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Multicatalytic and 26 S ubiquitin/ATP-stimulated proteases in maturing rabbit red blood cells

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Rabbit red blood cells of various ages were separated on Percoll gradients and the activities of two large cytosolic proteases were measured. Both the multicatalytic protease (MCP), assayed by hydrolysis of fluorigenic peptides, and the 26 S ubiquitin/ATP-stimulated protease, assayed by degradation of ubiquitin–lysozyme conjugates, declined 3-fold or less during maturation of rabbit reticulocytes to erythrocytes. The ability of MCP to hydrolyze three classes of peptides decreased in parallel indicating that the 20 S protease is not significantly remodeled during red blood cell maturation.

Multicatalytic protease; Ubiquitin; 26 S Protease; ATP-dependent proteolysis; Erythrocyte

1. INTRODUCTION

There have been a number of studies on the proteolytic capabilities of maturing red blood cells. McKay et al. [1] found that the breakdown of amino acid analog-containing proteins decreased with reticulocyte maturation. Speiser and Etlinger [2] reported that energy-dependent proteolysis of hemoglobin and casein was lost in extracts from mature erythrocytes. Likewise, it has been reported that proteolysis in rabbit blood was directly proportional to the number of reticulocytes being very low in mature erythrocytes [3,4].

Since ubiquitin-mediated proteolysis is a major energy-dependent pathway for protein destruction, it has been the specific focus of attention in several more recent studies. In this proteolytic pathway, the carboxyl terminus of ubiquitin is activated by an enzyme, E1, and transferred as a reactive thiol ester to a series of ubiquitin carrier proteins, termed E2s. These, in turn, can transfer ubiquitin to lysine residues on protein substrates some of which are then degraded (see [5,6] for reviews.) Both Raviv et al. [7] and Pickart and Vella [8] have shown that certain E2s are considerably less abundant in mature red blood cells. Raviv et al. [7], further showed that the reduced concentration of E2s is accompanied by impaired proteolysis of bovine serum albumin (BSA) and they were able to restore proteolysis of BSA in red cell extracts by addition of E2s and the ubiquitin protein ligase, E3. This observation would suggest that the 26 S protease, which degrades ubiquitin conjugates [9], is present in mature red cells. Here, we show that this is indeed the case since degradation of ubiquitin–lysozyme conjugates decreased less than 3-fold upon conversion of reticulocytes to erythrocytes. In addition, we demonstrate that 3 proposed separate peptidase activities (trypsin-like, chymotrypsin-like, and the 'glutamyl-site') of another large cytosolic protease, the multicatalytic proteinase (MCP), all decrease in parallel during red cell maturation.

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2. EXPERIMENTAL PROCEDURES

2.1. *Reticulocytosis induction and isolation of red blood cells*

A slight reticulocytosis was induced in white New Zealand rabbits (3 kg) by daily withdrawal of 25 ml of blood over a 4-day period. Phlebotomy rather than injection of phynylhydrazine was used because Rapaport and Dubiel have demonstrated significant differences in proteolytic capacity depending upon the method used to produce reticulocytosis [10]. Blood collected on the fourth day, which by methylene blue staining contained about 10% reticulocytes, was used in all experiments. White cells were removed by passage over a cellulose column [11].

2.2. *Cell separation*

Red blood cells were separated on Percoll density step gradients prepared from 7 solutions of Percoll, ranging in density from 1.079 to 1.107 in 10 mM triethanolamine, pH 7.0 supplemented with 5 mM glucose, 117 mM NaCl and 1.5 mg/ml bovine serum albumin [12]. The gradient was formed by pumping 4 ml of each Percoll solution into a 25 × 89 mm Beckman centrifuge tube. Between 1.5 and 2.0 ml of a 50% suspension of red cells in phosphate-buffered saline (PBS) containing 1 mg/ml glucose were layered onto the gradient and centrifuged in the cold for 45 min at 420 × *g*. The cells were collected by aspiration and washed 3 times with PBS-glucose at 4°C. Direct microscopic hemacytometry was used to construct a standard curve for cell number versus turbidity at 750 nm or hemoglobin content.

