Int. J. Dev. Biol. 44: 421-428 (2000)

Original Article

m-calpain levels increase during fusion of myoblasts in the mutant muscular dysgenesis (mdg) mouse

SANDRA JOFFROY*[1,2], NATHALIE DOURDIN[3], JEAN-PAUL DELAGE[4], PATRICK COTTIN[3], JEANINE KOENIG[1,5], and JEAN-JACQUES BRUSTIS[3]

[1] Laboratoire de Neurobiologie Cellulaire. Université de Bordeaux II, Talence, France, [2] Present address: INSERM E 99-29: Physiologie mitochondriale - Université Bordeaux 2, Bordeaux, France, [3] Laboratoire de Biochimie et Technologie des Aliments, ISTAB-UA-INRA 429, Université Bordeaux I, Talence, France, [4] UFR STAPS Université Victor Segalen Bordeaux II, Talence, France and [5] Present address: Institut de Myologie, Hôpital Pitié, Salpétrière, Paris, France.

ABSTRACT Previous studies have led to the hypothesis of a possible role for the calcium-dependent neutral protease m-calpain in myoblast fusion in culture. To evaluate this hypothesis, we chose as our model, the "muscular dysgenesis" mouse (mdg), which presents *in vivo* and *in vitro* characteristics of an elevated process of fusion (Yao and Essien, 1975; Dussartre, 1993; Ashby *et al.*, 1993, Joffroy *et al.*, 1999). The aim of this study was to demonstrate using myoblast cell lines and muscle biopsies from this *mdg* mutant, that the amount of m-calpain increases significantly as multinucleated myotubes are formed. Using immunoblot analysis, it was shown that the m-calpain concentration in a dysgenic cell line (GLT) increased 3-fold compared to what it was upon the introduction of the differentiation medium. On the other hand, in a normal cell line (NLT), the concentration of m-calpain did not vary significantly. Thus, when the transition from myoblasts to myotubes was slow, and the absolute level of fusion was reduced, as in the NLT cell line, the level of m-calpain was stable. In contrast, when the process of fusion was precocious and fast, and the level of fusion was elevated, such as in the GLT cell line, the concentration of m-calpain increased during fusion. Moreover, when myoblast fusion was prevented by the addition of calpain inhibitor II, the process was reduced by approximately 93%. Taking into account these observations, it is clear from our data that the muscular dysgenesis mouse provides a relevant model to study myoblast fusion and that m-calpain is involved in this process.

KEY WORDS: *muscular dysgenesis, cell culture, m-calpain, calpain inhibitor, fusion.*

Introduction

Skeletal muscle development involves the formation of multinucleated myotubes. This phenomenon is thought to proceed by the induction of differentiation (acquisition of fusion competence) in myoblast cells, their aggregation, and the union of their plasma membranes. Various membrane proteins including N- and M-cadherins, N- and V-CAMs, and integrins participate in myotube formation, but the molecular mechanisms of muscle-cell fusion are poorly understood.

It has been demonstrated recently that calpains are implicated in the process of myoblast fusion. Calpains (calcium-activated neutral proteases, EC 3.4.22.17) are cytosolic cysteine proteases, regulated by Ca^{2+} and consisting of two subunits of 80 and 30 kDa, respectively. The two major isoforms, μ- and m-calpains, differ particularly in their calcium requirement for activation and have been found in many cell types (for a review, see Croall and Demartino, 1991). m-calpain activity significantly increases at the time of myoblast fusion, whereas μ-calpain expression is delayed and reaches a maximum during the later stages of myogenesis (Cottin *et al.*, 1994). At present, according to a number of studies (Schollmeyer, 1986a; Kumar *et al.*, 1992; Brustis *et al.*, 1994; Kwak *et al.*, 1993; Cottin *et al.*, 1994; Balcerzak *et al.*, 1995; Dourdin *et al.*, 1997; Dourdin *et al.*, 1999), it seems

Abbreviations used in this paper: BSA, Bovine serum albumin; DTT, Dithiothreitol; DMEM, Dulbecco's Modified Eagle Medium; EDTA, Ethylenediaminetetraacetic acid; EGTA, Ethylene glycol-bis (β-aminoethylether)N, N, N', N'-tetraacetic acid; GLT, DysGenic Line transfected with the Large T antigen; NLT, Normal Line transfected with the Large T antigen; PBS, Phosphate-buffered saline; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

***Address correspondence to:** Sandra Joffroy. INSERM E 99-29: Physiologie mitochondriale, Université Bordeaux II, 146 rue Léo-Saignat, F-33076 Bordeaux-Cedex, France. Tel.: 33 05 57 57 13 79. Fax: 33 05 57 57 16 12. e-mail: sjoffroy@phys-mito.u-bordeaux2.fr

0214-6282/2000/$20.00

Printed in Spain
www.ehu.es/ijdb

reasonable to suppose that m-calpain is involved in the formation of multinucleated myotubes.

In order to support the hypothesis that m-calpain is implicated in myoblast fusion, we chose as our model the "muscular dysgenesis" mouse (*mdg/mdg*), which presents *in vivo* a longer muscle than is normal (Dussartre, 1993; Joffroy *et al.*, 1999) and *in vitro* a greater number of myotubes having a larger number of nuclei compared to controls (Yao and Essien, 1975; Ashby *et al.*, 1993). Thus, this mutation is characteristically associated with an abnormally elevated process of fusion. The dysfunction in the dysgenic mouse is an absence of the excitation-contraction coupling (E-C) (Powell, 1973), which is a consequence of the mutation and affects the gene coding for the α_1 subunit of the calcic channel of the T- Tubule, that is, one of the molecular constituents of the triad (Chaudhari, 1992). In parallel with the defective E-C coupling, the ultrastructural organization of dysgenic muscle cells is disrupted *in vivo* (Platzer and Gluecksohn-Waelsch, 1972) and *in vitro* (Courbin *et al.*, 1989). The few myofilaments present are poorly organized in sarcomeres, and the triads do not contain foot- protein (Ferguson *et al.*, 1984) between the T- Tubule and sarcoplasmic reticulum membranes (Pinçon-Raymond *et al.*, 1985; Tanabe *et al.*, 1988).

