSA]; monoclonal anti-β-dystroglycan antibody, 43DAG/8D5 [IgG subclass 2a, see 23], diluted 1:10 with PBS/BSA or PBS/RSA; monoclonal anti-adhalin antibody, $IVD3_1$ [IgG subclass 1, see 24, 35], diluted 1:50 with PBS/BSA or PBS/RSA; monoclonal anti-α-dystroglycan antibody, IIH6 [IgM, see 18, 20], diluted 1:5 with PBS/BSA or PBS/RSA; rabbit affinity purified anti-laminin antibody [Sigma, code L-9393], diluted 1:500 with PBS/BSA or PBS/RSA). The sections were then washed twice in PBS and further incubated in 20 μl of the secondary antibody (rhodamine-conjugated rabbit anti-mouse serum, DAKO code R270), diluted 1:200 in PBS/BSA or PBS/RSA, for 1 h at room temperature. The slides were washed twice in PBS, once in distilled water, dried around the sections, and mounted with Uvinert before examination using a Nikon Labophot fluorescence-optics microscope. Control sections were processed as described above, except that tissue culture medium containing 10% fetal calf serum ($D_{10}$ tissue culture medium), normal rabbit serum, and an irrelevant IgG or IgM control of the same subclass as the primary mouse monoclonal antibody were used instead of primary antibody. All controls performed showed cryosections that were devoid of labeling by the secondary antibody.

### Analysis of Western Blots

Small blocks of muscle (20–25 mg) were homogenized in 20 volumes of SDS treatment buffer, and 30 μl aliquots were used for polyacrylamide gel electrophoresis and Western blotting as described for dystrophin analysis (33). The blots to be probed for β-dystroglycan were from 4–7% gradient gels, while the blots to be probed for α-dystroglycan and adhalin were from non-gradient 10% gels. In both cases the blots were blocked for 1 h at room temperature using a 10 mM Tris buffer containing 0.15 M NaCl and 0.05% v/v Tween 20 (TBST) plus 5% w/v skimmed dried milk powder. The blots were then incubated with primary antibody (anti-α-dystroglycan, neat; anti-β-dystroglycan, diluted 1:20; anti-adhalin, diluted 1:50 in TBST) for 1 h at room temperature. After washing, the blots were incubated with horseradish peroxidase-conjugated second antibody (DAKO P260), diluted 1:300 with TBST, for 1 h at room temperature. After washing, the blots were exposed to 0.05% w/v diaminobenzidine hydrochloride in PBS containing 0.025% v/v hydrogen peroxide for 5 minutes. After development of the bands, the blots were rinsed well, dried and mounted on filter paper.

Densitometry was used to obtain quantitative data from the

Western blots. The blots and dried gels were scanned at an optical resolution of 400 d.p.i. using an Epson GT8000 flat bed scanner. The scanned images were stored as 256 greyscale TIFF files. The band images contained in the TIFF files were quantified using the Bioscan Optimas image analysis software package, modified for Western blots by K. Davison. The measurement technique employed assessed the total density contained within the boundaries of each band (volume relative optical density). Line graphs were drawn to present the densitometry data in a visual form.

The relative optical density of each band was calculated for sets of both β-dystroglycan and dystrophin standards and venom-injected muscle samples, and graphs were plotted using a line of best fit from the values obtained. The dystrophin and β-dystroglycan standards were prepared by diluting homogenates of control muscle to produce a range of dilutions from 1.0 mg to 20.0 mg protein concentration. The standard graphs for both β-dystroglycan and dystrophin were each fitted by linear regression lines of $y = 1.09x - 3.80$ and $y = 1.00x - 1.36$, respectively, and which had correlation coefficients of $r = 0.99$ and $r = 0.995$, respectively ($p > 0.001$), indicating that densitometry was linear with the amount of protein loaded onto and transferred from the gels.

### Terminology Used in the Text

The terminology applied to membrane systems surrounding muscle fibers is often confusing. In this work the following terminology is used:

*Sarcolemma:* The sarcolemma describes the structure including the outermost components of the cytoskeleton, the plasma membrane, the basal lamina, and the reticular network external to the basal lamina.

*Plasma Membrane:* The plasma membrane is the electrically excitable phospholipid membrane lying between the basal lamina and the cytoskeleton of the muscle fiber. The plasma membrane contains specific external, internal and transmembrane proteins, and these proteins determine the properties of the different parts of the membrane.

*Basal Lamina:* The basal lamina lies external to the plasma membrane of the muscle fiber. It is composed primarily of collagen type IV, but also contains collagen type V, laminin and fibronectin. It persists during degeneration of myofibers and is presumed to act as a mechanical scaffold during muscle repair and regeneration.

## RESULTS

### Pathology of Muscle Following Injection with Snake Venom

The toxic constituents of the venom of the Australian tiger snake, *Notechis scutatus scutatus,* damage skeletal muscle. The characteristics of the process are well known: individual muscle fibers began to exhibit hypercontraction and disruption of the plasma membrane at approximately 1–3 h, and by 6 h myofibrils and sarcomeres became disorganized and severely hypercontracted. There was extensive disruption of the plasma membrane, with perivascular infiltration of phagocytic cells. By 24 h the muscle fibers consisted of tubes of basal lamina containing clumps of degenerating myofibrillar debris and phagocytic cells. The microcirculation and the satellite cells were preserved. There was an early (12–24 h) activation of surviving satellite cells, and muscle fibers began to regenerate within the preserved basal lamina tubes by 2–3 d. The process of regeneration was virtually complete by 14–21 d. It was interesting to note that the intrafusal fibers of the muscle spindles tended to be spared from the effects of the venom. A detailed description of venom-induced damage to skeletal muscle can be found in Harris and Cullen (36).

### Control Muscle

Immunofluorescence studies of transverse sections of control soleus muscles labeled with monoclonal antibodies against α- and β-dystroglycan and adhalin showed that all three glycoproteins were localized at or near the plasma membrane of the muscle fibers in a manner not dissimilar to dystrophin (Fig. 1). Labeling with the β-dystroglycan antibody was strong, distinct and continuous over the entire membrane (Fig. 1A). Labeling with the adhalin antibody was occasionally slightly discontinuous (Fig. 1B). Labeling with the α-dystroglycan antibody was punctate, leading to distinct "beading" (Fig. 1C). The respective patterns of labeling seen on longitudinal sections were similar to those seen on the transverse sections (not shown). Figure 1D demonstrates anti-dystrophin labeling at the plasma membrane, and Figure 1E shows cryosections of control muscle labeled with anti-laminin antibodies to illustrate the presence of the basal lamina.

### Degenerating Muscle

Signs of skeletal muscle breakdown were observed as early as 1 h following venom assault. The muscle fibers displayed foci of hypercontraction and stretching, and there was an influx of phagocytic cells. Immunolabeling of cryosections of muscle removed 6 h after the inoculation of venom showed that β-dystroglycan was already lost from the plasma membrane of the most severely damaged myofibers, and by 12 h the majority of fiber profiles were β-dystroglycan-negative. A small number of fibers (approximately 30%) retained some weak and discontinuous labeling (Fig. 2A). By 1 d post-injection, none of the fiber profiles were labeled for β-dystroglycan (Fig. 3A). Adhalin and α-dystroglycan, on the other hand, were still present and labeled the sarcolemma strongly, despite the advanced stages of degeneration and regardless of whether the fibers were in a hypercontracted, stretched or collapsed state (Fig. 2B, C, 3B, C). Interestingly, the intrafusal fibers of the spindles were positively labeled for β-dystroglycan, but negative for α-dystroglycan (Fig. 3A, C, open arrows). Dystrophin was present in all fibers at 6 h and was absent from many fibers by 12 h (Fig. 2D). By 24 h, the protein was essentially un-

