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Developmental Regulation of the Peptide Hydrolyzing Activities of the Proteasome in Myogenic Differentiation

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We examined a role of proteasome, the non-lysosomal multicatalytic protease complex, on the differentiation of chick embryonic myoblasts in culture. The peptide hydrolyzing activities of proteasome were found to change; the hydrolyzing activity against N-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (SLLVY-AMC) was prominent and increased with myogenic differentiation. Proteasome inhibitors, N-carbobenzoxy-Leu-Leu-norvalinal (MG115) and N-carbobenzoxy-Ile-Glu (O-t-butyl)-Ala-Leucinal (PSI), blocked membrane fusion of myoblasts as well as the SLLVY-AMC hydrolyzing activity. Those inhibitory activities of the agents occurred in parallel, but were reversible and both cell fusion and the peptidase activity were restored when the agents were withdrawn from the culture medium. On the other hand, the agents caused accumulation of the ubiquitinylated proteins in the cytoskeletal proteins. These results suggest that each of the peptide hydrolyzing activities of proteasome is independently regulated during the myogenic differentiation and the chymotrypsin-like activity may play an important role in that process.

Key Words: Proteasome, Myogenic differentiation, Ubiquitination

INTRODUCTION

Myogenic differentiation of skeletal muscle cells is characterized by fusion of mononucleated myoblasts into myotubes (Bischoff et al., 1969; O'Neill et al., 1972). Because myogenic differentiation accompanies dramatic morphological changes such as membrane fusion of myoblasts, it is plausible that membraneous and/or cytoskeletal components of the cells should be redistributed and reorganized during the differentiation. A number of reports have suggested that protein breakdown plays an important role in mediating myoblast fusion (Pauw et al., 1979; Fulton et al., 1981). Intracellular proteolysis was suggested to be involved in the myogenic cellular processes and therefore to be requisite for myoblast fusion (Shollmeyer, 1986; Kwak et al., 1993a).

The proteasome is a symmetrical ring-shaped particle with an unusually large mass of 600~800 kDa (20 S) and is composed of nonidentical subunits with small molecular masses of 21~32 kDa (Tanaka et al., 1988; Orlowski, 1990; Lee et al., 1993). This complex has multiple catalytic activities, including components that hydrolyze peptide bonds on the carboxyl side of basic (trypsin-like activity), acidic (peptidylglutamyl-peptide hydrolyzing activity), and hydrophobic (chymotrypsin-like activity) amino acids (Wilk et al., 1983; Rivett, 1989). In addition, it shows a latent proteolytic activity that can be activated by poly-L-lysine or fatty acids (Dahlmann et al., 1985; Tanaka et al., 1986). The proteasome is known to be the catalytic core of the 26 S protease complex, which is the ubiquitin/ATP-dependent proteolytic system plays a major role in the removal of abnormal and denatured proteins as well as short-lived proteins (Eytan et al., 1989; Ciechanover et al., 1994). The natural cellular substrates of the ubiquitin-dependent system include phytochrome, MATα2 repressor, cyclins, p53, and Myc etc (Rechsteiner, 1991). In order to study cellular function of the proteasome, specific inhibitors including a series of peptide-aldehydes have been developed (Palombella et al., 1994; Figueiredo-Pereira et al., 1994; Traenckner et al., 1994).

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In an attempt to clarify a role of proteasome in myoblast differentiation, we investigated the changes in the level of peptidase activities of the proteasome during the myogenic differentiation of chick embryonic myobalsts in culture. We also examined the effect of MG115 or PSI on myoblast fusion as well as on peptide hydrolyzing activities of the proteasome. Here we demonstrate that each of the peptide hydrolyzing activities of proteasome is independently regulated in the myogenic differentiation and that the SLLVY-AMC hydrolyzing activity may be involved in the differentiation of myoblasts. In addition, we show that the inhibitors cause accumulation of ubiquitinylated proteins in cytoskeletal fraction of the fusion-arrested myoblasts.