In the present investigation, we quantified the expression of m-calpain in a normal cell line, NLT [Normal (+/+) Line transfected with the Large T antigen], and in a dysgenic cell line, GLT [dysGenic (*mdg/mdg*) Line transfected with Large T antigen], (Powell *et al.*, 1996), and we demonstrated that the addition of m-calpain inhibitor to the culture medium of GLT lines blocks myoblast fusion. We also demonstrated that this protease is overexpressed *in vitro* in the GLT cell line and *in vivo* in the dysgenic muscle. We conclude that the dysgenic mouse provides a relevant model for the study of myoblast fusion, and our results also confirm the involvement of m-calpain in the process of fusion.

Results

Kinetics of myoblast fusion in GLT and NLT cell lines

As shown in Figure 1, which represents the NLT and GLT fusion curves, there was a significant difference between the kinetics of fusion in the two cell lines. Indeed, the curve showing the NLT kinetics was less steep than that showing the GLT, and, especially, the first appearance of fusion was delayed (to 5 days after the introduction of the fusion medium, corresponding to 120 h). Furthermore, fusion proceeded more slowly in the NLT cell line than in the GLT cell line. The GLT curve reflects a rapid rate of fusion: after 24 h, we observed 12% fusion, whereas in the NLT cell line, the fusion process had not yet begun. After 72 h, fusion had reached 45%, after 120 h 75%, and after 168 h 77% in the GLT cell line. In the NLT cell line cultured for 264 h in the fusion medium, fusion reached only 40%, whereas just 72 h were sufficient for the GLT cell line to reach 45%.

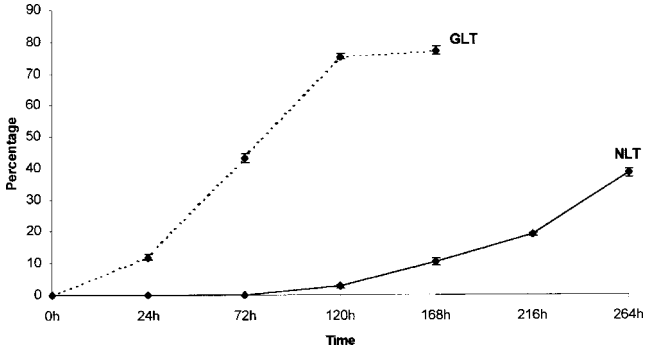

Fig. 1. Kinetics of myoblast fusion in control (NLT) and dysgenic (GLT) cultures. *Fusion was measured at various times and expressed as percentage of fusion. Percentage of fusion = (nuclei in myotubes/nuclei in myotubes + nuclei in single-cell myoblasts) x 100. Vertical bars denote standard errors of the mean (S.E.M.) for the three experiments.*

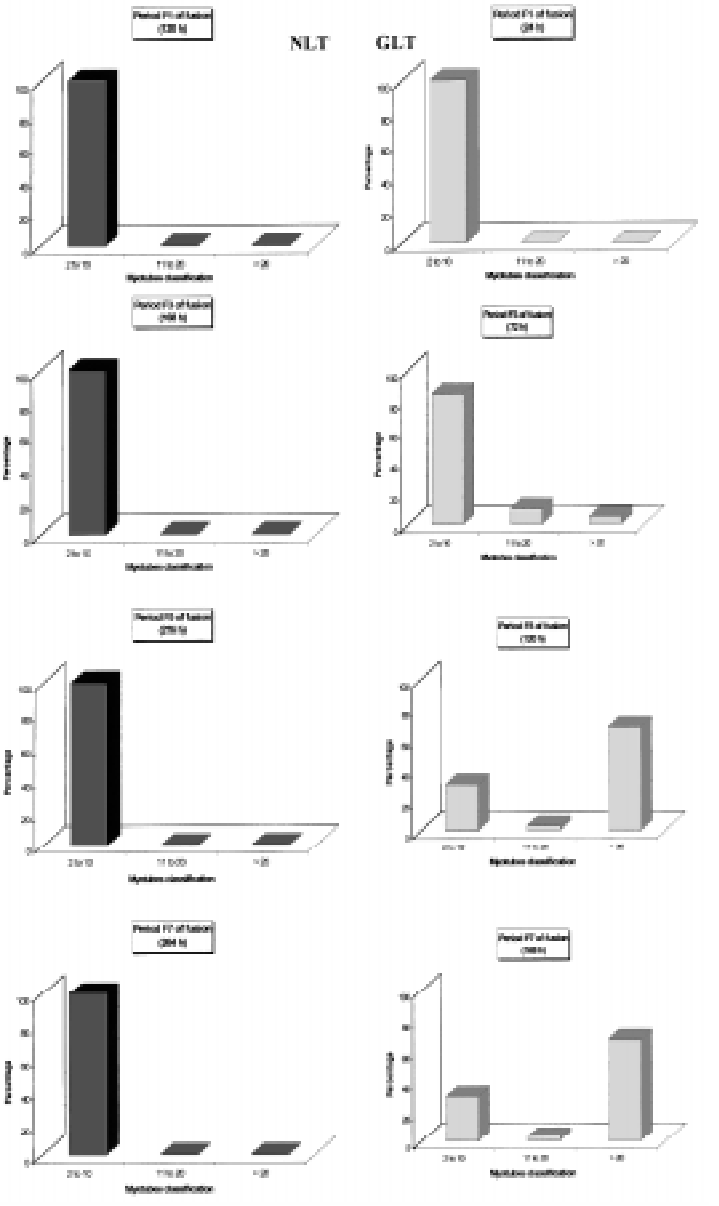

Fig. 2. Quantitative analysis of nuclei number contained in NLT and GLT cell lines during the early stages of differentiation. *The comparison was carried out using three categories of myotubes: those containing 2 to 10 nuclei, 11 to 20 nuclei, and more than 20 nuclei.*

Cell differentiation

In this study, the myotubes were classified into three groups according to their nuclei number: myotubes containing 2 to 10 nuclei, myotubes containing 11 to 20 nuclei, and myotubes containing more than 20 nuclei.

As shown in Figure 2, whatever the stage of early differentiation, 100% of the myotubes from the NLT cell-line cultures belonged to the first category of myotubes containing 2-10 nuclei/myotube.

In contrast, in the GLT cell line, 24 h after the introduction of the fusion medium, 100% of the myotubes contained 2 to 10 nuclei, whereas after 72 h, this proportion decreased to 85%, while 10% of the myotubes contained 11 to 20 nuclei, and 5% of myotubes contained more than 20 nuclei. After 120 h or 168 h, the proportion in this last category increased further. Indeed, at these late stages, small myotubes (with 2-10 nuclei) were weakly represented, constituting only 3% of the myotubes, compared to 67% that contained more than 20 nuclei and 30% that were of intermediate size (with 11-20 nuclei).