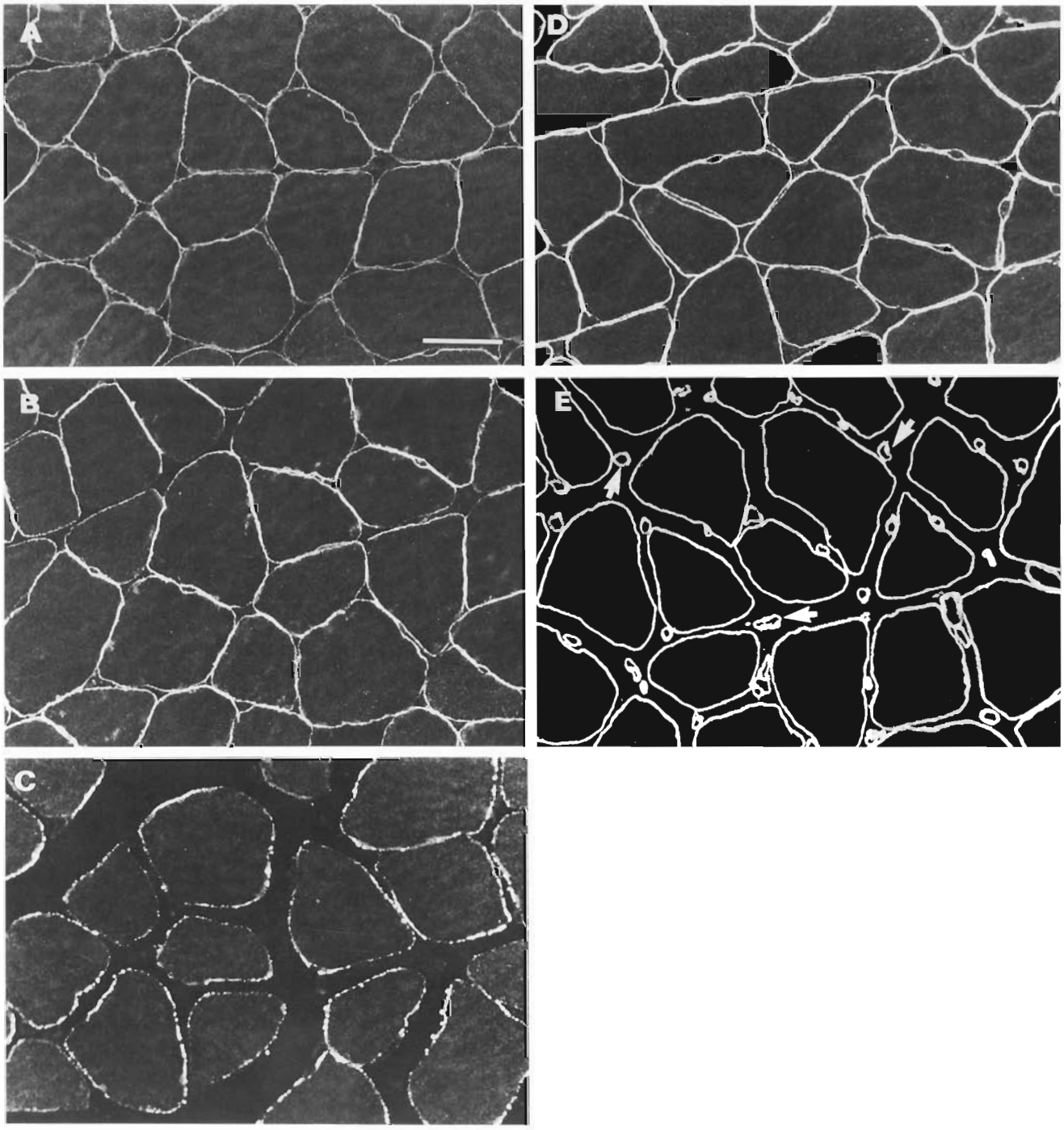


**Fig. 1.** Transverse cryosections from control rat soleus muscle labeled with anti-β-dystroglycan (A), anti-adhalin (B), anti-α-dystroglycan (C), anti-dystrophin (D) and anti-laminin (E) antibodies, and visualized by fluorescence microscopy. All the antibodies labeled at the periphery of the muscle fibers, β-dystroglycan, dystrophin and laminin continuously, adhalin slightly less so, and α-dystroglycan in a punctate fashion. Laminin was also present in the microvasculature (E, arrows). Bar = 25 μm.

detectable (Fig. 3D), except for the presence of labeling at the membrane of the intrafusal muscle fibers of the spindles. This suggested that the plasma membrane had been degraded. Laminin, however, was present at all stages of degeneration, confirming that the basal lamina remained intact despite the total destruction of the muscle fibers (Figs. 2E, 3E). Quantitative Western blotting (see below) showed quite clearly that 50% of β-dystroglycan was lost by 1 h, whereas the time for loss of 50% of dystrophin was approximately 4 h.

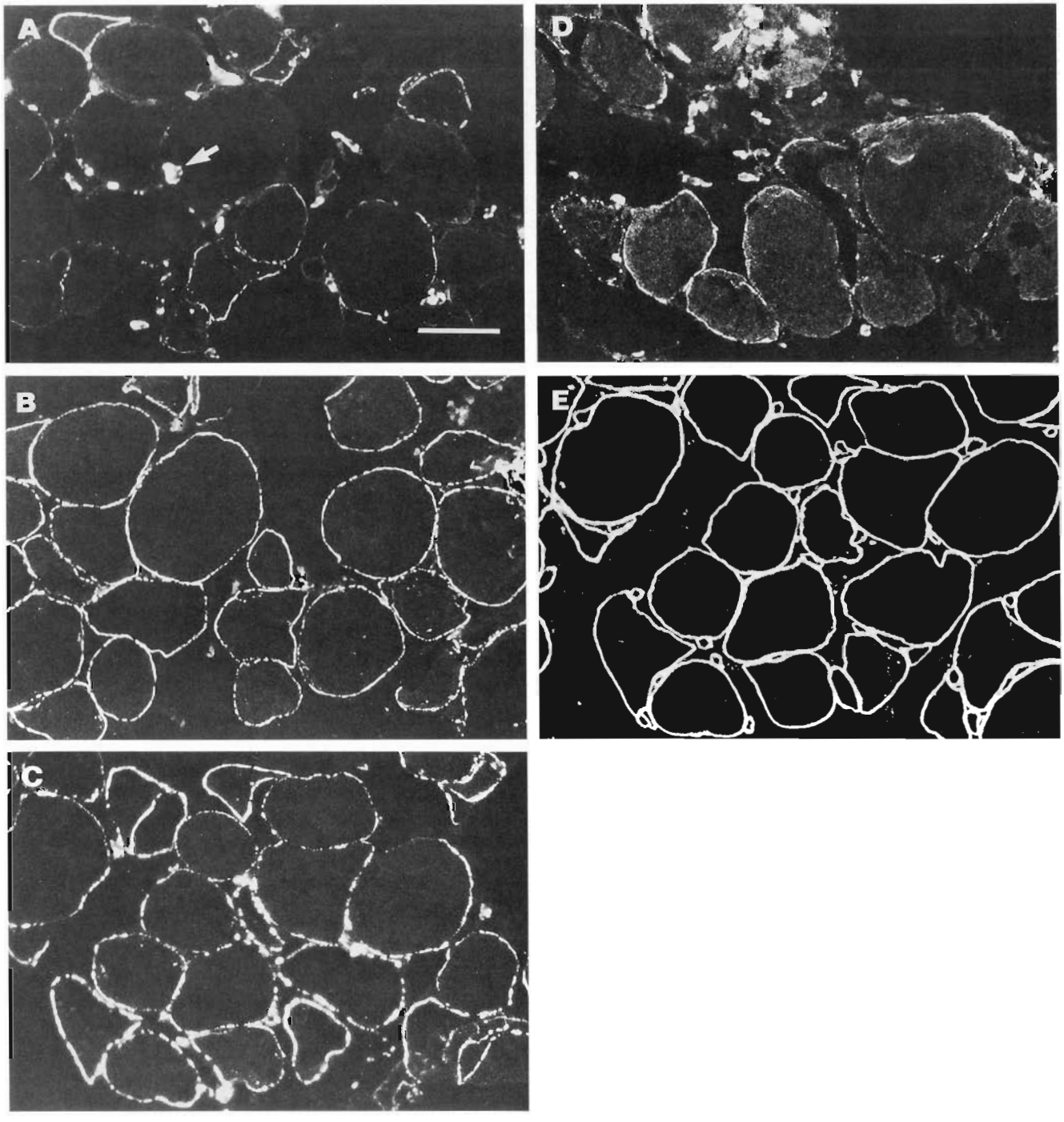


**Fig. 2.** Transverse cryosections of rat soleus muscle 12 h after the injection of venom, labeled with anti-β-dystroglycan (A), anti-adhalin (B), anti-α-dystroglycan (C), anti-dystrophin (D) and anti-laminin (E) antibodies, and visualized by fluorescence microscopy. β-Dystroglycan and dystrophin had been lost from the plasma membrane of most fibers, and the remaining positively labeled fibers demonstrated only weak labeling. Adhalin, α-dystroglycan and laminin labeling remained intact in all fibers. Phagocytic cells were visible in many of the degenerating fibers (arrows). Bar = 25 μm.

