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Cell-penetrating inhibitors of calpain block both membrane fusion and filamin cleavage in chick embryonic myoblasts

Kyu Bong Kwak[a], Jun-ichi Kambayashi[b], Man Sik Kang[a], Doo Bong Ha[a] and Chin Ha Chung[a]

[a]*Department of Molecular Biology and SRC for Cell Differentiation, College of Natural Sciences, Seoul National University, Seoul 151-742, South Korea* and [b]*Department of Surgery II, Osaka University Medical School, Osaka 553, Japan*

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Benzyloxycarbonyl(Z)-Leu-nLeu-H (calpeptin) and Z-Leu-Met-H, cell-penetrating inhibitors of calpain, were found to block myoblast fusion without any effect on cell proliferation and alignment along their bipolar axis. They also inhibited the accumulation of creatine kinase during myogenesis. These effects were dose-dependent, and could be reversed upon removal of the drug from the culture medium. Furthermore, treatment of the inhibitors prevented the hydrolysis of filamin, which is sensitive to cleavage by calpain in vitro and interferes with actin–myosin filament formation by cross-linking F-actin molecules. On the other hand, leupeptin, which can also inhibit calpain in vitro but can not penetrate into cells, showed little or no effect on both myoblast fusion and filamin cleavage. These results suggest that calpain may play an important role in cytoskeletal reorganization that is requisite for myoblast fusion. The role of calpain on the expression of muscle-specific proteins remains unknown

Calpain; Filamin, Calpeptin, Myoblast fusion

1. INTRODUCTION

A prominent event in myogenesis is the membrane fusion of mononucleated myoblasts to multinucleated myotubes [1,2]. This process absolutely requires influx of Ca^{2+} [3] and accompanies various cellular events, such as redistribution of membrane proteins and reorganization of cytoskeletons [4,5]. A number of reports have suggested that protein breakdown plays an important role in mediating myoblast fusion [6–9]. It has been shown that Ca^{2+}-activated thiol-protease requiring millimolar Ca^{2+} (m-calpain: henceforth referred to as calpain) is relocalized from cytosol to membrane during the fusion [7,8]. In addition, both the activity and protein levels of calpain have recently been shown to increase dramatically during the early period of myogenesis [9]. On the other hand, the activity level of calpastatin, an endogenous inhibitor of the protease [10], remains similar during the entire period of the cell culture. Therefore, calpain has been suggested to be a candidate protease involved in myoblast fusion.

Filamin (250 kDa), which facilitates actin microfilament assembly and interferes with actin–myosin filament formation [11,12], has been shown to be cleaved in cultured myoblasts to 240 kDa, in a manner similar to in vitro cleavage of the protein by calpain [9,13]. Furthermore, the hydrolysis of filamin is most evident at the period of myoblast fusion. Therefore, it has been suggested that calpain may play an important role in cytoskeletal reorganization that is requisite for the cell fusion [9]. In an attempt to clarify the role of calpain, we examined the effect of cell-penetrating inhibitors of calpain on myoblast fusion and cleavage of filamin in cultured cells.

Correspondence address. C.H. Chung, Department of Molecular Biology, College of Natural Sciences, Seoul National University, Seoul 151-742, South Korea.

2. MATERIALS AND METHODS

2.1. *Materials*

Calpeptin and Z-Leu-Met-H were provided by Dr. Kambayashi [14]. The 80 kDa catalytic subunit of calpain, filamin, and creatine kinase were purified to apparent homogeneity as described previously [9]. Antibodies against the purified proteins were prepared by injecting each protein into albino rabbits. Culture agents were obtained from Gibco and ^{125}I-labeled protein A was from New England Nuclear.

2.2. *Cell culture*

Myoblasts from breast muscle of 12-day-old chick embryos were prepared as described previously [9,15]. The cells were plated on collagen-coated culture dishes at a concentration of 5×10^5 cells/ml in Eagle's essential medium containing 10% horse serum, 10% chick embryo extract and 1% antibiotic/antimycotic solution. One day after cell seeding the culture medium was changed with the same medium but containing 2% embryo extract. When needed, calpain inhibitors were added to the culture medium at the time of medium change.

