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Developmental Changes of Proteolytic Activities of 26S Protease Complex and 20S Proteasome in Chick Embryonic Muscle

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The multicatalytic 20S proteasome consisting of 12-15 subunits of 22-35 kDa is the catalytic core of the ATP/ubiquitin-dependent 26S protease complex that also is comprised of multiple subunits of 22-110 kDa. In order to determine whether the proteolytic activities change during muscle development, the enzyme preparations were obtained from 11-, 14- and 17-day old chick embryonic muscle using a BioGel A-1.5m column. The 26S complex preparation from 14- or 17-day old muscle hydrolyzed both N-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Suc-LLVY-AMC) and ubiquitin-lysozyme conjugates about 50% as well as that from 11-day old muscle. In addition, the activity of 20S proteasome against Suc-LLVY-AMC also decreased by about 20-30%. However, the protein level of 26S complex remained constant during the entire development period, while that of 20S proteasome increased 5- to 6-fold, as analyzed by nondenaturing polyacrylamide gel electrophoresis followed by immunoblot analysis using the antibodies raised against the purified enzymes. Thus, the specific activity of 20S proteasome against the peptide must decrease rather dramatically during the muscle development. These results suggest that the development-dependent changes in the proteolytic activities of both 20S proteasome and 26S protease complex from embryonic muscle are due to alterations in the expression of certain subunits in the enzymes that are responsible for their specific catalytic functions but not to overall changes in the enzyme amounts.

KEY WORDS: 26S Protease Complex, 20S Proteasome, Muscle Development

The degradation of most proteins in eukaryotic cells requires a constant supply of metabolic energy (Goldberg and St. John, 1976; Hershko and Ciechanover, 1982). A major energy-dependent system for protein breakdown is the ubiquitin pathway, and this system involves in selective and rapid degradation of highly abnormal proteins and certain short-lived regulatory proteins (Rechsteiner, 1991; Glotzer *et al.*, 1991; Nishizawa *et al.*, 1992). Proteins to be degraded by this pathway are ligated through their lysine amino groups to the polypeptide ubiquitin. This modification targets them for proteolysis by an ATP-dependent enzyme system that is specific for ubiquitin-conjugated proteins. ATP is thus required

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for both ubiquitin-protein ligation as well as for conjugate breakdown.

An ATP-dependent protease responsible for the degradation of ubiquitinated proteins was first described by Hough *et al.* (1986). Purification of this enzyme from rabbit reticulocytes and chick skeletal muscle revealed that it has an apparent size of about 1,300 kDa and a sedimentation coefficient of 26S (Hough *et al.*, 1987; Waxman *et al.*, 1987; Lee *et al.*, 1993). This 26S protease complex contains a characteristic set of 10-20 polypeptides (40-110 kDa) and probably all the subunits (20-32 kDa) of 20S proteasome. Both 26S protease complex and 20S proteasome exhibit at least three distinct endopeptidase activities, cleaving bonds on the carboxyl side of hydrophobic, acidic and basic amino acid residues (Rivett, 1989; Lee *et al.*, 1993), although 20S proteasome, but not 26S complex, also has a latent proteolytic activity that can be activated by poly-L-lysine or fatty acids (Dahlmann *et al.*, 1985; Tanaka *et al.*, 1986). Furthermore, anti-20S proteasome antibody can precipitate the proteolytic activities of 26S complex (Matthews *et al.*, 1989; Lee *et al.*, 1993). Therefore, 20S proteasome has now been recognized as the proteolytic core of 26S protease complex.

Recently, 20S proteasome consisting of multiple subunits has been shown to undergo functional and structural change during the course of the development of tissues and organisms. In developing *Drosophila* and chick embryonic muscle, the subunit patterns of 20S proteasome are changed and these alterations have been suggested to be in part due to post-translational modification, such as protein phosphorylation (Haass and Kloetzel, 1989; Ahn *et al.*, 1991). The subunit pattern of chicken 20S proteasome also differs from one tissue to the other and the expression of a number of its subunits changes during development of the tissues (Hong *et al.*, 1994). In addition, it has been shown that cell-specific accumulation of 20S proteasome occurs during the embryogenesis of *Drosophila* (Klein *et al.*, 1990). These studies imply that the proteolytic activities of 20S proteasome and perhaps with those of 26S complex may be under developmental control and involved in the process(es) related to embryonic development, such as nuclear functions and morphogenic events.