### Regenerating Muscle

At no stage during the degenerative phase or the early stages of regeneration was there a loss of laminin labeling, confirming the preservation of the basal lamina tubes of the muscle fibers. The first signs of regeneration were seen as early as 2 d following venom assault, with multinucleate myotubes being formed within the old basal lamina tube of degenerated muscle fibers. At 3 d following venom assault, the new myotubes began to lay down new basal lamina within the old, loosely folded basal

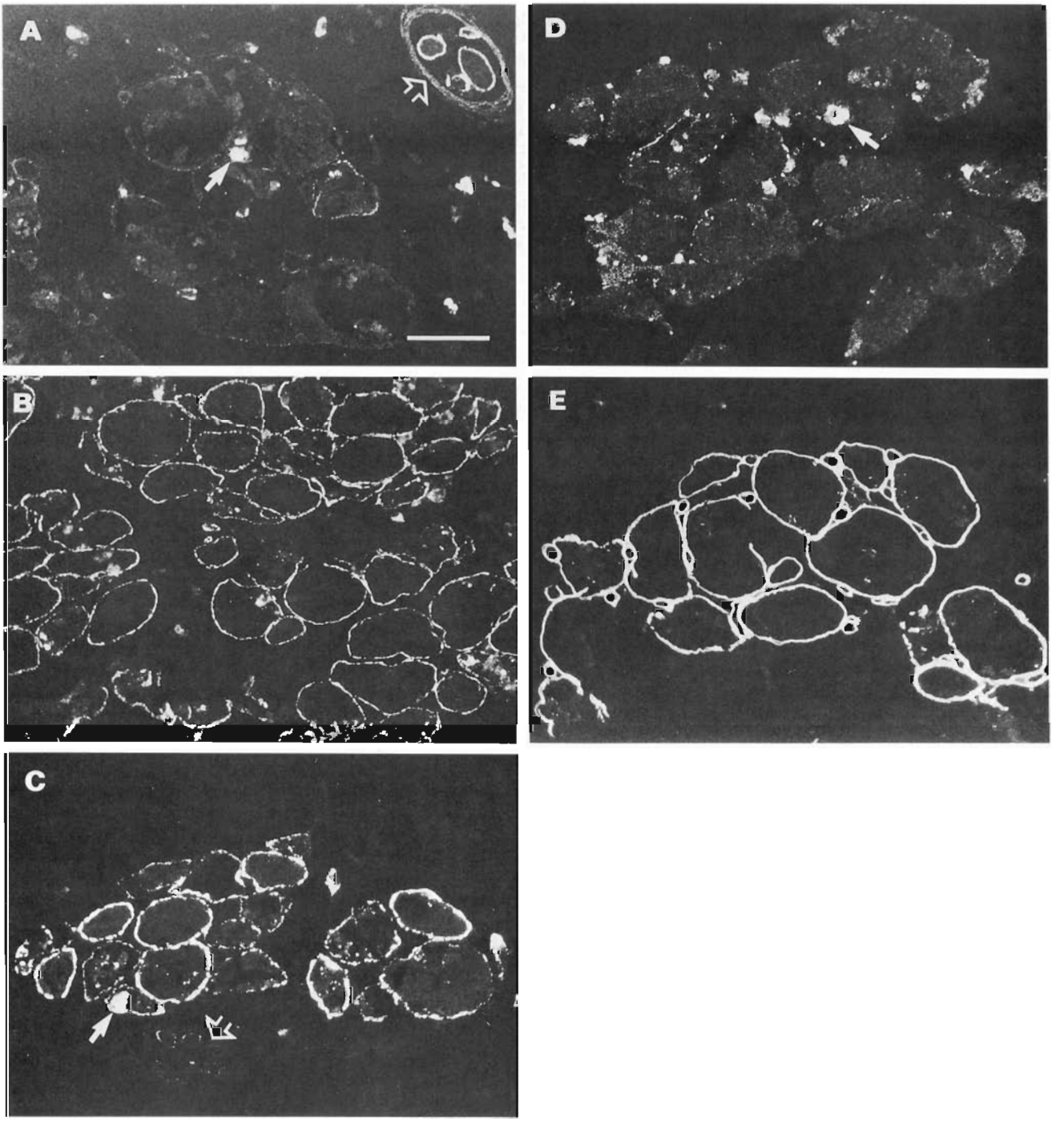


**Fig. 3.** Transverse cryosections of rat soleus muscle 24 h after the injection of venom, labeled with anti-β-dystroglycan (A), anti-adhalin (B), anti-α-dystroglycan (C), anti-dystrophin (D) and anti-laminin (E) antibodies, and visualized by fluorescence microscopy. Anti-β-dystroglycan labeling was almost completely absent in the degenerating muscle fibers, and only approximately 20% of fibers showed the presence of anti-dystrophin labeling. Adhalin, α-dystroglycan and laminin were all still present at the sarcolemma, as illustrated by strong labeling. It was interesting to note that the intrafusal fibers of spindles labeled strongly for β-dystroglycan (A, open arrow), but weakly, if at all, for α-dystroglycan (C, open arrow), which was opposite of the labeling pattern seen in the extrafusal fibers. Phagocytic cells were still visible around and within the degenerating fibers (solid arrows). Bar = 25 μm.

