Developmental Regulation of Proteolytic Activities and Subunit Pattern of 20 S Proteasome in Chick Embryonic Muscle*

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Joon Young Ahn†, Seung Oh Hong†, Kyu Bong Kwak†, Shin Sung Kang†, Keiji Tanaka†, Akira Ichihara†, Doo Bong Ha†, and Chin Ha Chung‡

From the †Department of Molecular Biology and SRC for Cell Differentiation, College of Natural Sciences, Seoul National University, Seoul 151-742, Korea, the †Department of Biology, Kyungpook National University, Taegu 702-701, Korea, and the ‡Institute for Enzyme Research, Tokushima University, Tokushima 770, Japan

The proteolytic activities of the 20 S proteasome were found to change in their levels during the development of chick embryonic muscle. The peptide-cleaving activities against N-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin and N-benzoyloxycarbonyl-Ala-Arg-Arg-4-methoxy-2-naphthylamide gradually decreased with the time of development. On the other hand, the casein-degrading activity in the presence of poly-L-lysine markedly increased from embryonic day 11 and reached a maximal level by day 17. These changes appeared to be tissue-specific because little or no change in any of the proteolytic activities was observed with developing embryonic brain, while dramatic alterations occurred in the extents of the peptide hydrolyses in liver. Furthermore, a number, but not all, of the proteasome subunits in embryonic muscle were changed in their amounts during the development. These results suggest that the alterations in the proteasome activities and subunit pattern are developmentally regulated and may be correlated.

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; Orlowksi, 1990). This complex exhibits at least three distinct endopeptidase activities, cleaving bonds on the carboxyl side of hydrophobic, acidic, and basic amino acid residues (Rivett, 1989). In addition, it shows a latent proteolytic activity that can be activated by sodium sulfate fractionation. The purified anti-proteasome antibody was covalently attached to CNBr-activated Sepharose 4B and used to immunoprecipitate the proteasome from chick embryonic muscle. IgGs were isolated from the antisera and used to immunoprecipitate the proteasome from chick embryonic muscle. The assays were then performed as described (Seol et al., 1989). Antisera against proteasome and creatine kinase were prepared by injecting the purified proteins into albino rabbits. IgGs were isolated from the antisera by sodium sulfate fractionation.

Of additional importance is that the multifunctional proteasome can undergo functional and structural changes during the course of development of tissues and organisms. A recent report has shown that in developing Drosophila, the proteasome undergoes changes in its subunit pattern, and post-translational modification is at least in part responsible for the changes (Haass and Kloetzel, 1989). It has also been shown that cell-specific accumulation of the complex occurs during the embryogenesis of Drosophila (Klein et al., 1990). These studies imply that the multicatalytic activities of proteasome may be under developmental control and involved in the processes related to embryonic development, such as nuclear functions and morphogenetic events (Klein et al., 1990).

An attempt to elucidate the role of the proteasome in muscle development, we investigated the changes in the level of proteolytic activities of the enzyme complex in variously aged muscle tissues of chick embryo. We also examined whether the proteasome undergoes changes in its subunit pattern during muscle development. In the present studies, we demonstrated that the proteolytic activities of proteasome are under developmental control. We also showed that the enzyme complex undergoes development-specific alterations in its subunit pattern.

EXPERIMENTAL PROCEDURES

Materials—Proteasome and creatine kinase were purified from adult chick skeletal muscle to apparent homogeneity as described (Tanaka et al., 1986; Eppenberger et al., 1967). Antisera against proteasome and creatine kinase were prepared by injecting the purified proteins into albino rabbits. IgGs were isolated from the antisera by sodium sulfate fractionation. The purified anti-proteasome antibody was covalently attached to CNBr-activated Sepharose CL-4B (Axen et al., 1967). [3H]Casein was prepared as described by Jenoff and Dearborn (1979). [3H]Formaldehyde and [125I]protein A were purchased from Du Pont-New England Nuclear. The synthetic peptide substrates of proteasome were obtained from Peptide Institute Inc., Japan. All other reagents were purchased from Sigma.

Preparation of Embryonic Muscle Extracts—Extracts of chick embryonic breast muscle, brain, and liver were prepared from 8-, 11-, 14-, and 17- and 20-day-old chick embryos. The tissues were washed and homogenized in 50 mM Tris-HCl buffer (pH 7.8) containing 1 mM dithiothreitol, 0.1 mM EDTA, and 20% (v/v) glycerol. The homogenates were then centrifuged for 1 h at 12,000 × g, and the supernatants were dialyzed against the same buffer and kept frozen at -70 °C until use.