Immunodetection and quantification of m-calpain: an *in vitro* study

In rat primary cell cultures, it has been recently demonstrated that the amount of m-calpain increases as a function of myoblast fusion (Cottin *et al*., 1994). The goal of these experiments was to quantify the 80 kDa m-calpain band on cultured cell lines as a function of the early stages of myogenesis. Consequently, myoblasts cultured in fusion medium for different lengths of time were used: 4 h, 120 h, 168 h, 216 h, and 264 h, for the control cell line, NLT, and 4 h, 24 h, 72 h, 120 h, and 168 h for the GLT cell line. Western-blot studies using monospecific antibodies directed against m-calpain, followed by densitometric quantification (Fig. 3 A,B), showed a significant increase in m-calpain content at the beginning of fusion (24 h) in GLT lines. Indeed, the amount of m-calpain measured at this time was about 1.5 times higher than it was 4 h after the introduction of the fusion medium, corresponding to 3 days after initial plating. Furthermore, this increase was observed until day 7, when m-calpain expression reached a maximal value: The amount measured on day 7 (264 h) was about 3 times higher than at the 4 h stage. In contrast, in the case of the NLT cell line, the amount of m-calpain was constant throughout the early stages of myogenesis (Fig. 4 A,B).

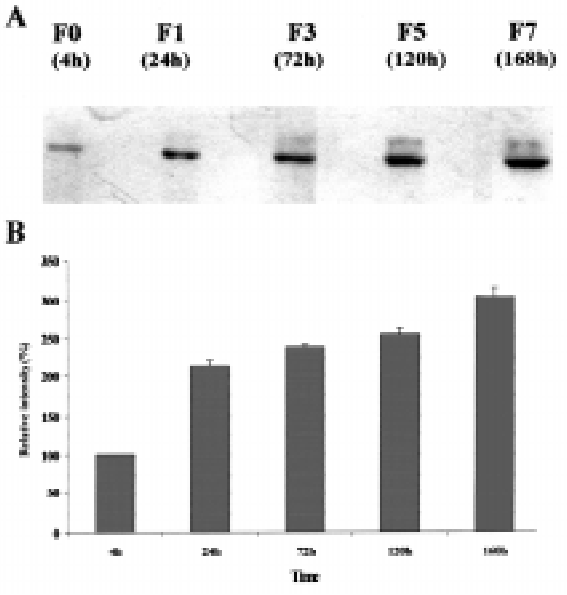

Fig. 3. m-calpain levels during myoblast fusion in the GLT cell line. (A) *Immunoblot analysis of m-calpain during the stage of myoblast fusion in the GLT cell line. Protein from different stages (4 h, 24 h, 72 h, 120 h, and 168 h after the introduction of the fusion medium) was subjected to SDS-PAGE (10%) and transferred for blotting.* **(B)** *Quantification of m-calpain during the stage of myoblast fusion in the GLT cell line. The apparent density of the bands on the membranes, observed after immunodetection for each stage, was estimated by scanning with a video densitometer. Results were expressed as a percentage compared to the cell content measured 4 h after introduction of the fusion medium. Data are representative of three experiments. The bars denote standard errors of the mean.*

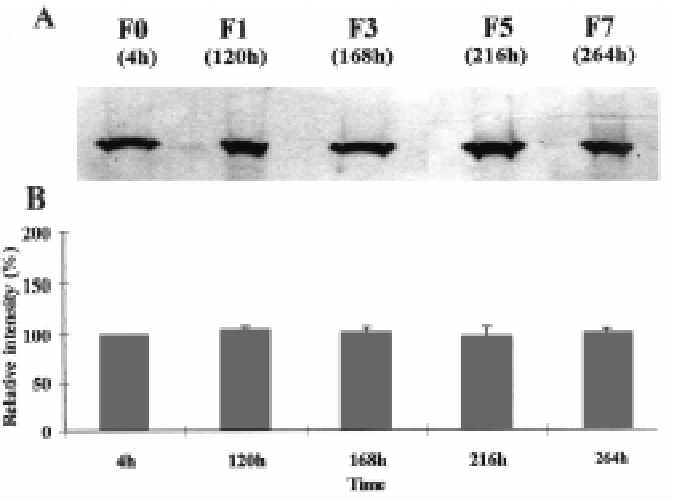

Fig. 4. m-calpain levels during myoblast fusion in the NLT cell line. (A) *Immunoblot analysis of m-calpain during the stage of myoblast fusion in the NLT cell line. Protein from different stages (4 h, 120 h, 168 h, 216 h, and 264 h after introduction of the fusion medium) was subjected to SDS-PAGE (10%) and transferred for blotting.* **(B)** *Quantification of m-calpain during the stage of myoblast fusion in the NLT cell line. The same experimental procedure was used in the NLT cell line as in the GLT cell line (see legend Fig. 3B).*

Effect of synthetic calpain inhibitor

Calpain inhibitor II blocks myoblast fusion in a dose-dependent manner (data not shown). After 72 h in the proliferation medium (Fig. 5A) with 10 μg/ml of calpain inhibitor II added, fusion was reduced by approximately 93% without affecting cell viability (Fig. 5B). When the culture medium containing this exogenous factor was removed and replaced by a fusion-stimulating medium, the myoblasts were still able to fuse and form multinucleated myotubes (Fig. 5C).

Fine structure of E14 and E19 muscles

At stage E14 (14-day-old mouse embryos), the control and dysgenic embryonic muscles presented the same spatial organization. In particular, the organization of thin and thick myofilaments was partial and peripheral (Fig. 6 A,B), as has been already observed by Ontell and Kozeka (1984). The only difference observed between the two populations was the high polynucleation that characterized the dysgenic myotubes (Fig. 6B). At stage E19, the dysgenic muscle preserved somewhat the same organization as was observed at stage E14 (Fig. 7B). The myogenic cells were of large diameter, and they remained close to one another and were surrounded by cells that could be identified with difficulty as fibroblasts. In contrast, the control muscle at stage E19 displayed a completely different spatial organization, with myotubes organized in clusters and separated by significant spaces (Fig. 7A).