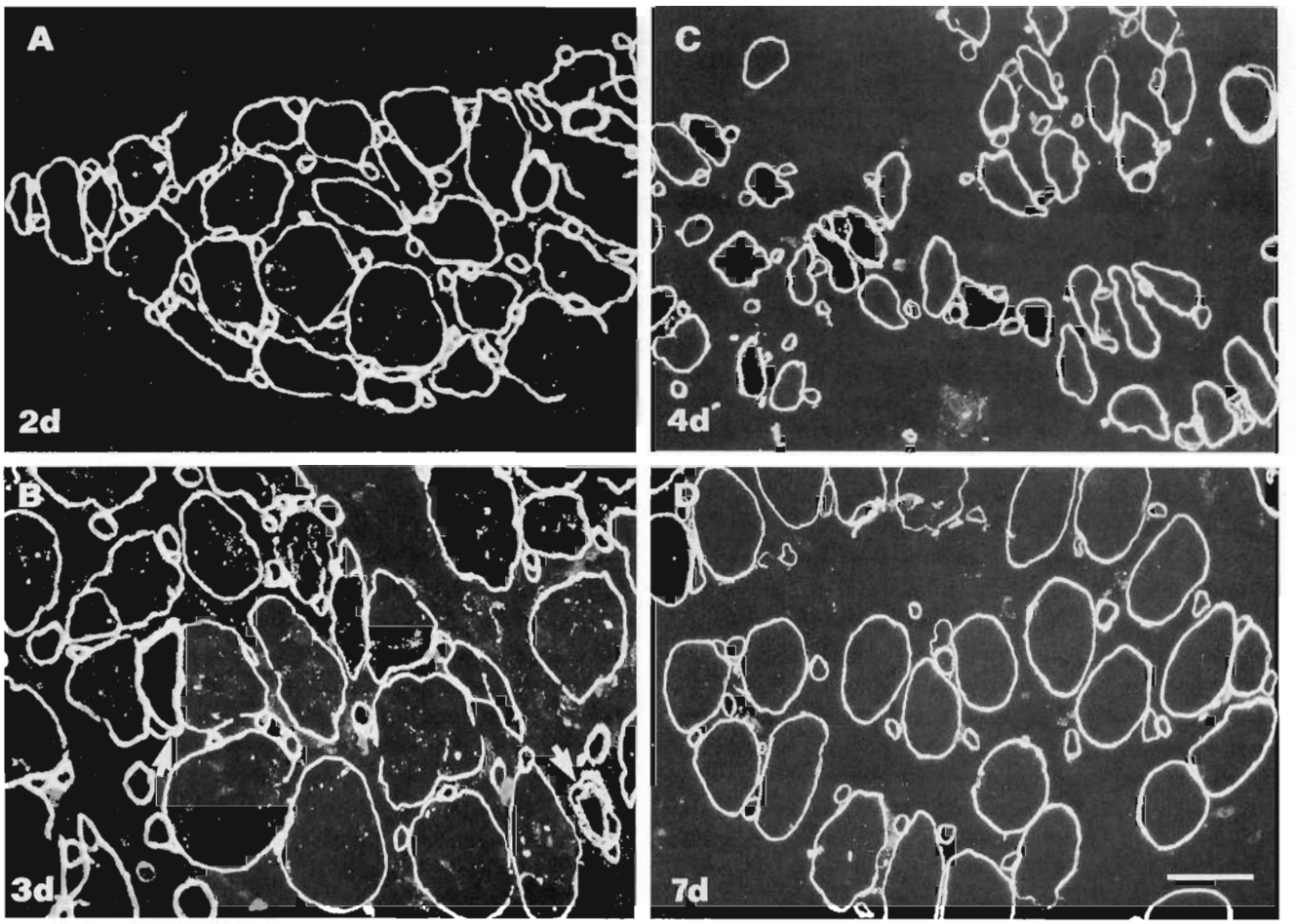


**Fig. 4.** Transverse cryosections of rat soleus muscle 2 d (A), 3 d (B), 4 d (C) and 7 d (D) after the injection of venom, labeled with anti-laminin antibodies and visualized by fluorescence microscopy. Laminin was present in the basal lamina throughout regeneration, as shown by strong, complete labeling at the fiber periphery at all stages. At 3 d, duplication of the basal lamina could be seen in a number of fibers (B, arrows), indicating the production of new basal lamina within the old basal lamina tube. By 4 d, each regenerating fiber had its own individual basal lamina. Bar = 25 μm.

lamina tube, leading to duplication of basal lamina profiles (Fig. 4B, arrow). By 4 d, the old basal lamina tube had been replaced by new basal lamina tightly surrounding each myofiber (Fig. 4C).

β-Dystroglycan was first localized to the plasma membrane of regenerating myotube profiles at 2 d (Fig. 5A). Labeling in these myotubes was rather weak and patchy, and appeared initially at those regions of plasma membrane most closely associated with the old basal lamina tube. This suggests that the basal lamina exerts some influence over the expression of this glycoprotein (see 37). By 3 d, around 75% of fiber profiles showed labeling, especially at the outside of bundles of myotubes (Fig. 5B, solid arrows). The diameter of the individual myotubes had increased within these bundles. By 4 d all the fibers manifested strong, distinct labeling of the whole plasma membrane (Fig. 5C). At 7 d the myofibers had increased in diameter, and the labeling was indistinguishable from normal (Fig. 5D).

Adhalin and α-dystroglycan were still present at the perimeter of all the myotube bundles at 2 d, even though labeling was weak. There was, however, no labeling of the individual myotubes comprising the bundles (Figs. 6A, 7A). Some of the myotubes still contained phagocytic cells (Figs. 6A, 7B, solid arrows). Three days after the administration of venom the size of the myotube bundles had increased, but the majority of the labeling was still confined to the region of the old basal lamina tube (Figs. 6B, 7B), although in some bundles labeling of individual myotubes was becoming evident (Figs. 6B, 7B). By 4 d, anti-adhalin and anti-α-dystroglycan antibodies strongly labeled individual regenerating myofibers (Figs. 6C, 7C), and by 7 d the labeling was indistinguishable from normal (Figs. 6D, 7D).

There was no localization of dystrophin to the plasma membrane of the regenerating myotubes before 4 d after the injection of venom (Fig. 8), and anti-dystrophin labeling of the plasma membrane was not uniformly pos-

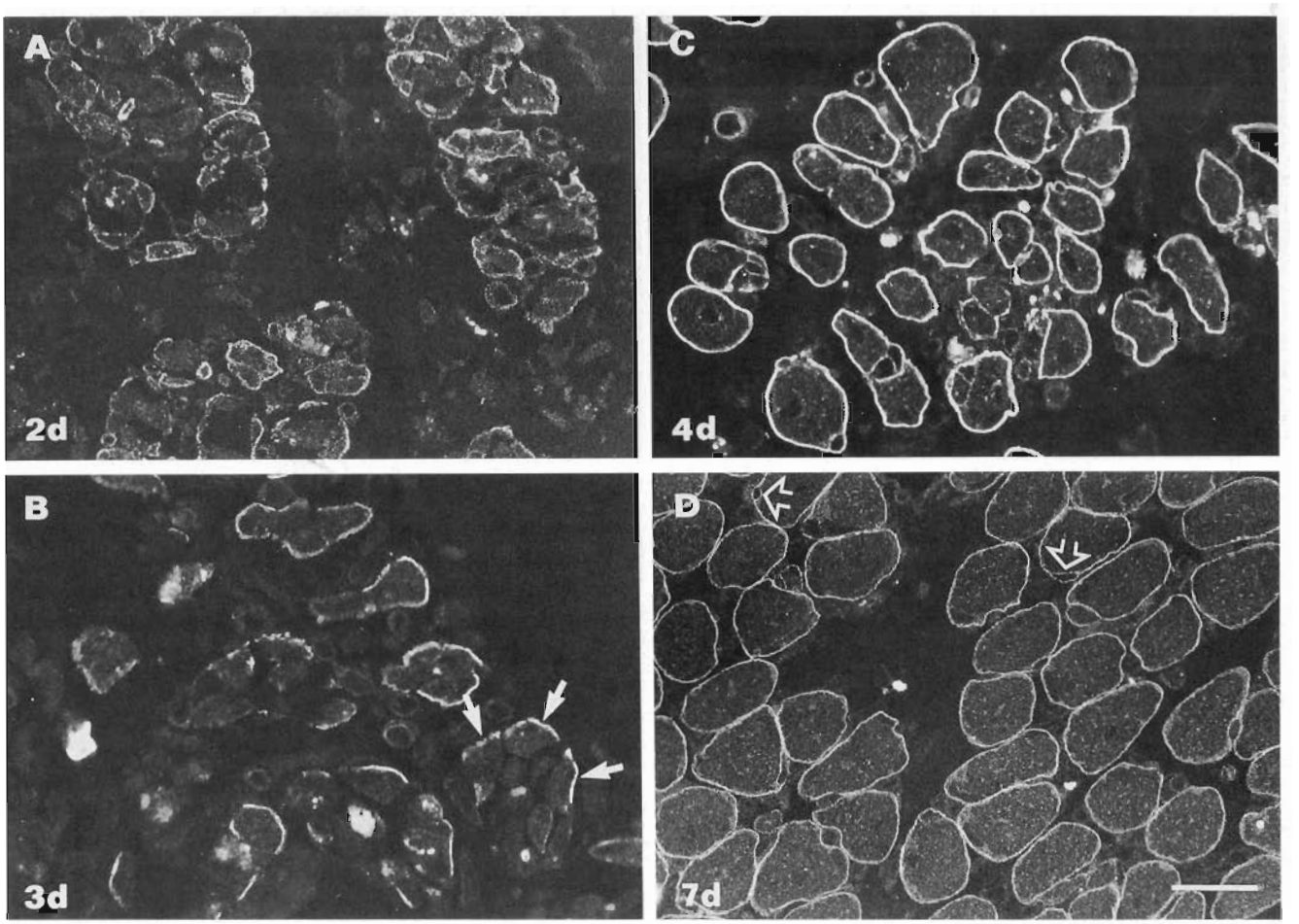


**Fig. 5.** Transverse cryosections of rat soleus muscle 2 d (A), 3 d (B), 4 d (C) and 7 d (D) after the injection of venom, labeled with anti-β-dystroglycan antibodies and visualized by fluorescence microscopy. Regenerating myotubes displayed β-dystroglycan labeling as early as 2 d. By 3 d it was clear that initial expression of this glycoprotein occurred at those areas of the plasma membrane which were adjacent to the old basal lamina tube (B, solid arrows). At 4 d, all regenerating fibers exhibited strong, complete labeling of the plasma membrane. At 7 d it was apparent that β-dystroglycan labeled both above and below the satellite cells (D, open arrows). Bar = 25 μm.

itive until 5–7 d. Analysis of the Western blots (see below) showed that 50% of β-dystroglycan had been re-expressed by approximately 3.5 d, whereas the half-time for re-expression of dystrophin was 4.5 d.