MATERIALS AND METHODS

1. Cell culture

Myoblasts from the breast muscle of 12-day-old chick embryo were prepared as previously described (Kim et al., 1992). 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% (v/v) horse serum, 10% (v/v) chick embryo extract, and 1% (v/v) antibiotic/antimycotic solution. One day after cell seeding, the culture medium was changed with the same medium but containing 2% embryo extract. When needed, MG115 or PSI was added to the culture medium at the time of medium change. MG115 and PSI were generously gifted from Dr. Keiji Tanaka. At the appropriate time of culture, the cells were washed three times with ice-cold phosphate-buffered saline (PBS) and immediately fixed using a mixture of 95% ethanol, 40% formaldehyde and acetic acid (20:2:1 by volume). The fixed cells were stained with hematoxylin solution and observed under a microscope. Degree of myoblast fusion was determined as previously described (Kim et al., 1998).

2. Preparation of the proteasome-enriched fraction

The proteasome-enriched fraction from myoblasts was prepared as described (Hedge et al., 1993) with a little modification. Myoblasts cultured in the presence or absence of proteasome inhibitors were washed three times with ice-cold PBS, harvested by centrifugation, and kept frozen at -70℃ until use. The cells were disrupted by sonication for 30 sec in 50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 1 mM dithiothreitol and 0.5 mM ATP. The cell extracts were centrifuged for 30 min at 15,000 x g to remove cell debris and microsomal fraction. Supernatants were collected, and then subjected to ultracentrifugation at 100,000 x g for 6 hr. The proteasome-enriched pellet fraction was resuspended in the same buffer but containing 20% (v/v) glycerol.

3. Assay of peptide hydrolyzing activities of the proteasome

The cleavage of fluorogenic peptide was determined by incubating the reaction mixtures (0.1 ml) containing 10 μg of enzyme sample and 0.1 mM of peptide substrate in a buffer (50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 1 mM dithiothreitol, and 20% glycerol) at 37℃ for 1 hr. The reaction was stopped by adding 0.1 ml of 10% SDS and 0.8 ml of 100 mM Tris-HCl (pH 9.0). The release of fluorophores was then measured as described (Ahn et al., 1991). The used peptide substrates were SLLVY-AMC for chymotrypsin-like activity, Arg-Arg-4-methoxy-β-naphthylamide (ARR-MNA) for trypsin-like activity, and N-carbobenzoxy-Ala-N-carbobenzoxy-Leu-Leu-Glu-β-naphthylam ide (LLE-NA) for peptidylglutamyl peptidase activity. All the fluorogenic peptides were obtained from Sigma.

4. Extraction of cytoskeletal proteins

Cytoskeletal proteins which are insoluble in 0.5% (v/v) Triton X-100 solution were obtained from cultured myoblasts as described (Kwak et al., 1993b). Myoblasts cultured for the appropriate periods were washed three times with PBS, harvested and then kept frozen at -70℃ until use. The cells were disrupted with mild sonication. Proteins were then extracted in 10 mM Tris-HCl buffer, pH 7.8, containing 140 mM NaCl and 0.5% (v/v) Triton X-100 for 30 min at room temperature. After incubation, the mixtures were centrifuged at 15,000 x g for 5 min and the pellets were washed three times with the same buffer. The washed pellets were then resuspended in the same buffer by sonication. The resolved proteins were referred to as the cytoskeletal proteins. Proteins were assayed as described by Bradford (1976).

5. Immunoblot analysis

Creatine kinase and myosin heavy chain were purified from adult chick muscle as described (Eppenberg et al., 1967; Margossian et al., 1973). Antisera against the purified proteins were prepared by injecting into albino rabbits.

Anti-ubiquitin antibody was obtained from Sigma. At the indicated culture time, cells were harvested and disrupted by sonication. The cell extracts were separated by electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate (SDS) (Laemmli, 1970). The proteins in the gels were transferred onto polyvinylidene fluoride (PVDF) membranes and reacted with the specific antisera and then with anti-rabbit IgG conjugated with horseradish peroxidase (Towbin et al., 1979).

RESULTS

To determine if the peptide hydrolyzing activities of the proteasome change in the differentiation of the chick embryonic myoblasts in culture, the proteasome-enriched fractions were prepared from the cells at different differentiation stages and three peptidase activities were assayed. In the early time of culture myoblasts proliferate in the growth medium, then by changing with differentiation medium, cells start to fuse each other to form myotubes. Myoblast fusion vigorously occurs from 24 hr to 48 hr of culture. As shown in Fig. 1, the hydrolyzing activity against SLLVY-AMC increased with culture time and reached a maximal level at 48 hr of culture. However, other two peptide hydrolyzing activities were slightly changed or little detected under the same reaction conditions. By the immunoblot analysis against anti-proteasome antibody, however, we could not find significant differences in the amount of the enzyme or in the subunit pattern of proteasome (data not shown).