In an attempt to elucidate the roles of 20S proteasome and 26S protease complex in muscle development, we investigated the changes in the proteolytic activities of the enzyme complexes and their overall protein levels in variously aged muscle tissues of chick embryo.

Materials and Methods

Materials

26S protease complex and 20S proteasome were purified to apparent homogeneity from chick skeletal muscle as described previously (Ahn *et al.*, 1991; Lee *et al.*, 1993). Antisera against the enzymes were prepared by injecting each of the purified proteins into albino rabbits. IgGs were isolated from the antisera by sodium sulfate fractionation (Johnstone and Thrope, 1982). ^{125}I-Protein A and Na^{125}I were purchased from Amersham, N-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Suc-LLVY-AMC) and other peptide substrates from Peptide Institute (Osaka, Japan), and BioGel A-1.5m from BioRad. All other chemicals were obtained from Sigma unless otherwise indicated.

Preparation of embryonic muscle extracts

Embryonic muscle tissues (5 g each) were excised out from 11-, 14- and 17-day old chick embryos. They were minced and homogenized using a Polytron in 15 ml of buffer-A [25 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT and 2 mM ATP] containing 0.25 M sucrose. The homogenized samples were centrifuged at 70,000 $\times$ g for 1 h and the resulting supernatants were referred to as embryonic muscle extracts.

Assays for proteolysis

The cleavage of fluorogenic peptides was determined by incubating reaction mixtures (0.1 ml) containing appropriate amounts of enzyme samples, 25 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.5 mM DTT, 1 mM ATP, and 0.1 mM peptide substrate with and without 0.02% SDS at 37°C

for 30-60 min. The reaction was then stopped by adding 100 μl of 1% SDS and 800 μl of 0.1 M Na-borate (pH 9.1), and release of fluorophores was measured as described (Ahn *et al.*, 1991). Hydrolysis of ^{125}I-labeled-ubiquitin-lysozyme conjugates was assayed as described (Hough *et al.*, 1986; Kanayama *et al.*, 1992). Protein concentration was measured as described by Bradford (1976) with bovine serum albumin as a standard.

Immunoblot analysis

Polyacrylamide gel electrophoresis under nondenaturing conditions was performed using 4.5% (w/v) slab gels (Hough *et al.*, 1987). After electrophoresis, proteins were electro-transferred onto nitrocellulose filters and reacted with anti-IgGs raised against the purified 26S protease complex and 20S proteasome. The filters were then reacted with ^{125}I-protein-A, washed, dried, and exposed to X-ray films (Fuji).

Results

In order to obtain both 26S protease complex and 20S proteasome, the muscle extracts prepared from 11-, 14- and 17-day old chick embryos were centrifuged for 12 hr at 100,000 × g. The precipitate was suspended in 6 ml of buffer-A containing 20% glycerol. The suspension was loaded onto a BioGel A-1.5m column (1.5 × 45 cm) equilibrated with the same buffer, and proteins were eluted at a flow rate of 15 ml/hr. Elutions of 26S complex and 20S proteasome were monitored by assaying their abilities to cleave the peptide substrate, Suc-LLVY-AMC. As shown in Fig 1A, two peaks of the peptide-hydrolyzing activity were evident. Of these, the first peak that eluted in the fractions corresponding to about 1,300 kDa also hydrolyzed ubiquitin-lysozyme conjugates to acid-soluble products in the presence of ATP and Mg^{2+} (Table 1). Thus, it appears clear that 26S protease complex is responsible for the peptidase as well as the ubiquitin-conjugate degrading activity of the first peak.

When the peptide-degrading activities in Fig. 1A were again assayed in the presence of SDS, both the activity peaks were completely abolished (Fig. 1B). In contrast, a new SDS-activatable peptidase peak was evident in the fractions corresponding to 750 kDa. These results clearly indicate that 20S proteasome is responsible for the SDS-activated activities. The second peak of the peptidase activity in Fig. 1A had an apparent molecular mass of less than 100 kDa as analyzed by gel filtration on a Sephacryl S-300 column (data not shown), and therefore must differ from any of 20S proteasome and 26S complex.