One difference between the labeling for dystrophin and adhalin, α- and β-dystroglycan in mature (i.e. 7 d postinjection) muscle fibers was the presence of anti-DAG labeling both beneath and above some satellite cells, whereas dystrophin only seemed to label beneath such cells (Figs. 5D, 6D, 7D, 8D, open arrows). It is difficult to understand the relationship between the various membrane proteins and the plasma membrane and/or basal lamina in such locations. There is no evidence to date to suggest that the antibodies label nonspecifically. With respect to adhalin, α- and β-dystroglycan, perhaps the plasma membrane of such satellite cells, as well as the plasma membrane of the myofiber, express these glycoproteins.

### Analysis of Western Blots

We were unable to detect bands of α-dystroglycan and adhalin on the Western blots using the antibodies available. Observation of the gels from which the blots were transferred showed that proteins comparable with the size of these two glycoproteins were indeed present prior to blotting. We presume, then, that the antibodies we used were incapable of recognizing the blotted proteins. It is relevant to note that the anti-adhalin antibody has, so far, been shown only to recognize non-reduced protein in isolated membrane preparations, and the antibody against α-dystroglycan similarly only recognizes the glycoprotein in isolated membrane preparations (SL Roberds, personal communication). We considered the possibility of isolating sarcolemma so that we could obtain definitive data from Western blots of adhalin and α-dystroglycan but calculated that we would need the soleus muscles of 400

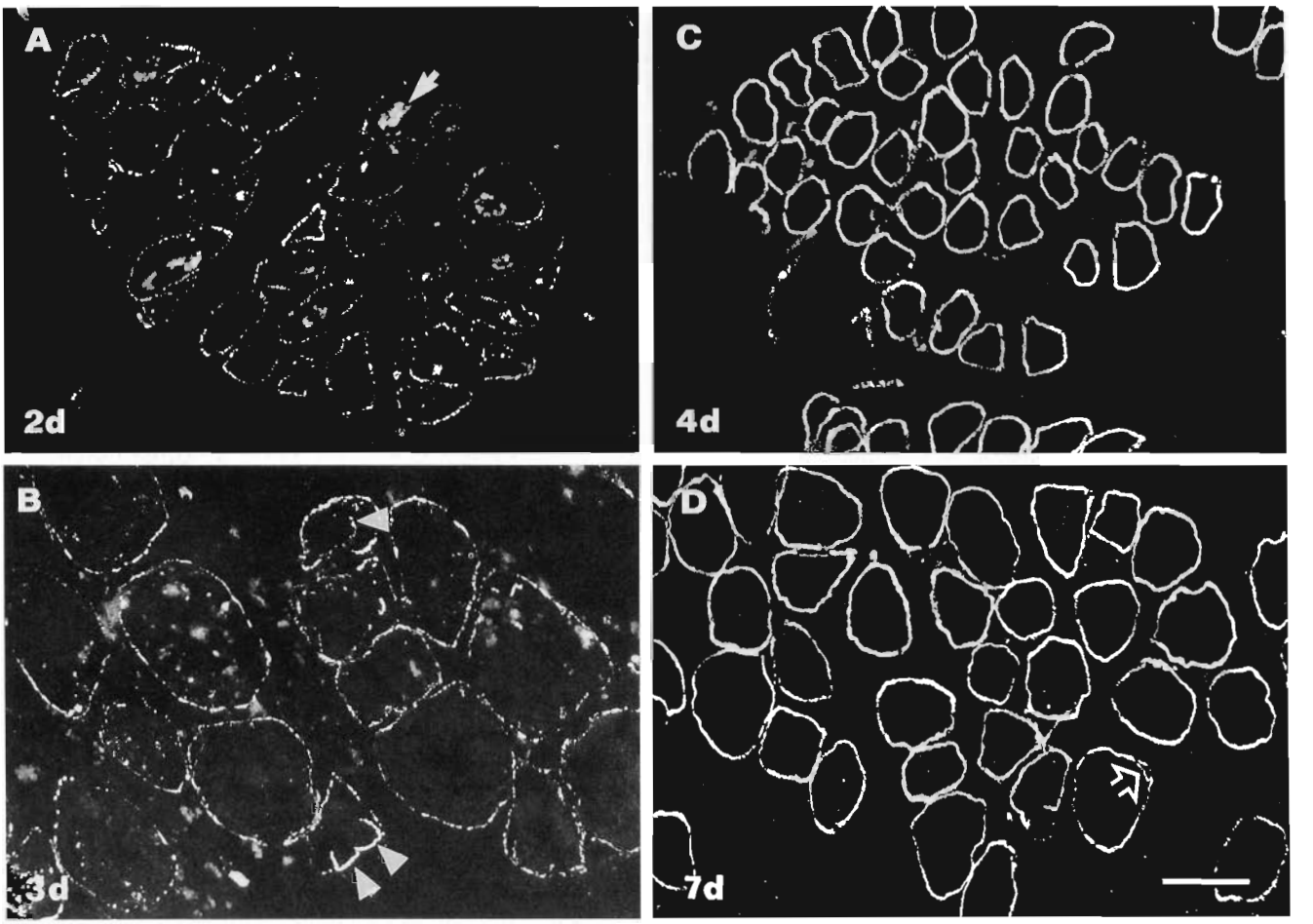


**Fig. 6.** Transverse cryosections of rat soleus muscle 2 d (A), 3 d (B), 4 d (C) and 7 d (D) after the injection of venom, labeled with anti-adhalin antibodies and visualized by fluorescence microscopy. Labeling was present in all fibers at all stages during the regenerative phase, although at 2 d the labeling was weak. Phagocytic cells were still visible at this stage (A, solid arrow). By 3 d, labeling of individual myotubes within the old basal lamina tube could be identified (B, arrowheads). At 4 d, each regenerating fiber showed complete sarcolemmal labeling. At 7 d it was apparent that adhalin labeled both above and below the satellite cells (D, open arrow). Bar = 25 μm.

rats per antibody. We did not feel that this was feasible, either ethically or logistically.

β-Dystroglycan began to break down as early as 1 h after the injection of venom, with degradation progressing steadily until no protein was detectable at 12–24 h (Fig. 9A). There was evidence of the full size protein reappearing at 2 d, with the density of the β-dystroglycan bands increasing as regeneration proceeded. Levels corresponding to control muscle were evident at 21 d. Dystrophin, in contrast, began to degrade slightly later and became undetectable at 1–2 d (Fig. 9B). Re-expression of dystrophin began at 3 d; there was then a rapid increase in abundance before control levels were reached at 7–21 d.

## DISCUSSION

This work investigated the fate of dystrophin and the DAG in rat skeletal muscle throughout a cycle of degeneration and regeneration. The entire process of muscle breakdown and reformation was initiated by the inoculation of a snake venom that, characteristically, leaves intact the basal lamina (together with its major constituent proteins, like acetylcholinesterase and laminin), satellite cells and microcirculation, but which destroys the plasma membrane (and its constituent proteins, such as the acetylcholine receptors) (36). The preservation of the basal lamina and the destruction of the plasma membrane were confirmed by the demonstration of continued labeling for laminin (a basal lamina protein) despite the rapid loss of dystrophin (a protein associated with the plasma membrane).