We then examined the effect of MG115 or PSI, the inhibitors of proteasome, on peptide hydrolyzing activities of the proteasome *in vitro*. The agents were added in the proteasome-enriched fraction with increasing concentrations and the peptide hydrolyzing activities were assayed. Both inhibitors specifically inhibited the SLLVY-AMC hydrolyzing activity than ARR-MNA in a dose-dependent manner

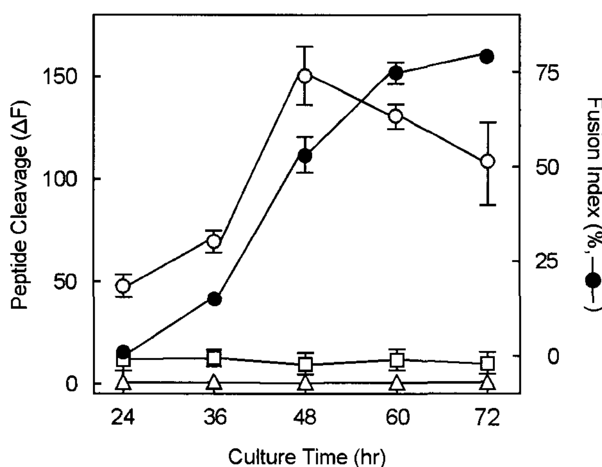

Fig. 1. Peptide hydrolyzing activities of the proteasome in the course of differentiation of chick embryonic myoblasts in culture. At the indicated culture time, cells were harvested and the proteasome-enriched fractions were obtained as described in "Materials and Methods". Then the hydrolyzing activities against the three peptides, SLLVY-AMC (○-○), ARR-MNA (□-□), and LLE-NA (△-△), were assayed. For the assay of fusion (●-●), cells were fixed, stained in a hematoxylin solution and observed under a microscope.

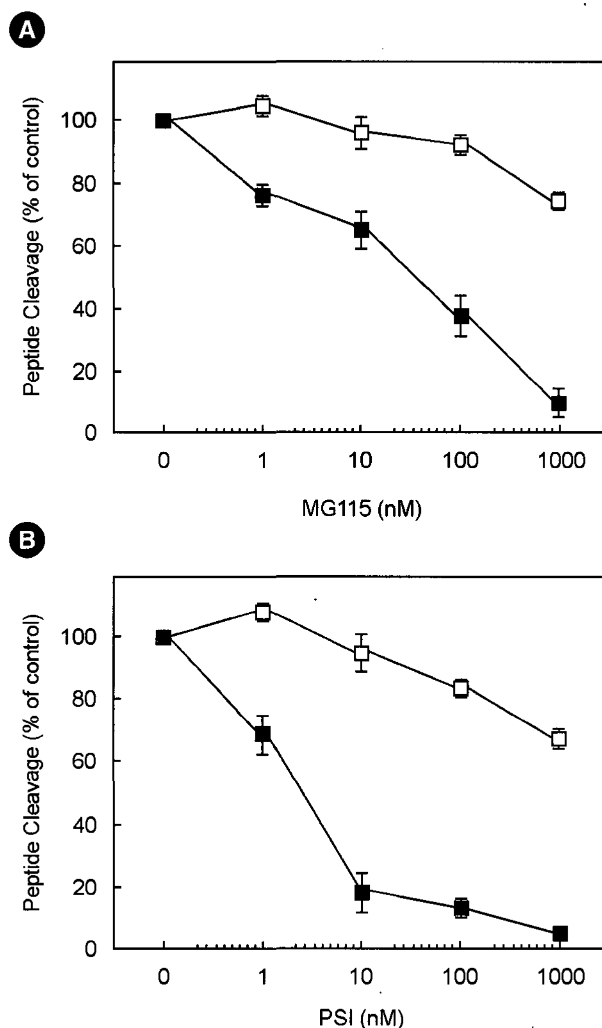

Fig. 2. Inhibition of peptide hydrolyzing activity of the proteasome in the MG115- or PSI-treated cells *in vitro*. The proteasome-enriched fractions were obtained from 72 hr-cultured myoblasts. MG115 **(A)** or PSI **(B)** were added to the fractions with increasing concentrations and SLLVY-AMC (■-■) or ARR-MNA (□-□) hydrolyzing activities were assayed.