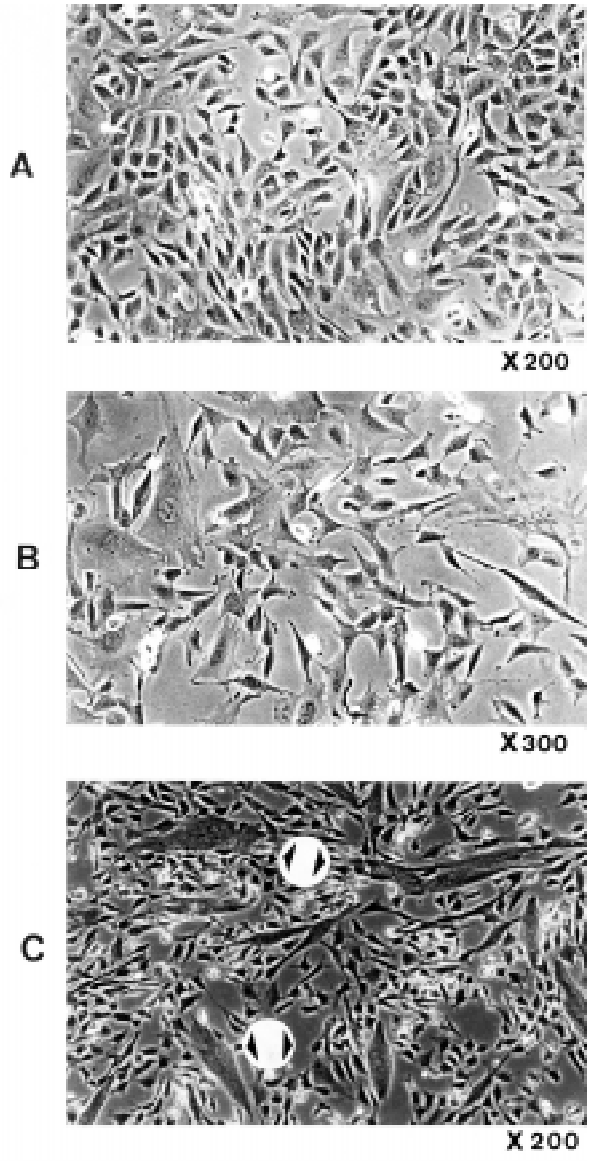

Fig. 5. Effect of calpain inhibitor II on myoblast fusion in the GLT cell line. (A) *Phase-contrast micrograph of the GLT cell line after 72 h in the proliferation medium (x 200).* **(B)** *Phase-contrast micrograph of the GLT cell line 24 h after the addition of calpain inhibitor II (10 μg/ml) to the fusion medium. At this time in the experiment, fusion was reduced by approximately 93% ± 4.7 (x 300).* **(C)** *Phase-contrast micrograph of the GLT cell line after the removal of calpain inhibitor II and its replacement by fusion medium (48 h after treatment with inhibitor II). At this time, myoblasts were still able to fuse and to form multinucleated myotubes (double arrows, x 200).*

Moreover, the diameter of the multinucleated cells in the control muscles was smaller than in the dysgenic muscle. The fibroblasts were recognizable by a clear morphology with long filopods loaded with reticulum and arranged around the myotube clusters.

The difference between the development of the dysgenic and control *gastrocnemius lateralis* muscles suggests that differentiation in the two cell types did not follow the same rhythm. The presence of such large myotubes in the dysgenic muscle might be explained by the high incidence of abnormal polynucleation. Thus, polynucleation might be correlated with the rate of maturation of the muscle. All these observations lead us to suppose that m-calpain, which is known to be involved in the fusion process, may be overexpressed in the dysgenic muscle. For this reason, we decided to quantify the activity of this protease *in vivo* in the dysgenic muscle.

Immunodetection and quantification of m-calpain: an *in vivo* study

The *in vivo* study using hindlimb muscles from dysgenic and control mice, showed the same results as was observed in the GLT and NLT cell lines: in *mdg/mdg* mice, m-calpain was overexpressed compared to the control. Indeed, in dysgenic mice the amount of this protease was doubled (Fig. 8 A,B).

Discussion

At the time of fusion, biochemical and immunological analyses have revealed major alterations in the myoblast cell surface (Paw and David, 1979). These observations raise the possibility that

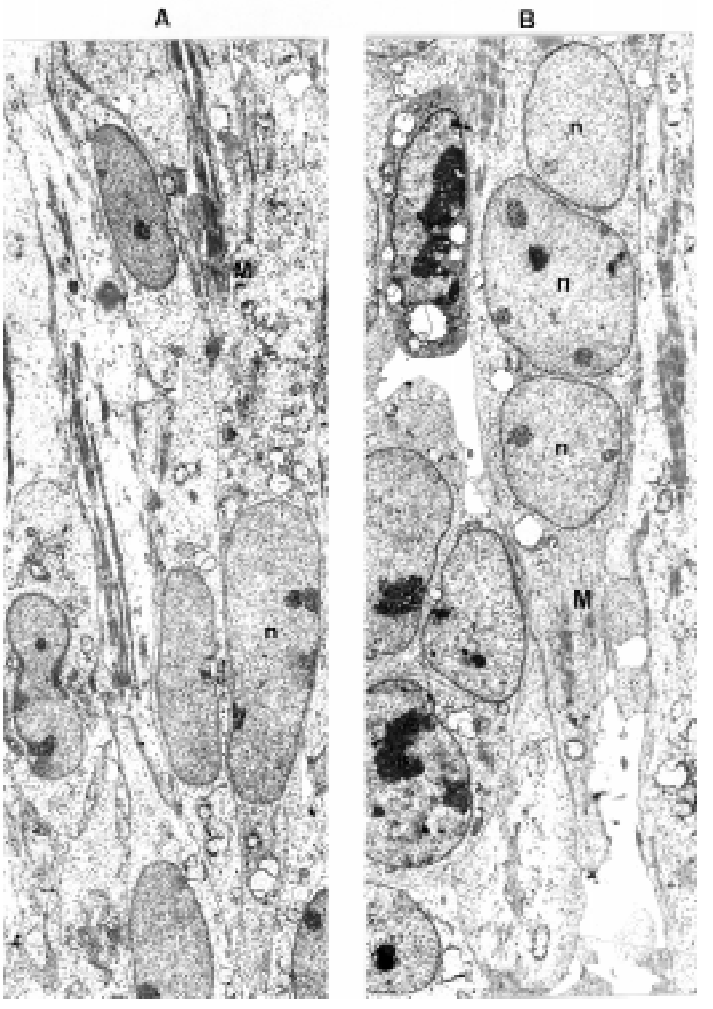

Fig. 6. Ultrastructure of (A) normal and (B) dysgenic muscle tissue (*gastrocnemius lateralis* at E14 (14-day-old mouse embryos)). *The normal and dysgenic myotubes (M) present the same ultrastructure, the organization of thin and thick myofilaments is partial and peripheral. Note the regrouping of nuclei (n) and the polynucleation in the dysgenic embryo (x 4500).*