Of interest was the finding that the peptidase and ubiquitin-conjugate degrading activities of 26S

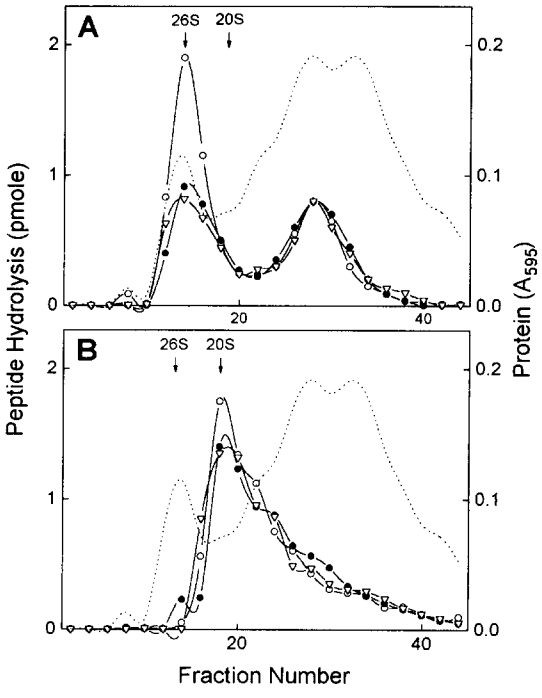

Fig. 1. Separation of 26S protease complex and 20S proteasome in muscle extracts from variously aged chick embryos by a BioGel A-1.5m column. The extracts were prepared as described in Materials and Methods. The same amounts of the extracts (200 mg each) obtained from 11- (○), 14- (●) and 17-day old muscle (▽) were loaded on a BioGel A-1.5m column (1.5 × 45 cm) equilibrated with buffer-A containing 20% glycerol. Fractions of 1.5 ml were collected at a flow rate of 15 ml/h. Aliquots (30 μl) of the fractions were assayed for the hydrolysis of Suc-LLVY-AMC in the absence (A) and presence of 0.02% SDS (B). Elution of proteins was indicated by dotted lines.

Table 1. ATP-dependent hydrolysis of ^{125}I-ubiquitin-lysozyme conjugates by 26S protease complex from variously aged chick embryonic muscle.

Embryonic days	% Hydrolysis	% Relative activity
11	13.6	100
14	5.7	42
17	6.2	46

The fractions containing 26S protease complex from the BioGel column (see Fig. 1A) were pooled and assayed for the ATP-dependent hydrolysis of ^{125}I-ubiquitin-lysozyme conjugates in the presence and absence of 5 mM $MgCl_2$ as described (Kanayama *et al.*, 1992). Reaction mixtures (0.1 ml, final volume) contained 5 μg of the 26S enzyme preparations, 2 mM ATP, 1 mM DTT and 50 mM Tris-HCl (pH 8.5). Incubations were performed at 37°C for 2 h, and radioactivities released into acid-soluble products were measured using a scintillation counter (Packard). The activity of 26S complex was then determined by subtracting the conjugate protein hydrolyzed in the absence of $MgCl_2$ from that seen in its presence.

complex from the 14- or 17-day old embryonic muscle were about 50% lower than those from 11-day old muscle (see Fig. 1A and Table 1). The SDS-activated peptidase activity of 20S proteasome also decreased although not to a significant extent (see Fig. 1B). To determine whether the activity levels of 26S complex and 20S proteasome correlate with their protein levels, the fractions under the respective peaks were pooled and the same aliquots of them were subjected to gel electrophoresis under a nondenaturing condition followed by immunoblot analysis using the anti-IgGs raised against each of the purified enzymes. As shown in Fig. 2A, the protein level of 26S complex was more-or-less similar regardless to the age of the embryonic muscle. Therefore, it appears that the decreases in the peptidase and ubiquitin-conjugate degrading activities of 26S protease complex are rather due to alterations in its subunit pattern, such as a decrease in the expression of certain specific subunits that are responsible for the proteolytic activities, but not to changes in overall expression of the enzyme complex.