(Fig. 2). Inhibiting activity of PSI is more potent than that of MG115; the half maximal inhibition of MG115 obtained at concentration of 30~40 nM and that of PSI obtained at 2~3 nM under the same conditions.

For further study of a role of the proteasome on myoblast fusion, the proteasome inhibitors were added in the culture medium at 24 hr of culture and cultured for the next 48 hr. Under normal conditions, myoblasts changed to myotubes by spontaneous membrane fusion at 72 hr (Fig. 3A). At the same culture time, however, myoblast fusion was dramatically inhibited by the addition of MG115 or PSI (Fig. 3B and 3C). The inhibitory activities of the agents are powerful; 15 nM of PSI or 100 nM of MG115 are enough for complete inhibition of cell fusion. The cells are bipolar as much like as the myoblasts just before cell fusion, but they do not fuse each other. The fusion inhibition by the

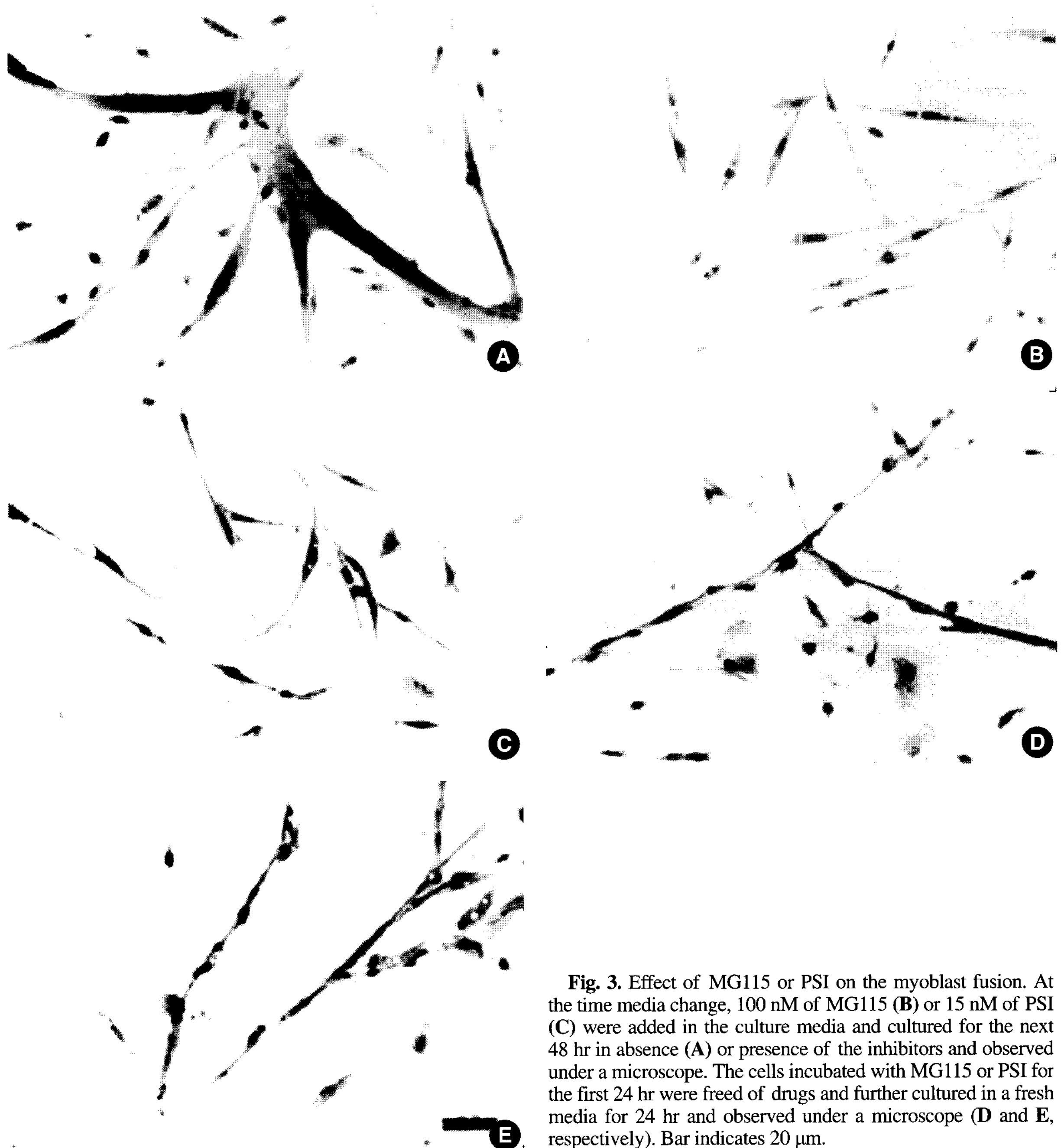

Fig. 3. Effect of MG115 or PSI on the myoblast fusion. At the time media change, 100 nM of MG115 (**B**) or 15 nM of PSI (**C**) were added in the culture media and cultured for the next 48 hr in absence (**A**) or presence of the inhibitors and observed under a microscope. The cells incubated with MG115 or PSI for the first 24 hr were freed of drugs and further cultured in a fresh media for 24 hr and observed under a microscope (**D** and **E**, respectively). Bar indicates 20 μm.