2.3 *Immunoblot analysis*

Myoblasts cultured for appropriate periods were harvested in 20 mM Tris-HCl buffer (pH 7) containing 5 mM EDTA. The cells were disrupted by sonication for 30 s, and their extracts were obtained by centrifugation at $500 \times g$ for 10 min. Aliquots of the extracts were electrophoresed in 6 or 12% (w/v) polyacrylamide gels in the presence of SDS as described [9]. After the electrophoresis, proteins in the gels were transferred to nitrocellulose filters, and reacted with the antibody raised against the purified filamin or creatine kinase and then with ^{125}I-protein A. The filters were then dried and autoradiographed [16].

3. RESULTS AND DISCUSSION

To investigate the effect of calpeptin on myogenic differentiation, myoblasts that had been cultured for 24 h were treated with the agent, and were observed under a microscope 48 h after the treatment. As shown in Fig. 1, the cells treated with calpeptin failed to form myotubes, unlike the control cells. When the agent was removed from the culture, the cell fusion began within 24 h. Of interest is the finding that the calpeptin-treated cells can still proliferate and align along their bipolar axis (Fig. 1B). Also, addition of the agent to the cells, which were already committed to membrane fusion, showed little or no effect (data not shown). Therefore, calpeptin appears to specifically and reversibly block myotube formation but allow proliferation and alignment of myoblasts.

We also examined the effects of other inhibitors of calpain on myoblast fusion. As shown in Fig. 2, Z-Leu-Met-H, another cell-penetrating inhibitor of calpain [14], inhibited cell fusion in a dose-dependent manner, as did calpeptin, however, a significantly higher concentration of Z-Leu-Met-H was required to obtain a half-maximal inhibition of the fusion than calpeptin. On the other hand, leupeptin, a peptide aldehyde inhibitor of thiol-proteases, including calpain [17], showed little or no effect on the fusion, nor did E-64, an oxirane inhibitor of thiol-proteases [18], show any effect (data not shown). The latter two agents, unlike calpeptin, contain a positively charged guanidium group at neutral pH [19], and therefore it is likely that the differential effects of the calpain inhibitors on myoblast fusion are due to their ability to be transported across the cell membrane.

Recently, we have shown that the activity and protein levels of calpain dramatically increase during the early period of myoblast fusion, and these increases almost coincide with the increased cleavage of the 250 kDa filamin to a 240 kDa product [9]. Because the fusion process is known to involve reorganization of cytoskeletons in myoblasts [4,5], and because limited proteolysis of cytoskeletal components is generally accepted as one of the potential mechanisms for regulating cytoskeletal organization [20–22], we have suggested that calpain may play an important role in cell fusion [9]. To clarify this possibility further, myoblasts that had been cultured for 24 h were treated with cell-penetrating inhibitors of calpain. After culturing the cells for a further 48

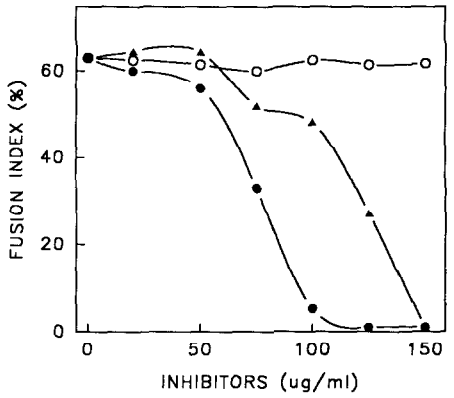

Fig. 2. Dose-dependent effect of calpain inhibitors on myoblast fusion. Myoblasts that had been cultured for 24 h were treated with increasing amounts of calpeptin (●), Z-Leu-Met-H (▲), and leupeptin (○). After the treatment, the cells were cultured for a further 48 h, stained, and observed. Fusion index is defined as the percentage of nuclei in myotubes compared to total nuclei.