In contrast, expression of 20S proteasome was found to dramatically increase as compared the

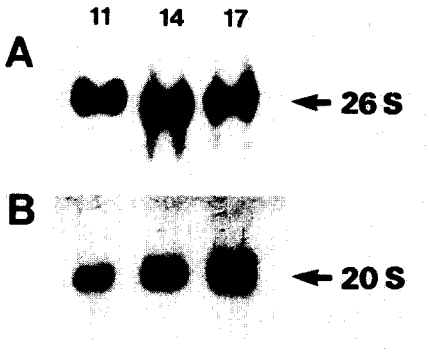

Fig. 2. Immunoblot analysis of 26S protease complex (A) and 20S proteasome (B) in the pooled BioGel fractions. The same aliquots (50 μg each) of the samples were subjected to polyacrylamide gel electrophoresis under a nondenaturing condition followed by immunoblot analysis as described in Materials and Methods. The numerals indicate the embryonic days.

enzyme levels in the developing muscle tissues (Fig. 2B). However, the increase in the enzyme level was not at all correlated with relatively insignificant changes in the peptidase activity, suggesting that the specific activity of 20S proteasome against Suc-LLVY-AMC should have decreased during the development of embryonic muscle. In order to quantitate more precisely the changes in the specific activity of 20S proteasome, the same aliquots of the enzyme fractions were again assayed for peptide hydrolysis. The resulting activities were then denominated by the amounts of 20S proteasome that were deduced by scanning the bands shown in Fig. 2B using a densitometer. Table 2 clearly shows that the specific activity of 20S proteasome markedly decreases during the developmental period (*i.e.*, at least from day 11 to day 17). Similar results were obtained for the other peptidase activities of 20S proteasome, such as those against N-carbobenzoxy (Cbz)-Ala-Arg-Arg-4-methoxy-β-naphthylamide (NA) and Cbz-Leu-Leu-Glu-NA (data not shown). These results suggest that expressions of 20S proteasome subunits responsible for the cleavage of three different peptides are strongly down-regulated during the development period.

Table 2. Hydrolysis of Suc-LLVY-AMC by 20S proteasome from variously aged chick embryonic muscle.

Embryonic days	Peptide hydrolysis (nmol)	Relative amounts of 20S proteasome	Specific activity of 20S proteasome
11	55.3	1.0	55.3
14	41.1	3.2	12.8
17	38.4	5.9	6.5

The fractions containing 20S proteasome from the BioGel column (see Fig. 1B) were pooled and assayed for the peptide hydrolysis. The reaction mixtures (0.1 ml) containing 5 μg of the 20S enzyme preparations, 0.1 mM Suc-LLVY-AMC, 25 mM Tris-HCl (pH 8), 1 mM ATP, 5 mM $MgCl_2$, 0.5 mM DTT and 0.02% SDS were incubated at 37°C for 30 min. Release of the fluorophores was then determined as described in Materials and Methods. The relative amount of 20S proteasome in the pooled BioGel fractions was determined by scanning the bands shown in Fig. 2B using a densitometer, and that from 11-day old muscle was expressed as 1.0 and the others as its relative values. The specific activity of 20S proteasome was calculated as the ratio of the amount of peptide hydrolyzed to the relative amount of the enzyme.

Discussion

The present studies demonstrate that the peptidase activities of both 26S protease complex and 20S proteasome and the ubiquitin-conjugate degrading activity of 26S complex decrease during the development of chick embryonic muscle. Because the protein level of 20S proteasome is rather increasing quite dramatically while that of 26S complex remains constant during the development period, it appears that the changes in the proteolytic activities of both the enzyme complexes are due to alterations in the expression of their specific subunits that are responsible for the respective activities. However, the relationship of the decrease in the proteolytic activities with the development of embryonic muscle remains totally unknown, particularly because the major event in muscle development accompanies the differentiation of mononucleated myoblasts into multinucleated myotubes (Bischoff and Holtzer, 1969; O'Neill and Stockdale, 1972), which process is known to require massive proteolytic function for mobilization and rearrangement of cytoskeletons and membrane proteins (Pauw and David, 1979; Fulton *et al.*, 1981; Kwak *et al.*, 1993).