Assays—Protein breakdown was measured using [3H]casein as the substrate in the presence and absence of 0.1 mg/ml poly-L-lysine. Reaction mixtures (0.1 ml) contained 100 μg of the muscle extracts, 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, and 10μg of [3H] casein (2 × 10^6 cpm). The assays were then performed as described (Seol et al., 1989). The cleavage of fluorogenic peptides was determined in similar reaction mixtures, except that they contained 20 μg of the muscle extracts and 0.1 mM peptide substrates. The release of fluorophores was then measured as described (Seol et al., 1989).
Proteins were assayed as described by Bradford (1976) using bovine albumin as a standard.

**Immunopurification of Proteasomes**—Each extract (180 mg) of 11- and 17-day-old embryonic muscle was loaded on a DEAE-cellulose column (1 × 3 cm) equilibrated with 50 mM Tris-Cl (pH 7.8) buffer containing 1 mM diithiothreitol, 0.1 mM EDTA, and 20% (v/v) glycerol. Proteins bound to the column were eluted with the buffer containing 0.3 M NaCl. The eluate was then applied to an anti-proteasome immunopurification column (1 × 3 cm). After washing the column extensively with the elution buffer, the proteasome was released with 0.1 M glycine HCl (pH 2.3) at a flow rate of 10 ml/h, neutralized to pH 7 with 1 M Tris, and concentrated using Centricon (Amicon Corp.).

**Electrophoretic Analysis**—Polyacrylamide gel electrophoresis was carried out in 12.5% (w/v) slab gels containing sodium dodecyl sulfate (SDS) (Laemmli, 1970). Two-dimensional gel electrophoresis was performed by following the method of O'Farrell (1975) with slight modification. In the first dimension, the affinity-purified proteasome was separated by isoelectric focusing on tube gels (0.2 × 10 cm) containing 8 M urea and Ampholine producing a pH gradient of 4–9. The resulting gels were equilibrated in 2% SDS and subjected to gel electrophoresis in 15% slab gel as above. Proteins were visualized by silver staining (Merrill et al., 1981).

Proteins in muscle extracts that had been separated by the electrophoresis in 10% slab gels were transferred onto nitrocellulose membranes. The membranes were incubated with anti-creatinine kinase, anti-IgG and then with 125I-protein A, dried, and exposed to x-ray films (Towbin et al., 1979).

**RESULTS**

**Developmental Regulation of Proteasome Activities**—To examine whether the proteolytic activities of the proteasome are under developmental control, muscle extracts were prepared from variously aged chick embryos and assayed for their ability to cleave Suc-Leu-Leu-Val-Tyr-AMC, Cbz-Ala-Arg-Arg-MNA, and [3H]casein. As shown in Fig. 1, the extents of the peptide hydrolyses gradually declined at later developmental stages. On the other hand, the casein hydrolysis in the presence of poly-L-lysine markedly increased, while that in its absence remained unchanged during the development (Fig. 2). We then tested whether the proteasome is indeed responsible for the peptide hydrolyses and poly-L-lysine-activated casein hydrolyses. The enzyme complex was precipitated by incubating the same muscle extracts with anti-proteasome anti-IgG and then with protein A-Sepharose. Table I shows that the immunoprecipitation strongly reduced the ability of the muscle extracts in the hydrolyses of both peptides and casein, while the incubation with preimmune IgG showed little or no effect. These results indicate that the proteolytic activities are revealed by proteasome and are under developmental regulation.

**Tissue-specific Changes in Proteasome Activities**—To examine whether the development-dependent alterations in the proteasome activities are tissue-specific, extracts of brain and liver were also prepared from the developing chick embryo and assayed for their ability to cleave the peptides and protein was determined as described in Figs. 1 and 2, respectively, but in the presence and absence of 20 µg of anti-proteasome anti-IgG or preimmune IgG. The casein hydrolysis by proteasome was estimated by subtracting the activity seen in the absence of poly-L-lysine from that seen in its presence. In the absence of the agent, either IgG showed little effect on the casein-degrading activity.

TABLE I

| Additions          | Relative activity against |  |
|--------------------|---------------------------|--|
|                    | Suc-LLVY-AMC             | 100  |
|                    | Cbz-ARR-MNA             | 100  |
|                    | [3H]Casein              | 100  |
| IgG from           |                          | 97    |
| preimmune serum    |                          | 102   |
| IgG from           |                          | 3     |
| immune serum       |                          | 22    |

* LLVY, leucine-leucine-valine-tyrosine.