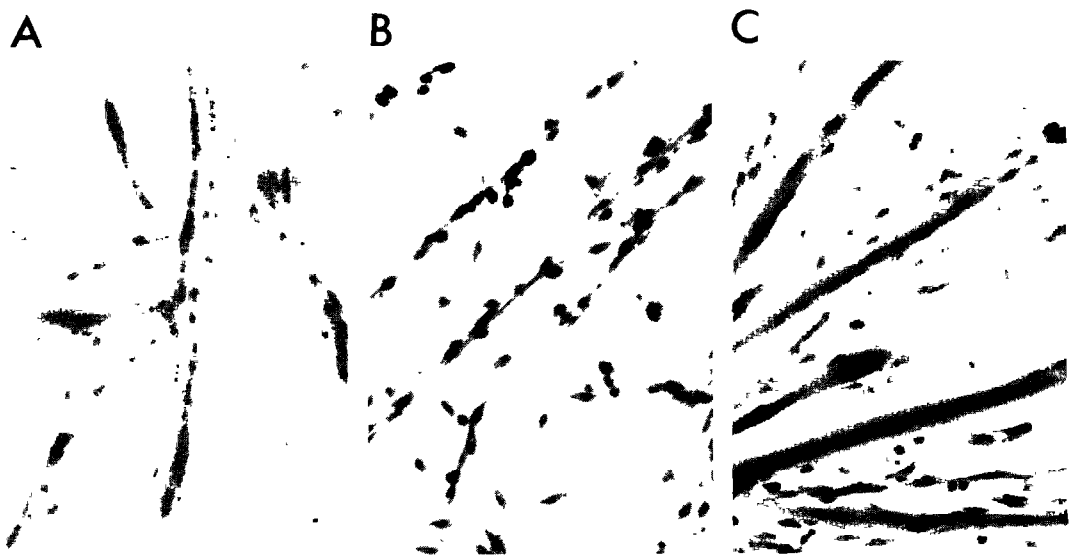

Fig. 1. Inhibition of myoblast fusion by calpeptin and its reversal. Myoblasts that had been cultured for 24 h were incubated for the next 48 h in the absence (A) and presence of 100 μM calpeptin (B). The cells incubated with calpeptin for 24 h were freed of the drug and further cultured for 24 h. They were then observed under a phase contrast microscope (C).

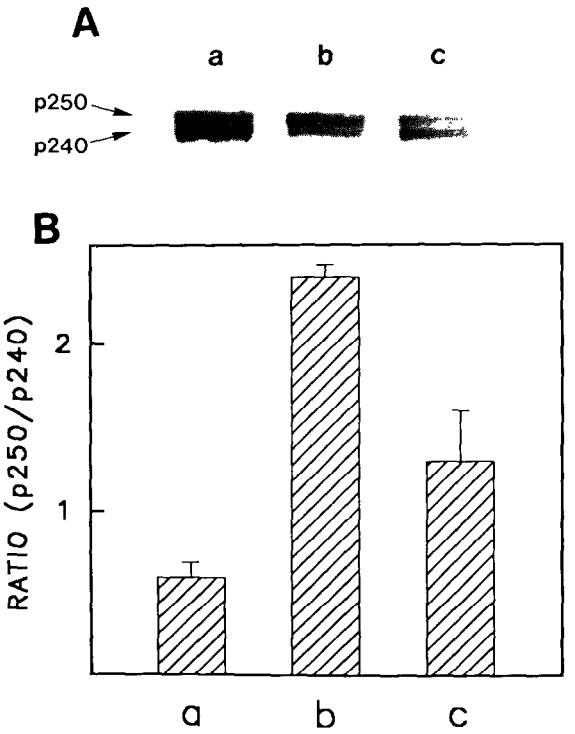

Fig. 3. Effect of cell-penetrating inhibitors of calpain on the cleavage of filamin. Myoblasts that had been cultured for 24 h were treated with 0 (lane a), 100 μM calpeptin (b) or 100 μM Z-Leu-Met-H (c). After the treatment, the cells were cultured for a further 48 h, harvested, and subjected to immunoblot analysis using anti-filamin antiserum as described in section 2 (A). The bands corresponding to 250 kDa (p250) and 240 kDa (p240) were then cut out, and their radioactivity counted (B).