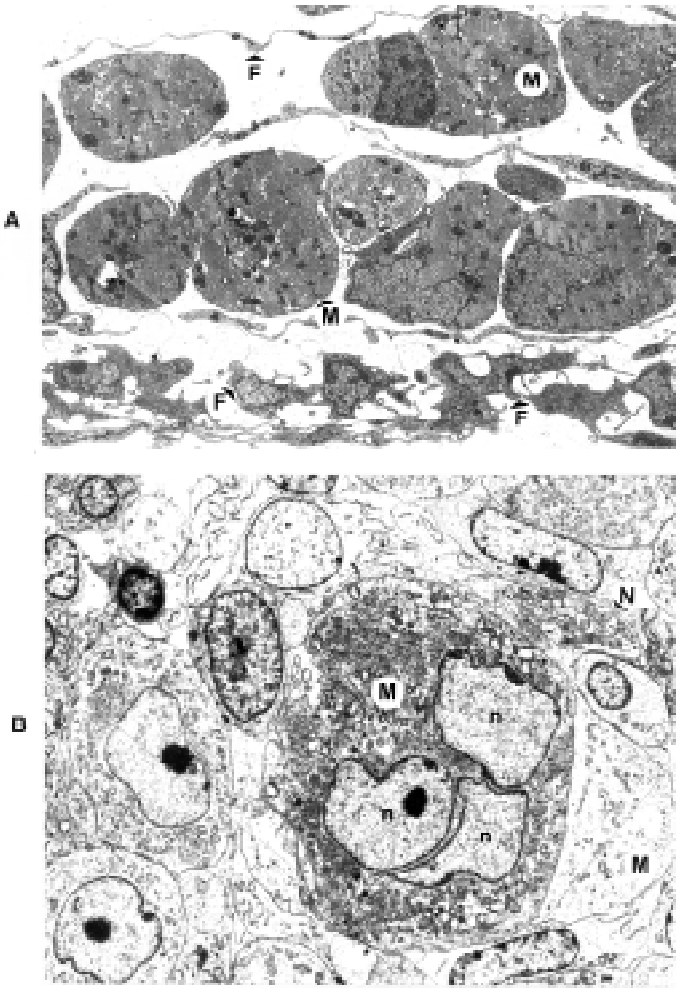

Fig. 7. Ultrastructure of (A) normal and (B) dysgenic muscle tissue (*gastrocnemius lateralis* at E19). *At this time, the dysgenic myotubes (M) present a large diameter, remain close to each other, and are surrounded by cells that can be identified with difficulty as fibroblasts. In contrast, the normal myotubes are organized in clusters and separated by significant spaces. Their diameter is also smaller than in the dysgenic muscle, and the fibroblasts (F) are recognizable by a clear morphology with long filopods loaded with reticulum and arranged around the clusters (x 4500).*

proteolysis may play an important role during myoblast fusion. It has been shown consistently that the fusion of primary rat myoblasts requires the activity of neutral metallo-endopeptidase (Couch and Strittmatter, 1983). In addition, a Ca^{2+}-activated neutral proteinase (m-calpain or CAPN2) has been reported to appear in myogenic cells at around the time of fusion (Kaur and Sanwall, 1981). Thus, as Schollmeyer suggested (1986b), the entry of Ca^{2+} into myoblasts before fusion could activate m-calpain that has been localized in the vicinity of the plasma membrane (Dayton *et al*., 1981). Since m-calpain exhibits a peripheral distribution in cells that are fusion competent and becomes membrane associated in fusing myoblasts (Schollmeyer, 1986a), it can be supposed that this thiol proteinase, which requires millimolar Ca^{2+}, plays a role in the process of myoblast fusion. Indeed, m-calpain activity strongly increases in cultured myoblasts during the early period of myogenic differentiation when μ-calpain (calpain I or CAPN1, which requires micromolar Ca^{2+}) is still not present in either proliferating or differentiated myoblasts (Kwak *et al*., 1993). Recently, m-calpain, well known as an intracellular enzyme, has been localized intercellularly (Adachi *et al*., 1990; Schmaier *et al*., 1990; Suzuki *et al*., 1990; Brustis *et al*., 1994) and, more particularly, associated with extracellular-matrix components (Adachi *et al*., 1990; Suzuki *et al*., 1990, Dourdin *et al*., 1999).

From the results obtained in the GLT and NLT cell lines with respect to fusion and the quantitative variation in m-calpain, several observations supporting the involvement of m-calpain at the time of fusion need to be emphasized.

When the transition from myoblasts to myotubes was slow and the absolute level of fusion was reduced, as in the NLT cell line, the amount of m-calpain present did not vary significantly. On the other hand, when the process was precocious and fast and the level of fusion was elevated, as in the GLT cell line, the amount of m-calpain increased as myogenesis progressed.

Results from our previous experiments using primary cultures of rat and a C2C12 cell line are in agreement with these results (Brustis *et al*., 1994; Elamrani *et al*., 1995). Indeed, the quantitative variations observed in the protease in the GLT cell line are similar to those observed in rat primary cell cultures, in which the process of fusion is both precocious and fast and reaches a level of 80% fusion. Studies on the C2C12 cell line, on the other hand, show an evolution of the fusion percentage and, similarly, an evolution in the m-calpain quantity resembling that observed in the NLT cell line. Indeed, in the C2C12 cell line, fusion occurs late, 72 hours after the introduction of the differentiation medium and reaches an absolute level of 60%. In the same way, the quantity of the protease present during the precocious stages of differentiation does not undergo any significant variation (data not shown). It seems therefore that