All of the generally accepted models describing the association between dystrophin, the glycoprotein complex and the plasma membrane (18, 38) indicate that the β-dystroglycan component of the dystrophin-glycoprotein complex is a primary candidate for the binding site

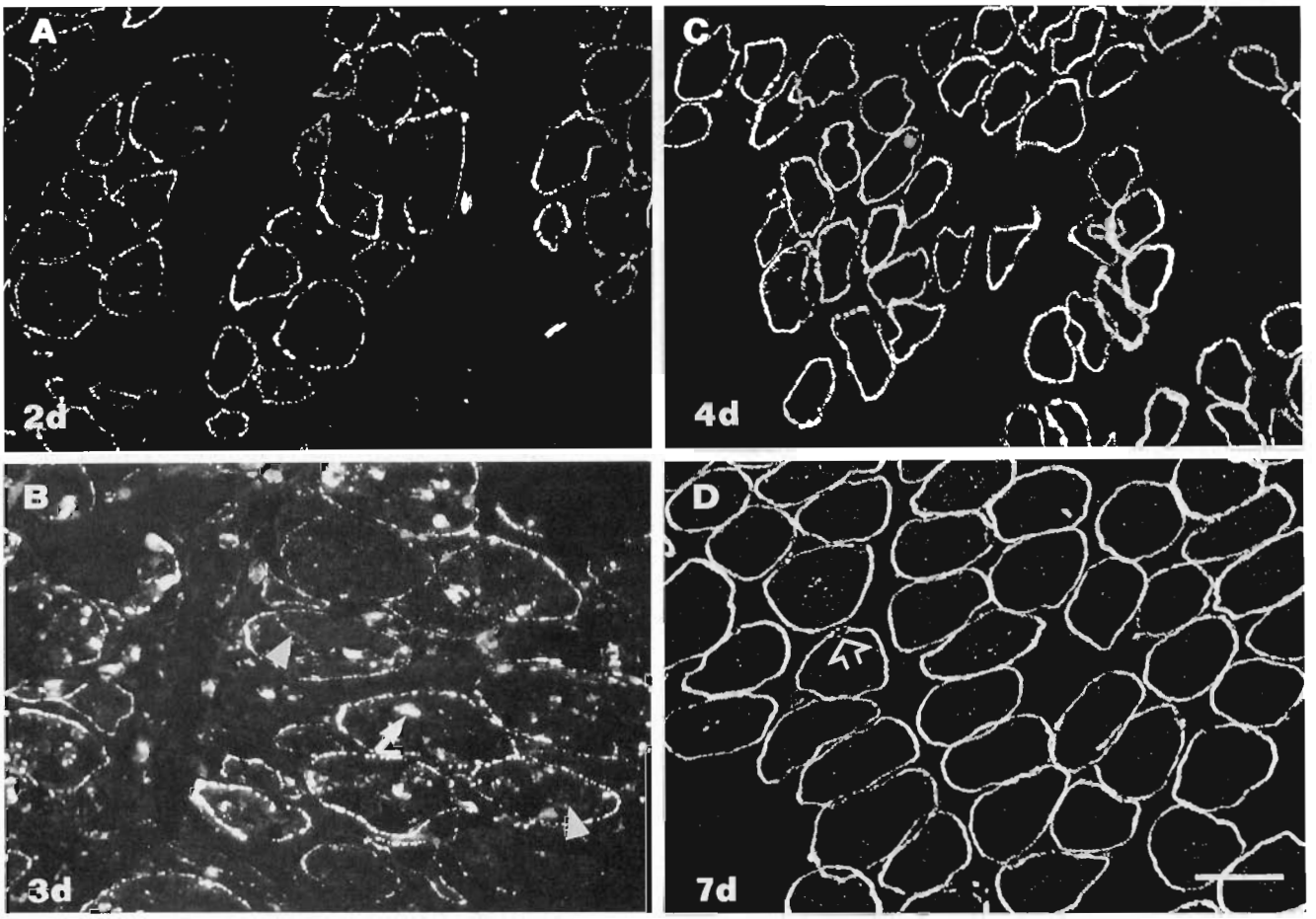


**Fig. 7.** Transverse cryosections of rat soleus muscle 2 d (A), 3 d (B), 4 d (C) and 7 d (D) after the injection of venom, labeled with anti-α-dystroglycan antibodies and visualized by fluorescence microscopy. Labeling was present in all fibers at all stages during the regenerative phase, although at 2 d the labeling was weak. By 3 d, labeling of individual myotubes within the old basal lamina tube could be identified (B, arrowheads). Phagocytic cells could still be seen at this stage (solid arrow). At 4 d, each regenerating fiber showed complete sarcolemmal labeling. At 7 d it was apparent that α-dystroglycan labeled both above and below the satellite cells (D, open arrow). Bar = 25 μm.

for dystrophin (19). The onset of muscle degeneration coincided with a rapid decline in the abundance of β-dystroglycan; only 1 h after the injection of venom, the muscle content of β-dystroglycan had been reduced by approximately 40%. This decrease occurred much faster than the reduction of dystrophin in the same tissue. The rate of loss of β-dystroglycan may be compared with the rate of loss of a number of other cytoskeletal and structural myofibrillar proteins under identical circumstances. The content of desmin and titin, for example, is reduced by 50% at 1 h and 3 h, respectively (39).

The fact that a single monoclonal antibody was used to determine the presence and abundance of β-dystroglycan could mean that the epitope to which the antibody binds is selectively and rapidly destroyed by proteases, in such a way as to leave the function of the protein essentially intact. We consider this possibility remote and, in the absence of additional monoclonal antibodies to alternative epitopes, we assume that the loss of β-dystroglycan binding reflects the loss of the protein.

New myotubes were recognizable 2 d after the administration of venom, but dystrophin was only detectable at the plasma membrane 4 d after venom assault. Its insertion into the membrane was not complete until 5–7 d after injection. β-Dystroglycan, to which dystrophin binds (19), was present at the plasma membrane at the very earliest stages of regeneration, i.e. 2 d after the injection of venom, when the plasma membrane was being reformed. The insertion of β-dystroglycan was complete by 4 d. Since dystrophin was not introduced into the membrane until 2 d after β-dystroglycan, it seems reasonable to infer that although the insertion of β-dystroglycan into the plasmalemma is independent of the expression of dystrophin, the attachment and distribution of dystrophin may be governed by the presence and distribution of β-dystroglycan. We cannot exclude the possibility, however,