2.3. *Lysate preparation*

An appropriate volume of each cell fraction was centrifuged in an

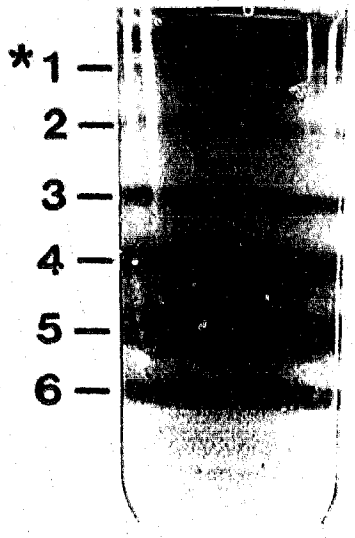

Fig. 1. Separation of rabbit red blood cells on a Percoll step gradient. Shown above is a 25 × 89 mm centrifuge tube following separation of 2.0 ml of a 50% suspension of blood cells from a slightly anemic rabbit on the seven layer step gradient described in section 2. Clean separation of the various layers permitted easy aspiration of cells at different stages of maturation.

Eppendorf microfuge for 20 min at 4°C such that a pellet containing 4×10^8 cells was obtained. The supernate was removed and 100 μl of water, containing 1 mM DTT and 50 μl of CCl_4 was added with vortexing. After 10 min on ice the lysate was centrifuged at 4°C for 20 min in an Eppendorf microfuge at maximum speed. The supernate (~ 120 μl) was removed, and 30 μl of glycerol were added.

2.4. *Enzyme Assays*

All fluorigenic substrates were present at 0.1 mM in a final volume of 0.1 ml of reticulocyte buffer [9]. The reaction was initiated by adding 10 μl of lysate, the reaction mix was incubated at 37°C for 10-20 min depending upon the substrate, and the reaction was stopped by adding 700 μl of cold 100% ethanol. After centrifugation, supernatant fluorescence was determined at 380/440 nm for MCA-peptides and 335/410 for NA-peptides using a Perkin-Elmer spectrofluorimeter. The fluorescence values were converted to nmoles of substrate hydrolyzed, and all assays were performed in linear ranges of enzyme versus product.

2.5. *Degradation of ubiquitin-lysozyme conjugates*

Ubiquitin-lysozyme conjugates were prepared as described [13]. The final reaction (115 μl) contained 50 μl of Ub–lysozyme conjugates (20 000 cpm/50 μl in 50 mM Tris, pH 7.5), 35 μl of reticulocyte buffer, 5 μl of ATP-regenerating system [9] or apyrase (4 U/μl) and 15 μl of extract. Samples including a no-enzyme blank were incubated at 37°C for 60 min before addition of 0.8 ml of bovine serum albumin at 10 mg/ml and 0.1 ml of 100% trichloroacetic acid. After 20 min on ice, all samples were centrifuged for 20 min at maximum speed in an Eppendorf centrifuge. Soluble radioactivity was determined by gamma spectroscopy.

2.6. *Native gel electrophoresis*

Native gel electrophoresis and in situ localization of proteases were performed as previously described [14].

2.7. *Materials*

Percoll, cellulose, Cbz-Leu-Leu-Glu-NA and apyrase were obtained from Sigma. The other fluorigenic peptides were obtained from Peninsula Laboratories (Belmont, CA). ^{125}I was obtained from New England Nuclear Corp.

3. RESULTS

Rabbit red blood cells can be reproducibly fractionated into populations at various stages of maturation by sedimentation in Percoll step gradients. Fig. 1 shows an example of such a separation and Table I presents the density distribution of a population of red blood cells from a slightly anemic rabbit (6% reticulocytosis). It can be seen from the data in Table I that reticulocytes are confined to the upper four fractions of the step gradient.

Extracts were prepared from an equal number of cells in each fraction and 5 fluorigenic peptides were employed to assay proteolytic activity in cells of different ages. Native gel electrophoresis and peptide overlay [14] were used to determine whether the peptides surveyed were cleaved only by the 26 S and 20 S proteases. According to the data in Fig. 2, the 5 peptides, identified in the legend to Fig. 2, are specific for the two large protease complexes.