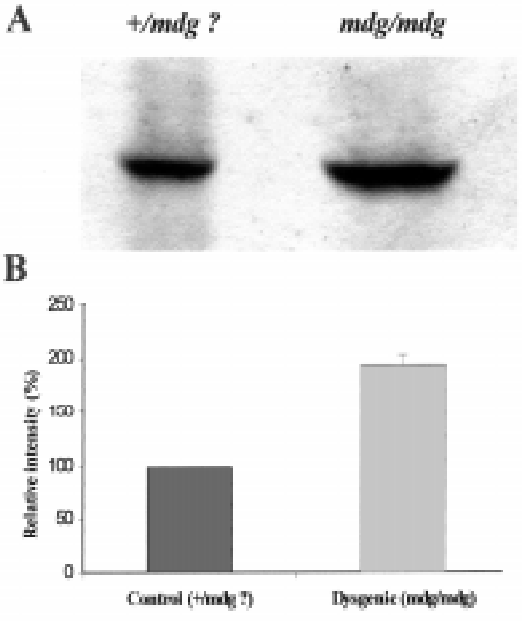

Fig. 8. (A) *Immunoblot analysis of m-calpain in dysgenic and control muscle tissue (E19).* **(B)** *Quantification of m-calpain in control and dysgenic muscle tissue. The apparent density of the bands on the membranes, observed after immunodetection, was estimated by scanning with a video densitometer. Results were expressed as a percentage compared to the normal data. Data are representative of three experiments. The bars denote standard errors of the mean.*

an augmentation of the enzyme quantity is concomitant with fast growth in the rate of fusion.

Otherwise, the quantification of signals corresponding to the protease, observed in all of the membranes obtained from the different experiments, suggests that the quantity of enzyme present in the NLT and C2C12 cell lines at the beginning of differentiation, although not varying in the later stages, is more important (by a factor of 2) than the quantity observed at the same stage in the GLT cell line and in the primary cultures (Kwak *et al.*, 1993; Cottin *et al.*, 1994; Elamrani *et al.*, 1995). It seems as well that the rapidity and amplitude of fusion are bound directly to the concentration of m-calpain present in myogenic cells just before the beginning of myogenesis, and to variations in this concentration during the process of fusion.

In dysgenic-mouse muscle experiments, it is clear that the quantity of m-calpain is significantly more important than in the experiments with control mice. It is important to note that fusion is nearly finished in control mice at the time of birth, but that it remains very important in dysgenic mice at the same stage. Indeed, dysgenic muscle at birth still presents features of the embryonic phase. Thus, these results seem to confirm a major role played by m-calpain during the process of fusion.

In conclusion, myoblast fusion appears to require m-calpain activity. Fusion is probably a multiple-step process involving proteases such as calpain, metallo-endoproteases, proteasome, and certain other enzymes (cathepsins) or the ADAMS (Gardrat *et al.*, 1997; Ebisui *et al.* 1995; Béchet *et al.*, 1991; Yagami-Hiromasa *et al.*, 1995). Consequently, achieving a loss of function of m-calpain by gene knockout probably would be the next logical step to prove a cause-effect relationship in the phenomenon of fusion.

Materials and Methods

Preparation of m-calpain

m-calpain was purified from 2 kg of fresh rabbit muscle as previously described by Garret *et al.*, 1988) and stored at 2°C in 20 mM Tris-HCl (pH 7.50) containing 2 mM EDTA, 2 mM EGTA, 1 mM DTT, and 1 mM NaN_3.

Purification of anti-m-calpain

Rabbit muscle m-calpain antibodies were purified as reported by Cottin *et al.* (1994), Penny *et al.* (1985), and Hatanaka *et al.* (1984). Different types of control experiments with respect to the sensitivity and specificity of the antibodies were carried out using the immunoblot method (Cottin *et al.*, 1994).

Animals

The muscular dysgenesis mice kindly provided by Dr J.A. Powell (Smith College, Northampton, Mass.) were bred by brother-sister matings from the original breeding pairs. For our study, newborn homozygous mutant dysgenic *mdg/mdg* mice and normal +/*mdg?* (*+/+* or +/*mdg*) littermates were obtained from dated pregnancies from heterozygous matings; no differences between +/+ control mice and +/mdg mice have been reported. The mice were sacrificed by ether anesthesia.

Dissection

Whole hindlimbs were dissected from the newborn mice, and the skin and connective tissue were carefully teased away. The entire hindlimb was removed at the haunch, rapidly frozen in isopentane prechilled with liquid nitrogen, and stored at -80°C.

Cell culture

The NLT and GLT lines of immortalized mouse skeletal myoblasts were obtained from J.A. Powell (Smith College, Northampton, Mass.) and cultured according to procedures previously described (Powell *et al.*, 1996). In most experiments, the cells proliferated in growth medium containing 80% Dulbecco's Modified Eagle Medium (DMEM) supplemented with glucose (Gibco) (1 g/l), fungizone (2.5 μg/ml), penicillin (5 units/ml), streptomycin (5 μg/ml), hygromycin B (100 μg/ml), 10% fetal calf serum (Boehringer), and 10% horse serum (Gibco). To induce fusion, the cells were grown to 80% confluence, corresponding to 3 days of culture, and then the growth medium was replaced with DME medium (without hygromycin B), supplemented with 2% horse serum and composed by 12.6 mM K^+ Hanks Balanced Salt Solution to prevent the myotubes from pulling off (fusion medium). In most experiments, the GLT cells were plated at 5.10^4 per ml and the NLT cells at 2.10^4 per ml in 35 mm culture dishes that had been previously covered with 0.1% gelatin. The cultures were grown at 37 °C in a humidified atmosphere containing 7.5% CO_2, and the medium was changed daily.

Treatment with a synthetic inhibitor

When it was necessary for the experiments, the normal medium (fusion medium) was supplemented with a synthetic inhibitor (calpain inhibitor II, synonym = N-acetyl-leucyl-leucyl-methioninal) purchased from Boehringer with a concentration of 10 μg/ml, as previously described by Dourdin *et al.* (1999). Control cultures were grown using the same treatment medium, but without synthetic inhibitor.

Reversibility test

The treatment with calpain inhibitor II was performed 24 h after initial plating. The reversibility of the inhibitory effect was investigated 24 h after the treatment. After washing the cells with prewarmed phosphate-buffered saline (Ca^{2+}, and Mg^{2+} free) for 1 min, the buffer was replaced by a fusion medium capable of stimulating myoblast fusion. After culturing for 48 h, the extent of fusion was evaluated by visual inspection of the cells.