h, they were harvested and subjected to immunoblot analysis using anti-filamin antiserum. Fig. 3 shows that the treatment of the inhibitors significantly reduced the conversion of the 250 kDa filamin to the 240 kDa product in the cells. Nearly identical data were obtained for cells treated with calpeptin for 24 h (data not shown). Of interest is the finding that calpeptin, which blocks the fusion more efficiently than Z-Leu-Met-H, reveals a more potent effect on the reduction of filamin cleavage than the latter agent. In contrast, leupeptin, which did not interfere with the fusion (see Fig. 2), showed little or no effect on the hydrolysis of filamin (data not shown). These results further support our suggestion that calpain plays an important role in cytoskeletal reorganization that is requisite for myoblast fusion.

It is noteworthy, however, that the specificities of calpeptin and Z-Leu-Met-H are not just restricted to calpain. Both agents are also capable of inhibiting other thiol-proteases, such as cathepsin B [14,19]. Furthermore, lysosomotrophic amines, such as chloroquine and ammonium chloride, have been reported to interfere with myoblast fusion [23], however, it appears less likely that lysosomal proteases are involved in cytoskeletal reorganization, due to their subcellular location. In

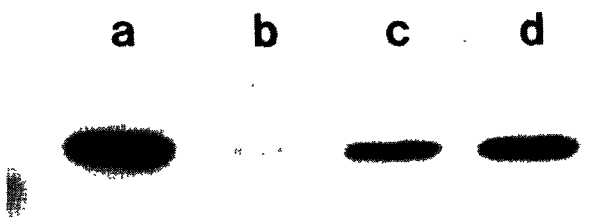

Fig. 4. Effect of cell-penetrating inhibitors of calpain on the accumulation of creatine kinase. Myoblasts that had been cultured for 24 h were treated with 0 (lane a), 100 μM calpeptin (b) or 100 μM Z-Leu-Met-H (c). After the treatment, the cells were cultured for a further 48 h. The cells cultured for 24 h in the presence of 100 μM calpeptin were freed of the drug and further cultured for another 24 h (d). They were then harvested and subjected to immunoblot analysis using anti-creatine kinase antiserum.

addition, the calpain inhibitors do not contain any primary amine group that protonates at acidic pH for accumulation inside lysosomes, unlike the lysosomotrophic agents [24]. Furthermore, cathepsin B is unable to convert filamin into a 240 kDa product under the conditions tested for in vitro hydrolysis of the protein by calpain (data not shown). Nevertheless, a possibility that other unknown, cytosolic thiol-protease(s) as well as lysosomal proteases, including cathepsin B, may be involved in myoblast fusion can not be entirely excluded.

During myogenic differentiation, myoblast fusion is known to occur concurrently with the induction of muscle-specific proteins, such as myosin, α-actin and creatine kinase, although these two processes can be uncoupled [25,26]. To examine whether calpeptin may also affect biochemical differentiation, the cells were cultured and subjected to immunoblot analysis as above but using the anti-creatine kinase antiserum. Fig. 4 shows that accumulation of the protein is strongly hampered by calpeptin and less effectively by Z-Leu-Met-H. Calpain is known to hydrolyze in vitro protein kinase C, hormone receptors, epidermal growth factor and several calmodulin-binding proteins, and therefore is thought to modify signal transduction, the final step for regulation of gene expression in animal cells [27,28]. Thus, it appears possible that calpain may also play a role in regulation of the expression of muscle-specific proteins through limited proteolysis, although this possibility is totally speculative at present.

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