ARR, alanine-arginine-arginine.
Table II

| Embryonic days | Relative activity against Suc-LLVY-AMC<sup>a</sup> | Cbz-ARR-MNA<sup>b</sup> | [³H]Casein |
|----------------|-----------------------------------------------|-------------------------|-------------|
|                 |                                               |                         |              |
| Experiment A: in brain |                                               |                         |              |
| 8               | 100                                           | 100                     | 100          |
| 11              | 109                                           | 80                      | 83           |
| 14              | 110                                           | 87                      | 86           |
| 17              | 107                                           | 81                      | 98           |
| 20              | 92                                            | 90                      | 104          |
| Experiment B: in liver |                                               |                         |              |
| 8               | 100                                           | 100                     | 100          |
| 11              | 76                                            | 140                     | 84           |
| 14              | 66                                            | 3,056                   | 84           |
| 17              | 58                                            | 2,670                   | 97           |
| 20              | 43                                            | 1,940                   | 83           |

<sup>a</sup> LLVY, leucine-leucine-valine-tyrosine.

<sup>b</sup> ARR, alanine-arginine-arginine.

These results also suggest that the developmental regulation of proteasome activities is tissue-specific.

Developmental Regulation of Proteasome Subunit Pattern—To test whether the proteasome undergoes alterations in its subunit pattern during the embryonic muscle development, the enzyme complex was isolated from the muscle extracts using an immunoaffinity chromatography and was subjected to polyacrylamide gel electrophoresis in the presence of SDS. The proteasomes were visualized by silver staining. Lane c, the proteasome from adult chick muscle; lane d, size marker.

These results clearly indicate that the system we used exhibited the development-specific alterations in the cellular events, such as the induction of muscle-specific proteins.

DISCUSSION

The present studies demonstrated that both the peptide- and casein-degrading activities of the proteasome in chick embryonic muscle are under developmental control. Moreover, a number of subunits were found to change in their levels to significant extents with the time of muscle development. Therefore, it appears possible that the rise and fall of the proteolytic activities are correlated with the changes in the subunit pattern, although it is totally unknown whether the subunits with changes in their amounts are directly responsible for the alterations in the proteolytic activities. It is noteworthy that the changes in the spot intensity are rather confined to the subunits with less abundance. Therefore, it is interesting to speculate that the major subunits may play a role in the maintenance of a proteasome’s overall structure, while the minor spots may be directly or indirectly involved in the catalytic functions and therefore are under regulation as necessary.

A recent report has illustrated that Drosophila proteasome undergoes changes in its subunit pattern during the fly development, and yet its overall structure remained unchanged (Haass and Kloetzel, 1989). Furthermore, we have found that...
the changes in the subunit pattern of proteasome in developing chick embryonic liver are also limited to the subunits with minor abundance and yet accompanied by dramatic changes in the peptidase activities (Table II). It is also interesting to note that the proteasome subunits, whose primary structures are known, are found with relatively high abundance and that computer-assisted comparisons have failed to demonstrate any regions homologous to the amino acid sequences of active sites in other known proteases (Tamura et al., 1991).

Post-translational modification, such as protein phosphorylation, has been suggested to be responsible for the development-dependent diversification of the subunit pattern of Drosophila proteasome (Haass and Kloetzel, 1989). Any modification that brings the changes in the net charge of the proteasome subunits should also alter the position of the corresponding spots in an electrical field. However, the proteasome subunits in developing embryonic muscle undergo changes in their spot intensity, although limited to a certain number, but not in their overall position as analyzed by the two-dimensional gel electrophoresis, in which an isoelectric focusing was used for the first-dimensional separation of the subunits (Fig. 4). Therefore, the changes in the subunit pattern may be attributed to the development-specific alterations in the expression of the proteasome subunits in embryonic muscle.

Of particular interest was the finding that the proteasome activity on poly-L-lysine-activated casein hydrolysis dramatically increased during embryonic periods of days 11–17. Analysis of the number of nuclei in chick leg and pectoralis muscle has shown that by embryonic day 9, only 12% of all nuclei have fused to form myotubes, but that by day 18, about 80% of all the nuclei are in myotubes (Herrmann et al., 1970; Herrmann et al., 1970). In addition, it has been well documented that in using an in vitro culture system, the induction of muscle-specific proteins occurs concomitantly with the morphological changes of mononucleated myoblasts to multinucleated myotubes (Nadal-Ginard, 1979; Dunau and Goldman, 1988). Therefore, this increase occurred at about the same period and revealed the dramatic rise in the proteasome activity on poly-L-lysine-activated casein hydrolysis. These facts suggest an involvement of the proteasome in the skeletal muscle development. Intracellular proteolysis has been shown to be prerequisite for the differentiation of embryonic muscle cells, which accompanies the massive mobilization of membrane proteins and reorganization of cytoskeletal components (Fulton et al., 1981; Pauw and David, 1979). Therefore, it is possible that the increased proteolytic activity of the proteasome may be involved in the cellular processes. It is also possible that the rise and fall of the protein and peptide hydrolyses by the proteasome may lead to alterations in the level of unknown but crucial cell protein(s) that mediates the regulation of myogenic differentiation. However, all these possibilities are speculative at present, and further studies are required for the clarification of the role of the proteasomes in the development of chick embryonic muscle.

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