Knowing that all 5 peptides were diagnostic for the 26/20 S proteases, we assayed the hydrolysis of these peptides in extracts from erythroid cells at different stages of maturation. The data in Fig. 3 show that extracts from fully mature red cells (gradient fraction 5) degraded each peptide at about one-third the rate of extracts from reticulocytes (gradient fraction 1). Similar analysis of the energy-dependent degradation of ubiquitin–lysozyme conjugates revealed a comparable 2-fold decrease upon conversion of reticulocytes to mature red cells (Fig. 3). The 20 S or 26 S proteolytic ac-

TABLE I

Distribution of rabbit red blood cells in Percoll density gradients

Fraction	Cells/ml	Total	% of total	% Reticulocyte	Total reticulocytes
1	2.1×10^8	4.2×10^8	1.7	93%	3.9×10^8
2	3.6×10^8	7.1×10^8	2.9	59%	4.2×10^8
3	14.5×10^8	29.0×10^8	11.6	15%	4.3×10^8
4	22.5×10^8	45.0×10^8	18.0	7%	3.2×10^8
5	53.7×10^8	107.4×10^8	42.9	0%	--
6	28.5×10^8	57.1×10^8	22.8	0%	--
	Total cells =	249.8×10^8			15.6×10^8

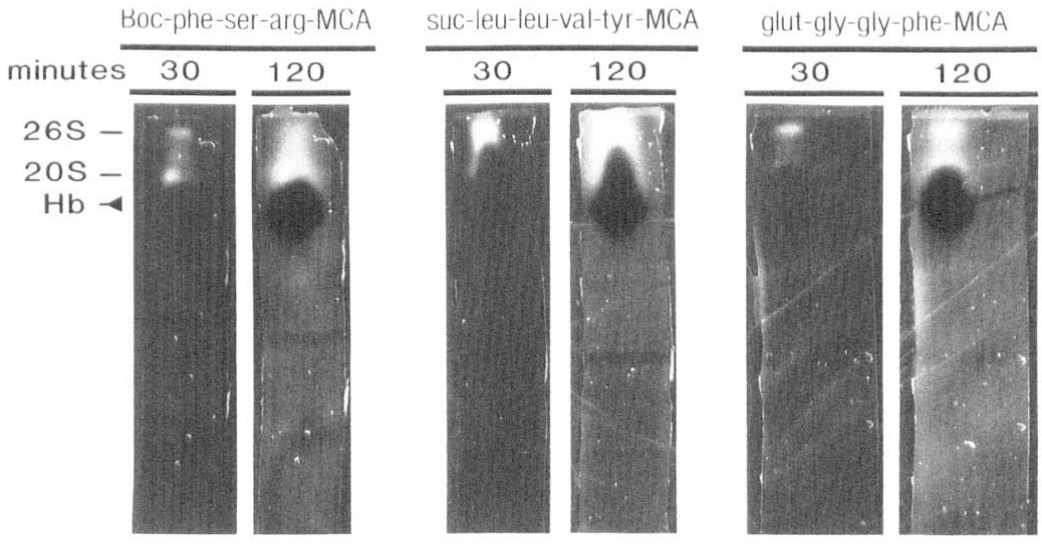

Fig. 2. In situ identification of 26 S and 20 S proteases after native gel electrophoresis of rabbit red cell extract. Extracts were prepared from mature red blood cells and analyzed on 4.5% native acrylamide gels [14]. The gels were then overlaid with the indicated substrates and photographed after 30 or 120 min. The white areas correspond to fluorescent MCA released upon hydrolysis of the respective peptide. The absence of MCA product in all regions of the gel except at sites to which the 26 S and 20 S proteases migrate is taken as evidence that the substrates are specific for these two large enzyme complexes. Equivalent results were obtained with Boc-Leu-Ser-Thr-Arg-MCA and Cbz-Leu-Leu-Glu-NA as well. The large dark area apparent in the samples incubated for 120 min is hemoglobin, which migrates with an anomalously large apparent molecular mass and can obscure fluorescence from the 20 S protease (e.g. middle panels).

tivity in older red cells cannot be attributed to reticulocyte contamination since the dotted line in Fig. 3 depicts the expected levels of protease activity assuming reticulocytes to be the sole source of 20 and 26 S enzymes. Clearly, the observed reductions in activity do not mirror the proportion of reticulocytes in each fraction. The data in Fig. 3 also demonstrate that there are parallel decreases in trypsin-like, chymotrypsin-like and the 'glutamyl-site' peptidase activities of MCP during red cell maturation.