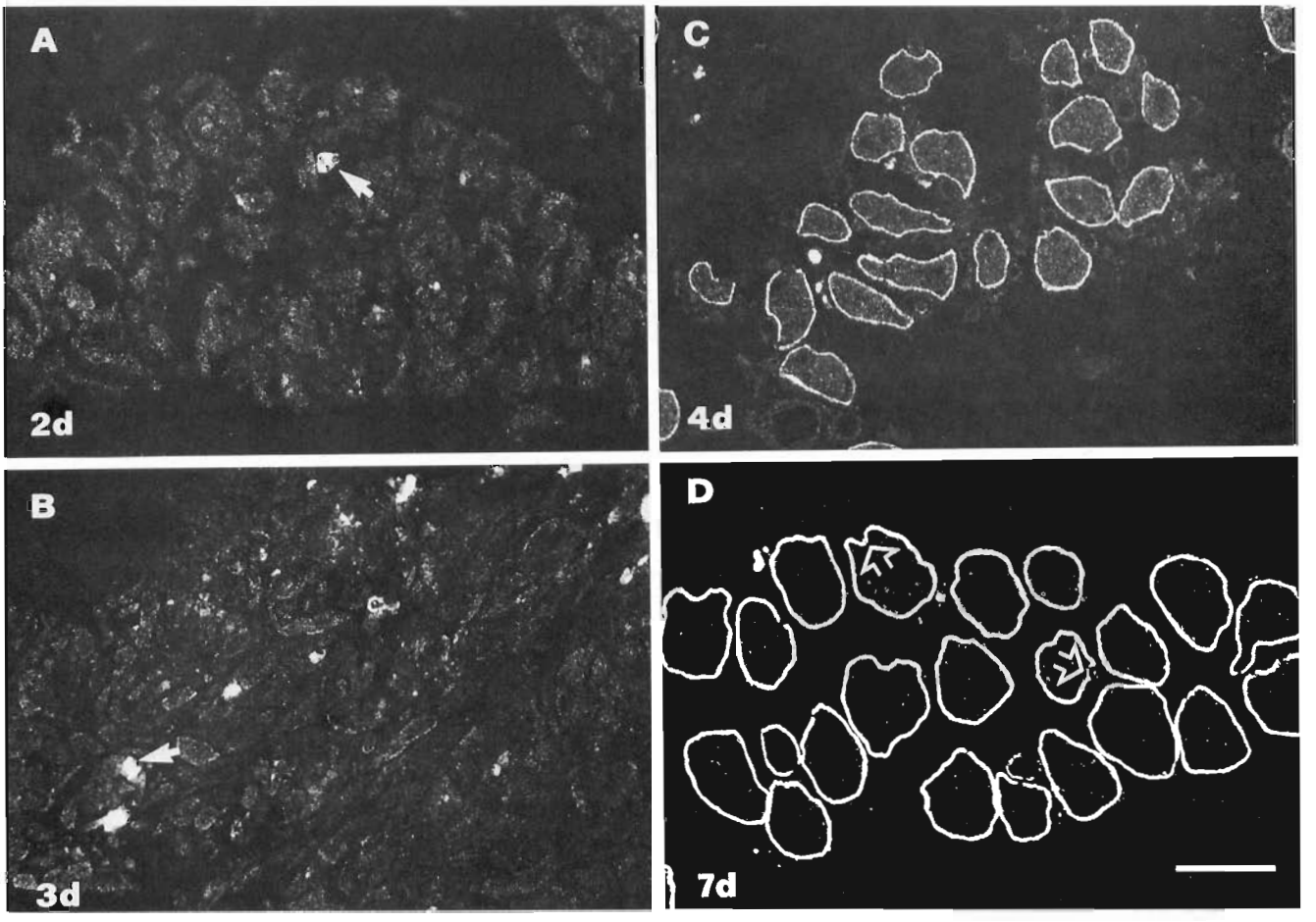


**Fig. 8.** Transverse cryosections of rat soleus muscle 2 d (A), 3 d (B), 4 d (C) and 7 d (D) after the injection of venom, labeled with anti-dystrophin antibodies and visualized by fluorescence microscopy. Regenerating myotubes could be identified from 2 d, but no dystrophin labeling was visible at 2 d or 3 d. Phagocytes were visible up to 3 d (A, B, solid arrows). Dystrophin labeling was first visible at the plasma membrane of the regenerating muscle fibers at 4 d (C). Unlike the DAG, dystrophin only labeled beneath the satellite cells at 7 d (D, open arrows). Bar = 25 μm.

that dystrophin may bind primarily to other component(s) of the DAG complex, such as syntrophin, the 59 kDa intracellular protein, and hence be linked via syntrophin to the rest of the sarcolemmal scaffolding.

Adhalin and α-dystroglycan remained localized at the sarcolemma throughout the entire cycle of degeneration and regeneration. This is consistent with the concept that the epitope-containing portion of the α-dystroglycan molecule remains attached to the basal lamina during muscle fiber breakdown and that adhalin remains attached to α-dystroglycan during this process. One interesting observation was the beaded appearance of α-dystroglycan compared to the smooth, continuous labeling seen with dystrophin and β-dystroglycan. An explanation for this phenomenon may be that since α-dystroglycan binds to laminin in the basal lamina, the labeling could possibly lie out of the plane of the section at intervals. Alternatively, contrary to the concept that there is a 1:1 ratio of α-dystroglycan to dystrophin, it is possible that there are fewer α-dystroglycan molecules associated with dystrophin molecules at the sarcolemmal interface than the original model suggested (18).

The differences in labeling patterns seen between α- and β-dystroglycan in the regenerating muscle fibers raises some interesting points. These two glycoproteins are encoded by a single 5.8 kb mRNA, which produces a 97 kDa precursor protein that is then post-translationally modified to result in two mature proteins of 41 kDa and 56 kDa (20). The 41 kDa protein is glycosylated to form a 43 kDa protein, β-dystroglycan; the 56 kDa protein is extensively glycosylated to form a 156 kDa protein, α-dystroglycan. Because α- and β-dystroglycan originate from the same gene, it might be expected that they are incorporated into the sarcolemma concomitantly. It is possible that this was the case, but since α-dystroglycan was continually present during muscle breakdown and subsequent reformation, we are unable to say definitively that this did occur. Another, possibly more likely, expla-

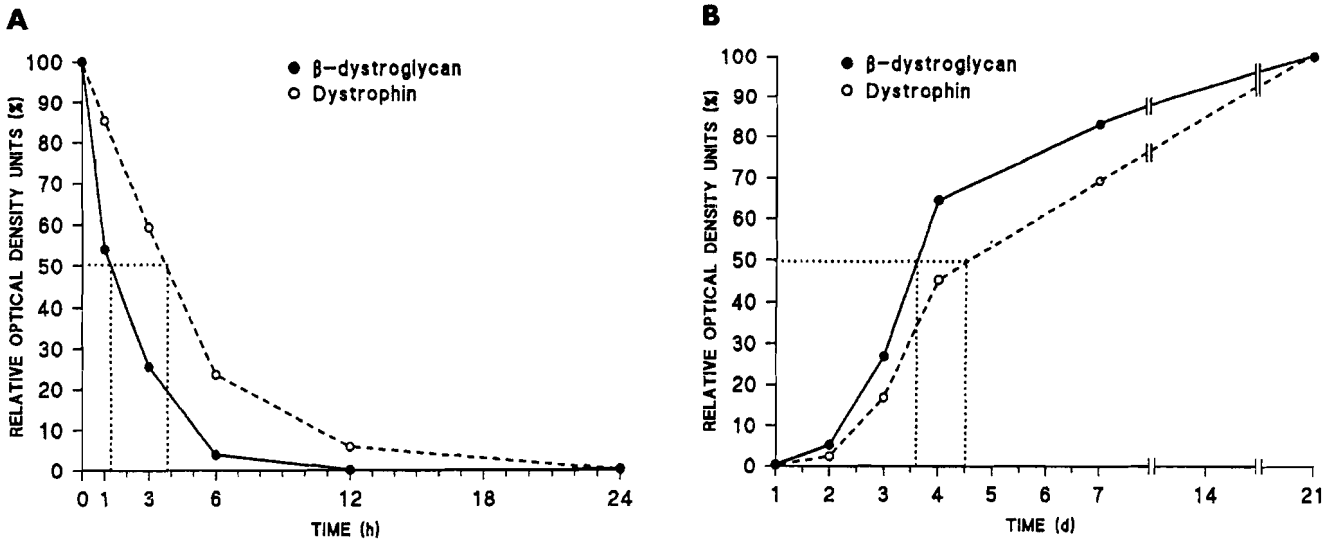


**Fig. 9.** Graphs illustrating data from densitometric assays made on Western blots probed with monoclonal anti-β-dystroglycan and anti-dystrophin antibodies showing the amounts of these two proteins present in the venom-injected muscle samples at each stage throughout the cycle of degeneration (A) and regeneration (B). β-Dystroglycan showed a steep decline from the time of venom injection, such that 50% had been lost by 1 h, and was absent by 12 h. Re-expression of β-dystroglycan was apparent by 2 d and accumulated rapidly, such that 50% was reached by 3.5 d and 100% by 21 d. Dystrophin loss and accumulation followed a similar pattern to that seen with β-dystroglycan, but the time scale was different. Four h had passed before 50% loss was recorded, re-expression was not visible until 3 d, and 50% was not reached until 4.5 d. By 21 d, levels of dystrophin were normal. Solid circles = β-dystroglycan; open circles = dystrophin.

nation for the differences seen in the labeling patterns is that the rate of processing and/or turnover of these two glycoproteins may differ. Methods including labeling of "old" and "new" α-dystroglycan are required to assess accurately the actual time of expression of this glycoprotein during muscle regeneration.

In addition to the relationship between the glycoproteins of the complex, the interdependence of the dystrophin and glycoprotein components of the complex was of interest. It was noted that β-dystroglycan was inserted into the sarcolemma up to 48 h before the insertion of dystrophin. There are several possible explanations for this difference, and the most likely are examined in turn: (i) During normal development, dystrophin and the components of the glycoprotein complex may follow different routes to the plasma membrane. As a cytoskeletal protein, dystrophin will probably be synthesized on cytoplasmic ribosomes, whereas the glycoproteins will be synthesized on ribosomes of the rough endoplasmic reticulum (RER), before being transported through the Golgi apparatus of the regenerating muscle cells for sorting and glycosylation. The implication of this would be that dystrophin and β-dystroglycan would only associate on reaching the sarcolemma. (ii) If transcription of the β-dystroglycan and dystrophin genes was initiated at approximately the same time, a delay of 24–48 h would not be unexpected, since it takes at least 16 h to transcribe and translate the extremely long dystrophin gene, but far less for the β-dystroglycan gene (40). Despite our lack of detailed knowledge on these points, the significant implication is that the DAG complex needs to be fully formed and established before the insertion of dystrophin into the plasmalemma, suggesting that the DAG act as a kind of "template" for the linkage with dystrophin, their positions possibly predetermined by signals originating in the basal lamina.

It was of considerable interest that the area of initial expression of the glycoproteins in regenerating myotubes was directly adjacent to the old basal lamina tube. The basal lamina, and in particular laminin, may, therefore, influence the expression and localization of these glycoproteins and dystrophin. It is also possible that α-dystroglycan, at least, may be involved in the attachment, spreading and growth of myoblasts along a substrate of laminin in the basal lamina (20). It is likely that the basal lamina has an important modulatory, if not regulatory, role in the expression of the glycoproteins of the dystrophin-glycoprotein complex.