Electrophoresis and immunoblotting procedures

The same procedures were used for both types of material, cell cultures and animals. After homogenization at 4 °C in sample buffer (0.05 M Tris-HCl, pH 6.80, SDS 0.01%, bromophenol blue, 30% glycerol, 1% DTT), muscular tissues or detached cells from the dishes were sonicated for 30 s at 0 °C. The samples were then boiled for 5 min. One hundred fifty μg of proteins from each sample were run on 10% (w/v) SDS-PAGE according to the method of Laemmli (1970). Fractionated proteins were then electrotransferred (3 h under 1.2A) onto nitrocellulose sheets (Amersham). After blocking the nonspecific sites with a TBS (tris-buffered saline) solution containing 5% BSA, the membranes were incubated with monospecific antibody (dilution 1/400) directed against m-calpain according to the method of Cottin *et al.* (1994). The identification of the antigen-antibody complex was realized via a second antibody coupled to horseradish peroxidase) with an ECL western-blotting detection kit (Amersham).

Quantification

Measurement of myoblast fusion

At various times, cultured cells were rinsed twice with cold PBS and fixed at room temperature in 4% paraformaldehyde prepared in PBS for 15 min. After staining with Hansen's hemalun (8 min), nuclei were counted, and the percentage of fusion was evaluated. Percentage fusion equals (number of nuclei in myotubes / total number of nuclei in single myoblasts and in myotubes) times 100.

Quantification by densitometric analysis

The apparent density of the bands on the membranes was estimated after scanning with a video densitometer (Bioprofile, Vilber-Lormat). The linearity response and the reproducibility of densitometry in the quantification were controlled by duplicate calibration curves using different concentrations of purified m-calpain. Under these conditions, densitometric quantification was quite linear and reproducible.

Protein quantification

Protein concentrations were determined using a BCA protein assay kit purchased from Pierce and with BSA as the standard.

Cell differentiation

To follow muscle-cell differentiation, a quantitative study of the number of nuclei in the myotubes was evaluated with three categories of myotubes defined: myotubes with 2 to 10, 11 to 20, and more than 20 nuclei.

Transmission electron microscopy

After rapid dissection of each *gastrocnemius* muscle from E14 and E19 control and dysgenic mice, the muscles were fixed at room temperature in a solution containing 2% paraformaldehyde and 0.4% glutaraldehyde in 0.05 M phosphate buffer for E14 and 0.08 M buffer for E19 muscles. The muscles were then rinsed in the same buffer and postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer. Fixed samples were dehydrated in graded alcohol and embedded in Epon resin. Ultra-thin sections of about 65 nm were stained with a saturated solution of uranyl acetate in 50% acetone followed by staining with 0.2% lead citrate solution. The observations were made with a Philips EM 201 electron microscope operated at 60 kV.

Acknowledgements

The research reported in this paper was supported by l'Institut National de la Recherche Agronomique (INRA, France) and by grants from the Association Française contre les Myopathies (AFM, France). We also thank Susan Duhon for correcting the English.

References

ADACHI, E., MUKAIYAMA, E.T., SASA,I K., HAYASHI, T., KAWASHIMA, S., KASAI, Y., HAYASHI, M. and HASHIMOTO, P.H. (1990). Immunohistochemical evidence of the extracellular localization of Calcium Neutral Protease (CANP) in rabbit skeletal muscle, lung and aorta. *Arch. Histol. Cytol.* 53: 413-422.

ASHBY, P.R., WILSON, S.J. and HARRIS, A.J. (1993). Formation of primary and secondary myotubes in aneural muscles in the mouse mutant peroneal muscular atrophy. *Dev. Biol.* 159: 519-528.

BALCERZAK, D., POUSSARD, S., BRUSTIS J.J., ELAMRANI, N., SORIANO, M., COTTIN, P. and DUCASTAING, A. (1995). An antisense oligodeoxyribonucleotide to m-calpain mRNA inhibits myoblast fusion. *J. Cell Sci.* 108: 2077-2082.

BECHET, D.M., FERRARA, M.J., MORDIER, S.B., ROUX, M.P., DEVAL, C.D. and OBLED, A. (1991). Expression of lysosomal cathepsin B during calf myoblast-myotube differentiation. Characterization of a cDNA encoding bovine cathepsin B. *J. Biol. Chem.* 266: 14104-14112.

BRUSTIS, J.J., ELAMRANI, N., BALCERZAK, D., SAFWATE, A., SORIANO, M., POUSSARD, S., COTTIN, P. and DUCASTAING, A. (1994). Rat myoblast fusion requires exteriorized m-calpain activity. *Eur. J. Cell Biol.* 64: 320-327.

CHAUDHARI, N. (1992). A single nucleotide deletion in the skeletal muscle-specific calcium channel transcript of Muscular Dysgenesis (mdg) mice. *J. Biol. Chem.* 267: 25636-25639.

COTTIN, P., BRUSTIS, J.J., POUSSARD, S., ELAMRANI, N., BRONCARD, S. and DUCASTAING, A. (1994). Ca^{2+}-dependent proteinases (calpains) and muscle cell differentiation. *Biochim. Biophys. Acta* 1223: 170-178.

COUCH, C.B. and STRITTMATTER, W.J. (1983). Rat myoblast fusion requires metallo-endoprotease activity. *Cell* 32: 257-265.

COURBIN, P., KOENIG, J., RESSOUCHES, A., BEAM, K and POWELL, J.A. (1989). Rescue of excitation-contraction coupling in dysgenic muscle by addition of fibroblasts *in vitro*. *Neuron* 2: 1341-1350.

CROALL, D.E. and DEMARTINO, G.N. (1991). Calcium-activated neutral proteases (calpain) system: structure, function and regulation. *Physiol. Rev.* 71: 813-847.

DAYTON, W.R., SCHOLLMEYER, J.V., LEPLEY, R.A. and CORTES, L.R. (1981). A calcium-activated protease possibly involved in myofibrillar protein turnover. Isolation of a low-calcium-requiring form of the protease. *Biochim. Biophys. Acta* 659: 48-61.

DOURDIN, N., BALCERZAK, D., BRUSTIS, J.J., POUSSARD, S., COTTIN, P. and DUCASTAING, A. (1999). Potential m-calpain substrates during myoblast fusion. *Exp. Cell Res.* 246: 433-442.

DOURDIN, N., BRUSTIS, J.J., BALCERZAK, D., ELAMRANI, N., POUSSARD, S., COTTIN, P. and DUCASTAING, A. (1997). Myoblast fusion requires fibronectin degradation by exteriorized m-calpain. *Exp. Cell Res.* 235: 385-394.