agents was reversible and myoblast fusion resumed with withdrawal of the agents from the culture medium (Fig. 3D and 3E).

We then examined if the inhibitors affect the SLLVY-AMC hydrolyzing activity *in vivo* and if the inhibition is related with the fusion inhibition. As shown in Fig 4, the peptidase activity of the proteasome from the inhibitor-treated cells decreased with the increasing concentration of the agents. As with *in vitro* assay, PSI was more potent than MG115. Half-maximal inhibition by MG115 or PSI is obtained around 90~100 nM or 7~8 nM, respectively. These concentrations are about 3~5 times higher than those obtained from the assay *in vitro* (see Fig. 2). This difference

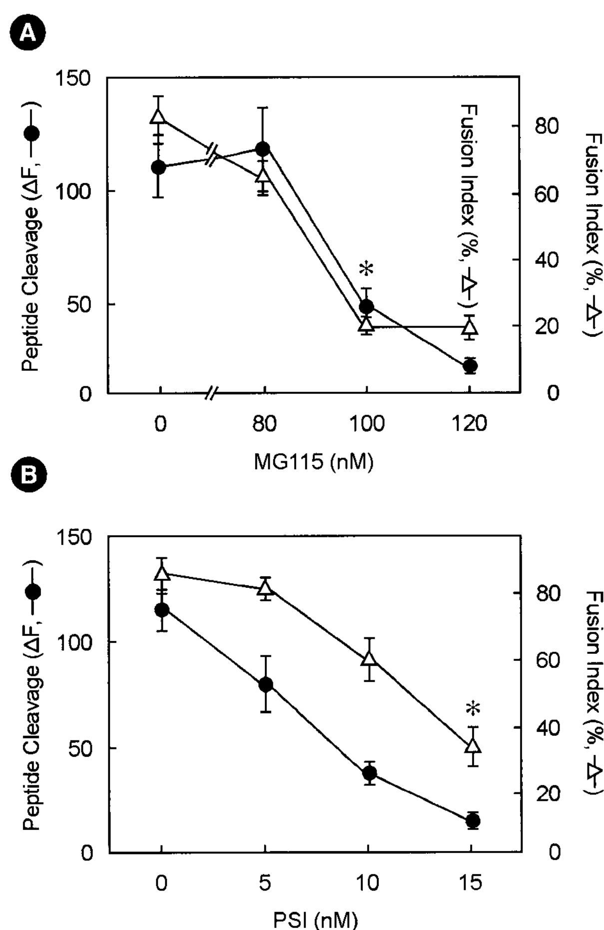

Fig. 4. Effects of MG115 or PSI on the SLLVY-AMC hydrolyzing activity and on the myoblast fusion. At 24 hr of culture, MG115 (**A**) or PSI (**B**) were added in the culture media with increasing concentrations and cultured for the next 48 hr. Cells were then harvested and assayed for their SLLVY-AMC hydrolyzing activities. Fusion index were also determined as described previously. Myoblasts incubated with the same agents for the first 24 hr were freed of them and cultured for the next 24 hr and assayed for their SLLVY-AMC hydrolyzing activities (*)