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## REFERENCES

1. Emery AEH. Duchenne muscular dystrophy. Second Edition. Oxford: Oxford University Press, 1993
2. Hoffman EP, Brown RH, Kunkel LM. Dystrophin: The protein product of the Duchenne muscular dystrophy locus. Cell 1987;51: 919–28

3. Koenig M, Hoffman EP, Bertelson CJ, Monaco AP, Feener C, Kunkel LM. Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organisation of the DMD gene in normal and affected individuals. Cell 1987;50:509–17
4. Arahata K, Ishiura S, Ishiguro T, et al. Immunostaining of skeletal and cardiac muscle surface membrane with antibody against Duchenne muscular dystrophy peptide. Nature 1988;333:861–3
5. Bonilla E, Samitt CE, Miranda AF, et al. Duchenne muscular dystrophy: Deficiency of dystrophin at the muscle cell surface. Cell 1988;54:447–52
6. Sugita H, Arahata K, Ishiguro T, et al. Negative immunostaining of Duchenne muscular dystrophy (DMD) and *mdx* muscle surface membrane with antibody against synthetic peptide fragment predicted from DMD cDNA. Proc Jpn Acad 1988;64:37–9
7. Zubrzycka-Gaarn EE, Bulman DE, Karpati G, et al. The Duchenne muscular dystrophy gene product is localized in sarcolemma of human skeletal muscle. Nature 1988;333:466–9
8. Vater R, Cullen MJ, Nicholson LVB, Harris JB. The fate of dystrophin during the degeneration and regeneration of the soleus muscle of the rat. Acta Neuropathol 1992a;83:140–8
9. Pons F, Augier N, Léger J, Mornet D, Léger JJ. Isolated dystrophin molecules as seen by electron microscopy. Proc Natl Acad Sci USA 1990;87:7851–5
10. Sato O, Nonomura Y, Kimura S, Maruyama K. Molecular shape of dystrophin. J Biochem 1992;112:631–6
11. Koenig M, Monaco AP, Kunkel LM. The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. Cell 1988;53: 219–28
12. Cullen MJ, Walsh J, Nicholson LVB, Harris JB. Ultrastructural localization of dystrophin in human muscle by using gold immunolabelling. Proc R Soc Lond B 1990;240:197–210
13. Bartlett RJ, Sharp NJH, Kornegay JN, Roses AD. Normal and abnormal dystrophin gene expression. In: Kakulas BA, Howell JM, Roses AD, eds. Duchenne muscular dystrophy: Animal models and genetic manipulation. New York: Raven Press Ltd, 1992:41–65
14. Klamut HJ, Bosnoyan LO, Worton RG, Ray PN. Expression of dystrophin minigene in myogenic cell cultures. In: Kakulas BA, Howell JM, Roses AD, eds. Duchenne muscular dystrophy: Animal models and genetic manipulation. New York: Raven Press Ltd, 1992:255–62
15. Campbell KP, Kahl SD. Association of dystrophin and an integral membrane glycoprotein. Nature 1989;338:259–62
16. Ervasti JM, Ohlendieck K, Kahl SD, Gaver MG, Campbell KP. Deficiency of a glycoprotein component of the dystrophin complex in dystrophic muscle. Nature 1990;345:315–19
17. Yoshida M, Ozawa E. Glycoprotein complex anchoring dystrophin to sarcolemma. J Biochem 1990;108:748–52
18. Ervasti JM, Campbell KP. Membrane organization of the dystrophin-glycoprotein complex. Cell 1991;66:1121–31
19. Suzuki A, Yoshida M, Hayashi K, Mizuno Y, Hagiwara Y, Ozawa E. Molecular organization at the glycoprotein-complex-binding site of dystrophin. Three dystrophin-associated proteins bind directly to the carboxy-terminal portion of dystrophin. Eur J Biochem 1994;220:283–92
20. Ibraghimov-Beskrovnaya O, Ervasti JM, Leveille CJ, Slaughter CA, Sernet SW, Campbell KP. Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. Nature 1992;355:696–702
21. Ohlendieck K, Campbell KP. Dystrophin-associated proteins are greatly reduced in skeletal muscle from *mdx* mice. J Cell Biol 1991;115:1685–94
22. Ohlendieck K, Matsumura K, Ionasescu VV, et al. Duchenne muscular dystrophy: Deficiency of dystrophin-associated proteins in the sarcolemma. Neurology 1993;43:795–800
23. Cullen MJ, Walsh J, Nicholson LVB. Immunogold localization of the 43-kDa dystroglycan at the plasma membrane in control and dystrophic human muscle. Acta Neuropathol 1994;87:349–54
24. Matsumura K, Tomé FMS, Collin H, et al. Deficiency of the 50K dystrophin-associated glycoprotein in severe childhood autosomal recessive muscular dystrophy. Nature 1992;359:320–2
25. Roberds SL, Leturcq F, Allamand V, et al. Missense mutations in the adhalin gene linked to autosomal recessive muscular dystrophy. Cell 1994;78:625–33
26. Roberds SL, Ervasti JM, Anderson RD, et al. Disruption of the dystrophin-glycoprotein complex in the cardiomyopathic hamster. J Biol Chem 1993;268:11496–9
27. Matsumura K, Tomé FMS, Ionasescu V, et al. Deficiency of dystrophin-associated proteins in Duchenne muscular dystrophy patients lacking COOH-terminal domains of dystrophin. J Clin Invest 1993;92:866–71
28. Hoffman EP, Garcia CA, Chamberlain JS, Angelini C, Lupski JR, Fenwick R. Is the carboxyl-terminus of dystrophin required for membrane association? A novel, severe case of Duchenne muscular dystrophy. Ann Neurol 1991;30:605–10
29. Clemens PR, Ward PA, Caskey CT, Bulman DE, Fenwick RG. Premature chain termination mutation causing Duchenne muscular dystrophy. Neurology 1992;42:1775–82
30. Helliwell TR, Ellis JM, Mountford RC, Appleton RE, Morris GE. A truncated dystrophin lacking the C-terminal domains is localized at the muscle membrane. Am J Hum Genet 1992;50:508–14
31. Récan D, Chafey P, Leturcq F, et al. Are cysteine-rich and COOH-terminal domains of dystrophin critical for sarcolemmal localization? J Clin Invest 1992;89:712–16
32. Harris JB, Johnson MA. Further observations on the pathological responses of rat skeletal muscle to toxins isolated from the venom of the Australian tiger snake, *Notechis scutatus scutatus*. Clin Exp Pharmacol Physiol 1978;5:587–600
33. Nicholson LVB, Davison K, Falkous G, et al. Dystrophin in skeletal muscle: I. Western blot analysis using a monoclonal antibody. J Neurol Sci 1989;94:125–36
34. Nicholson LVB, Johnson MA, Davison K, et al. Dystrophin or a "related protein" in Duchenne muscular dystrophy? Acta Neurol Scand 1992;86:8–14
35. Jorgensen AO, Arnold W, Shen ACY, Yuan S, Gaver M, Campbell KP. Identification of novel proteins unique to either transverse tubules (TS28) or to the sarcolemma (SL50) in rabbit skeletal muscle. J Cell Biol 1990;110:1173–85
36. Harris JB, Cullen MJ. Muscle necrosis caused by snake venoms and toxins. Electron Microsc Rev 1990;3:183–211
37. Dickson G, Azad A, Morris GE, Simon H, Noursadeghi M, Walsh FS. Co-localization and molecular association of dystrophin with laminin at the surface of mouse and human myotubes. J Cell Sci 1992;103:1223–33
38. Matsumura K, Campbell KP. Deficiency of dystrophin-associated proteins: A common mechanism leading to muscle cell necrosis in severe childhood muscular dystrophies. Neuromusc Disord 1993;3: 109–18
39. Vater R, Cullen MJ, Harris JB. The fate of desmin and titin during the degeneration and regeneration of the soleus muscle of the rat. Acta Neuropathol 1992b;84:278–88
40. Tennyson CN, Klamut HJ, Worton RG. The human dystrophin gene requires 16 hours to be transcribed and is cotranscriptionally spliced. Nature Genet 1995;9:184–90

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