DUSSARTRE, C. (1993). Etude du développement musculaire embryonnaire et de la différenciation myogénique *in vitro* chez un mutant murin: Muscular dysgenesis. Thèse pour le Doctorat de l'Université de Bordeaux II, mention Neurosciences et Pharmacologie.

EBISUI, C., TSUJINAKA, T., MORIMOTO, T., FUJITA, J., OGAWA, A., ISHIDOH, K., KOMINAMI, E., TANAKA, K. and MONDEN, M. (1995). Changes of proteasomes and cathepsins activities and their expression during differentiation of the C2C12 myoblasts. *J. Biochem.* 117: 1088-1094.

ELAMRANI, N., BRUSTIS, J.J., DOURDIN, N., BALCERZAK, D., POUSSARD, S., COTTIN, P. and DUCASTAING, A. (1995). Desmin degradation and Ca^{2+}-dependent proteolysis during myoblast fusion. *Biol. Cell* 85: 177-183.

FERGUSON, D.G., SCHWARTZ, H.W. and FRANZINI-AMSTRONG, C. (1984). Subunit structure of junctional feet in triads of skeletal muscle: a freeze-drying, rotary-shadowing study. *J. Cell Biol.* 99: 1735-1742.

GARDRAT, F., MONTEL, V., RAYMOND, J. and AZANZA, J.L. (1997). Proteasome and myogenesis. *Mol. Biol. Rep.* 24: 77-81.

GARRET, C., COTTIN, P., DUFOURCQ, J. and DUCASTAING, A. (1988). Evidence for Ca^{2+}-independent association calpain II and phospholipids vesicles. *FEBS. Lett.* 227: 209-214.

HATANAKA, M., YOSHIMURA, N., MURAKAMI, T., KANNAGI, R. and MURACHI T. (1984). Evidence for membrane-associated calpain I in human erythrocytes: detection by an immunoelectrophoretic blotting method using monospecific antibody. *Biochemistry* 23: 3472-3276.

JOFFROY, S., DELAGE, J.P., LETELLIER, T., ROSSIGNOL, R., MALGAT, M., POWELL, J.A., MAZAT, J.P. and KOENIG, J. (1999). Modification du métabolisme mitochondrial dans des fibroblastes issus de souris mutantes muscular dysgenesis. Evidence d'une étroite communication embryonnaire entre myoblastes et fibroblastes. Paper presented at the fourth Colloquium of Neurosciences Society: BC 21 (Marseille, Abstr.).

KAUR, H. and SANWALL, B.D. (1981). Regulation of the activity of a calcium-activated neutral protease during differentiation of skeletal myoblasts. *Can. J. Biochem.* 59: 743-747.

KUMAR, A., SHAFIQ, S., WADGAONKAR, R. and STRACHER, A. (1992). The effect of protease inhibitors, leupeptin and E64, on differentiation of C2C12 myoblasts in tissue culture. *Cell Mol. Biol.* 38: 477-483.

KWAK, K.B., CHUNG, S.S., KIM, O.M., KANG, M.S., HA, D.B. and CHUNG, C.H. (1993). Increase in the level of m-calpain correlates with the elevated cleavage of filamin during myogenic differentiation of embryonic muscle cells. *Biochim. Biophys. Acta* 1175: 243-249.

LAEMMLI, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.

PAW, P.G. and DAVID, J.D. (1979). Alterations in surface proteins during myogenesis of a rat myoblast cell line. *Dev. Biol.* 70: 27-38.

PENNY, I.F., TAYLOR, M.A., HARRIS, A.G. and ETHERINGTON, D.E. (1985). Purification and immunological characterization of two calcium-activated neutral proteinases from rabbit skeletal muscle. *Biochim. Biophys. Acta* 829: 244-252.

PINCON-RAYMOND, M., RIEGER, F., FOSSET, M. and LAZDUNSKI, M. (1985). Abnormal transverse tubule system and abnormal amount of receptors for a calcium channel inhibitors of the dihydropyridine family in skeletal muscle from mice with embryonic muscular dysgenesis. *Dev. Biol.* 112: 458-466.

PLATZER, A.C. and GLUECKSOHN-WAELSCH, S. (1972). Fine structure of mutant (muscular dysgenesis) embryonic mouse muscle. *Dev. Biol.* 28: 242-252.

POWELL, J.A. and FAMBROUGH, D. (1973). Electrical properties of normal and dysgenic mouse skeletal muscle in culture. *J. Cell Physiol.* 82: 21-38.

POWELL, J.A., PETHERBRIDGE, L. and FLUCHER, B.E. (1996). Formation of triads without the dihydropyridine receptor a subunits in cell lines from dysgenic skeletal muscle. *J. Cell Biol.* 134: 375-387.

SCHMAIER, H.S., BRADFORD, H.N., LUNDBERG, D., FARBER, A. and COLMAN, R.W. (1990). Membrane expression of platelet calpain. *Blood* 72: 1273-1281.

SCHOLLMEYER, J.E. (1986a). Possible role of calpain I and calpain II in differentiating muscle. *Exp. Cell Res.* 163: 413-422.

SCHOLLMEYER, J.E. (1986b). Role of Ca^{2+} and Ca^{2+} -activated protease in myoblast fusion. *Exp. Cell Res.* 162: 411-422.

SUZUKI, K., SHIMIZU, K., HMAMOTO, T., NAKAGAMA, Y., HAMAKU, T., YAMAMURO, T. (1990). Biochemical demonstration of calpains and calpastatin osteoarthritic synovial fluid. *Arthritis Rheum.* 33: 728-732.

TANABE, T., BEAM, K., POWELL, J.A. and NUMA, S. (1988). Restoration of excitation-contraction coupling and slow calcium current in dysgenic muscle by dihydropyridine receptor complementary DNA. *Nature* 336: 134-139.

YAGAMI-HIROMASA, T. SATO, T., KURISAKI, T. KAMIJO, K., NABESHIMA, Y. and FUJISAWA-SEHARA, A. (1995). A metalloprotease-disintegrin participating in myoblast fusion. *Nature* 377: 652-656.

YAO, M.L. and ESSIEN, F.B. (1975). DNA synthesis and the cell cycle in cultures of normal and mutant *(mdg/mdg)* embryonic mouse muscle cells. *Dev. Biol.* 45: 166-175.

Received: December 1999
Accepted for publication: March 2000