of efficiency might be due to limited cell membrane permeability and/or the potential reactivity of the peptides' aldehyde group (Traenckner et al., 1994). In addition, the inhibitors blocked fusion of myoblasts in a dose-dependent manner. At higher concentrations, cells detached from substrate and their viability was largely impaired (data not shown). Of interesting is that decrease in the degree of myoblast fusion occurred in parallel with the fall in the SLLVY-AMC hydrolyzing activity of proteasome. Furthermore, as described above, the inhibitory activities of the agents are reversible and the fusion resumption by the deprivation of inhibitors from culture media is concurrent with restoration of peptidase activity (* in Fig. 4). These results clearly demonstrate that the SLLVY-AMC hydrolyzing activity of proteasome may be associated with myoblast fusion.

During the myogenic differentiation, myoblasts can synthesize and accumulate muscle specific proteins such as creatine kinase, acetylcholine receptor, α-actin, myosin heavy chain, tropomyosin, etc. (Paterson et al., 1972; Turner et al., 1974; Shainberg et al., 1978). We examined if the proteasome inhibitors influence on the synthesis of muscle specific proteins. The immunoblot analyses against anti-creatine kinase or anti-myosin heavy chain antibodies have shown that they also block the accumulation of those pro-

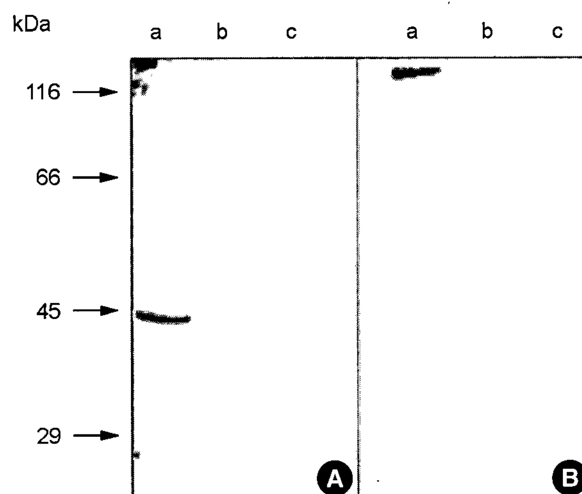

Fig. 5. Effect of MG115 or PSI on the synthesis of creatine kinase and myosin heavy chain. At 24 hr of culture, MG115 (lane b) or PSI (lane c) were added in the culture media and further cultured in the absence (lane a) or presence of the inhibitors for the next 48 hr. Then cells were harvested, subjected to electrophoresis, and stained with anti-creatine kinase antibody (**A**) or anti-myosin heavy chain antibody (**B**).

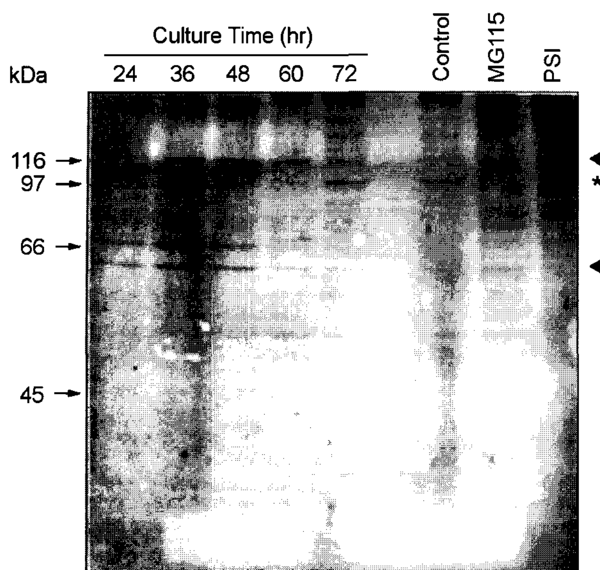

Fig. 6. Immunoblot analysis of the ubiquitinylated proteins. Myoblasts were harvested at the indicated culture time and cytoskeletal proteins were obtained as described in "Materials and Methods". The proteins were subjected to immunoblot analysis against anti-ubiquitin antibody. Control indicates control 72 hr cell extract; MG115, MG115-treated 72 hr cell extract; PSI, PSI-treated 72 hr cell extract. The arrowheads indicate 110-kDa and 60-kDa proteins and * indicates 97-kDa protein.

teins (Fig. 5). These results suggest that the inhibitors of proteasome block both morphological differentiation and biochemical differentiation of myoblasts.