Noteworth is the finding that the protein level of 26S protease complex remains unchanged while that of 20S proteasome increases by about 6-fold when compared the enzyme amount in 11-day embryonic muscle to that in 17-day old muscle. These findings suggest that the protein level of 20S proteasome in developing muscle is in much excess over that of 26S complex and hence appears to be unusual if 20S proteasome solely acts as the catalytic core of 26S complex. Therefore, the excess, residual 20S proteasome, that are not assembled into 26S complex, is likely to exert its unknown function(s) during the muscle development. One additional catalytic function of 20S proteasome, that is not shared with 26S complex, is the latent protein-degrading activity that can be dramatically activated by low concentrations of SDS or long chain fatty acids (Dahlmann *et al.*, 1985; Tanaka *et al.*, 1986). In addition, we have previously shown that, unlike its decreased peptidase activity found in the present studies, the SDS-activated casein hydrolysis by 20S proteasome increases several-fold during the muscle development (Ahn *et al.*, 1991). These results are clearly in accord with the present demonstration that the protein level of 20S proteasome increases to similar extent during the same period. Perhaps this latent activity of 20S proteasome may play an important role in myoblast differentiation, while the other activities of both 20S proteasome and 26S protease complex may not be directly involved in the myogenic process.

Nevertheless, it remains still possible that the peptidase activities of both 26S complex and 20S proteasome and the ATP-dependent, ubiquitin-

conjugate degrading activity of 26S complex may involve in certain earlier step for muscle development, such as by cleaving critical regulatory proteins with tight specificity, and therefore may be unnecessary at the later developmental periods. The regulatory factors, such as MyoD, myogenin and Myf-5, are known to play critical roles in myogenic differentiation of embryonic muscle cells (Weintraub, 1993) and to have short half-lives (Thayer *et al.*, 1989). Therefore, it would be interesting to determine whether these myogenic factors are sensitive to cleavage by 26S protease complex and/or 20S proteasome for possible regulation of muscle cell differentiation, particularly at the early step of muscle development. This possibility is presently under investigation.

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계배 근조직 발달과정에서의 26S 단백질 분해효소 복합체 및 20S proteasome의 단백질 분해활성의 변화

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22-35 kDa 크기의 12-15개 단위체로 구성된 20S proteasome은 22-110 kDa 크기의 다수의 단위체로 구성된 ATP/ubiquitin-의존성 26S 단백질 분해효소 복합체의 catalytic core이다. 이 두 효소의 근조직 발달동안의 단백질 분해활성 변화양상을 조사하기 위해서, BioGel A-1.5m column을 이용하여 각각 11, 14, 17일된 계배 골격근으로부터 이 효소들의 분획을 얻었다. 그 결과, 14, 17일된 근육에서 얻은 26S 복합체 분획은 11일된 근육에서 얻은 분획에서보다 Suc-LLVY-AMC와 ubiquitin-lysozyme conjugate에 대한 분해활성이 50% 정도 감소하였다. 또한, Suc-LLVY-AMC에 대한 20S proteasome의 분해활성도 20-30% 정도 감소하였다. 그러나, 동일한 분획들을 비변성 전기영동을 한 후, 두 효소에 대한 항체를 사용하여 immunoblot 분석을 한 결과, 26S 복합체의 단백질의 경우는 근조직 발달과정동안 일정한 수준으로 유지되는 반면에 20S proteasome은 5-6배 증가하였다. 따라서, Suc-LLVY-AMC에 대한 20S proteasome의 specific activity는 근조직 발달과정동안 현저히 감소함을 알수 있었다. 이러한 결과는, 계배 골격근 발달과정동안에 나타나는 20S proteasome과 26S 단백질 분해효소 복합체 분해활성 변화가 효소의 전체적인 양적 변화에서 보다는 특정한 효소활성기능을 담당하는 단위체들의 발현양상의 변화에서 기인된 것임을 추측케 하였다.