4. DISCUSSION

The multicatalytic proteinase (MCP) is a large 20 S enzyme complex formed from multiple (>10 in most higher species) subunits with molecular masses between 20 and 30 kDa [15,16]. The 700 kDa protease has been found in the cytoplasm and nucleus of all eucaryotic organisms examined thus far and in the archaebacterium, *Thermoplasma* as well [17]. There is a growing conviction among students of intracellular proteolysis that MCP plays a major role in the turnover of cytosolic/nuclear proteins. In fact, MCP subunits were proposed to form part of the even larger 26 S, ATP-stimulated protease that degrades ubiquitin conjugates [18]. Recent studies have confirmed this hypothesis [19,20].

Clearly, a major task for the future will be to determine the relative contributions of the 20 S and 26 S proteases to overall protein degradation within cells. Equally important, the natural substrates of these and other cytoplasmic proteases will have to be identified. Evidence has already been presented that the ubiquitin-dependent pathway is involved in the degradation of reticulocyte mitochondrial proteins [21,22] and in the degradation of excess globin chains [23].

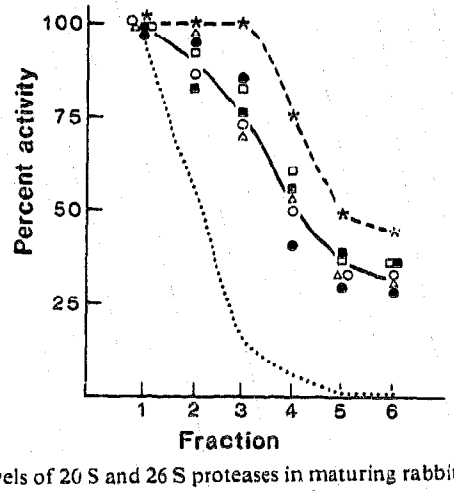

Fig. 3. Levels of 20 S and 26 S proteases in maturing rabbit red blood cells. Extracts prepared as described in methods were assayed for the multicatalytic protease using 5 fluorigenic peptides: Boc-Phe-Ser-Arg-MCA, (○); Boc-Leu-Ser-Thr-Arg-MCA, (●); Suc-Leu-Leu-Val-Tyr-MCA, (□); Glut-Gly-Glu-Phe-MCA, (■); Cbz-Leu-Leu-Glu-NA, (△). Extracts were also assayed for the degradation of ubiquitin–lysozyme conjugates (-*-*-). The data are expressed as enzyme activity per cell with fraction 1 (>90% reticulocytes) normalized to 100%. The dotted line (---) shows the percentage of reticulocytes in each fraction.

It is in this context that the studies presented above were initiated. Since the report by Speiser and Etlinger [2] that ATP-dependent proteolysis was diminished in extracts from mature rabbit red cells, there have been several papers stating implicitly or explicitly that oxidized proteins of erythrocytes are degraded by the 20 S protease [24-27]. The data in Figs 2 and 3 show clearly that the 26 S protease persists in mature red blood cells. Of course, its presence does not negate the possibility that the 20 S protease is actually responsible for destruction of oxidized proteins in erythrocytes. However, one cannot draw this conclusion on the erroneous supposition that the 26 S enzyme is not present. Also, one cannot eliminate a role for the 26 S protease because E3s, ubiquitin protein ligases, are scarce or absent in mature red blood cells. We have recently shown that histone H3 conjugates, formed by direct transfer of ubiquitin from various E2s to the histone, can be degraded by the 26 S protease [28]. Furthermore, in two studies we have demonstrated that ubiquitin can be transferred to several proteins in 'loaded' mature human red blood cells; one of the conjugates has a molecular mass expected for globin plus a single ubiquitin [29,30]. Taken together these observations indicate that it would be unwise to conclude that the ubiquitin-dependent proteolytic pathway is absent from mature red blood cells.

The current studies address another important issue regarding MCP. Kloetzel and his colleagues [31,32] have presented evidence that the subunit composition of MCP changes during Drosophila development. This raises the possibility that specific cell types assemble 20 S complexes containing different ratios of proteolytic activities. The data in Fig. 3 indicate that there is little change in the relative proteolytic activities of MCP during the transition of rabbit reticulocytes to erythrocytes. Thus, the enzymatic composition of MPC may vary among different tissues, but it does not appear to change significantly during maturation of erythroid cells.

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