Myoblast fusion process is known to be related with the reorganization of membraneous and/or cytoskeletal proteins and the activity of proteolysis may be involved in these process(es). Therefore, we investigated the ubiquitinylated proteins in cytoskeletal fraction of the differentiating myoblasts. As shown in Fig. 6, the pattern of immunoreactive proteins against anti-ubiquitin antibody was changed in the culture time. In particular, the intensity of 110- and 60-kDa proteins decreased with the culture time and they almost disappeared at 72 hr, while 97-kDa protein was detected only in 72 hr-myotube stage. We further examined whether the proteasome inhibitors affect the degradation or accumulation of ubiquitin-conjugated proteins. The agents caused accumulation of the high molecular weight ubiquitinylated proteins in the cytoskeletal fractions compared to the untreated control cell extracts. It is noteworthy that 110- and 60-kDa proteins, which disappeared in the fused myotubes, remained in the agents-treated cell extracts and 97-kDa protein was disappeared with the agents. At present time, it is uncertain whether the accumulation of ubiquitinylated proteins directly related with fusion inhibition by the proteasome inhibitors. However, when the myoblasts fusion has resumed with deprivation of the agents, the most of the ubiquitinylated proteins disappeared (data not shown).

DISCUSSION

In this study, we demonstrate that each of the peptidase activities of the proteasome is regulated in different and independent manners in the course of differentiation of chick embryonic myoblasts in culture. In particular, the SLLVY-AMC hydrolyzing activity was prominent and increased with myoblast fusion. The substrate SLLVY-AMC was used for assay of the chymotrypsin-like activity of proteasome (Figueiredo-Pereira et al., 1994). Of interest is the finding that the inhibition of SLLVY-AMC hydrolyzing activity leaded to impairment of myoblast fusion and caused accumulation of the ubiquitinylated proteins in cytoskeletal fractions. In the previous report that suggested exposure of HT4 cells, a mouse neuronal cell line, to PSI caused accumulation of ubiquitinylated proteins, the agent did not alter protein content or cell viability at concentration of 5 μM (Figueiredo-Pereira et al., 1994), which is about 50~100 times higher concentration than that we used in this study. Moreover, there was not any notice that the incubation of HeLa cells with 60 μM PSI affected cells' physiology (Traenckner et al., 1994). In this study, however, we have found that submicromolar concentration of the proteasome inhibitors can alter both morphological and biochemical differentiation of myoblasts. These different sensitivity to the agents suggest that proteolysis by proteasome in the differentiation of myoblasts is one of the important regulatory processes.

In the course of differentiation, myoblasts dramatically change their morphology to form fused myotubes. This means that considerable reorganization of membraneous and/or cytoskeletal proteins occurred during that process. Protein degradation by proteasome is supposed to need in that process. Therefore, it is suggested that the accumulation of ubiquitinylated proteins in the cytoskeletal fractions by the agents may impair the membrane fusion of myoblasts. even though we could not show the identity of the ubiquitinylated proteins in the agents-treated cells. The possible involvement of the ubiquitin system in the degrada-

tion of specific cellular proteins has been suggested in many cases including the activation of NF-κB (Lee, et al., 1993; Baeuerle et al., 1994; Palombella et al., 1994; Traenckner et al., 1994). Both MG115 and PSI inhibited accumulation of creatine kinase and myosin heavy chain, which are muscle specific proteins. These results suggest the proteasome may influence on the expression of certain gene(s) that is important for the regulation of differentiation state as well as on morphological reorganization of myoblasts at protein level. In this study, we demonstrate that proteolysis by the proteasome may play an essential role in myogenic differentiation. However, the cellular target(s) of proteasome by ubiquitin system is not uncertain at this time. More studies are needed to elucidate the cellular substrate(s) of proteasome in the regulation of myoblast differentiation and the identity of the ubiquitinylated proteins in this study.

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