PROTEIN A24 LYASE IS AN ISOPEPTIDASE

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1. Introduction

Chromosomal protein A24 was first described by its electrophoretic mobility on two-dimensional polyacrylamide gels [1]. It was identified as a conjugated chromatin protein composed of histone 2A [2] and a ubiquitin moiety [3–5] which was linked by an isopeptide bond from a carboxyl terminal glycylglycine to the ϵ-NH_2 of lysine 119 of histone 2A [6–8]. Protein A24 has been found to be an integral component of a subset of chromatin nucleosome core particles [9–11].

Levels of protein A24 markedly decreased during nucleolar hypertrophy [12–14] and in some active chromatin fractions where free ubiquitin was found [15–17]. Cleavage of the ubiquitin–histone 2A bond may be involved in some transcription systems [15]. Moreover, in chromatin the ubiquitin portion of protein A24 turns over throughout interphase [18,19]. Recently, an enzyme, protein A24 lyase, was described which cleaved both free and nucleosomal protein A24 and analysis of the products by gel electrophoresis implied that the enzyme catalyzed a deconjugation [20]. This report is the carboxyl-terminal sequence analysis of the ubiquitin released from purified protein A24 by the lyase. Inasmuch as the lyase released ubiquitin with an intact glycylglycine terminus. The results indicate that the site of cleavage in protein A24 is at the isopeptide linkage between the carboxyl terminal glycine residue 76 of ubiquitin and the ϵ-amino group of lysine residue 119 of histone 2A.

2. Experimental

2.1. *Solubilization of protein A24 lyase from rat liver nuclei*

All operations were performed at 0–4°C. Sucrose–Mg^{2+} liver nuclei [21] from 3 Holtzman rats were suspended in 18.5 ml 0.05 M Tris (pH 7.9)/8% glycerol/5 mM Mg acetate/0.1 mM Na_2 EDTA/1 mM dithiothreitol/1 mM PMSF (TGMEDP-8%). A 4 M ammonium sulfate solution (1.5 ml) was added followed by stirring for 15 min and sonication to low viscosity. After centrifugation at 100 000 × *g* for 60 min, ammonium sulfate was reduced to 0.1 M by addition of 40 ml TGMEDP-8%. After stirring for 15 min and centrifugation as above, addition of ammonium sulfate to 80% saturation yielded a precipitate which was collected by centrifugation at 10 000 × *g* for 15 min. The pellet was suspended in 0.05 M Tris (pH 7.9)/25% glycerol/5 mM Mg acetate/0.1 mM EDTA/0.5 mM dithiothreitol/0.5 mM PMSF in 0.1 M NaCl followed by dialysis twice against 1 liter of the same buffer. After centrifugation at 10 000 × *g* for 10 min, the supernatant was used as protein A24 lyase [20].

2.2. *Polyacrylamide–SDS gel electrophoresis*

As described in [20] samples were analyzed by one-dimensional 12% acrylamide/0.1% SDS/0.1 M sodium phosphate (pH 7.1)/6 M urea gel electrophoresis. The gels were stained in 0.22% Coomassie brilliant blue-R/30% methanol/10% acetic acid and destained in 33% methanol/10% acetic acid.

2.3. *Isolation of ubiquitin from rat erythrocytes*

Isolation of ubiquitin was by a modification of the procedures in [22,23]. Blood from 6 rats was placed in 200 ml ice-cold phosphate-buffered saline (0.15 M NaCl/0.01 M sodium phosphate (pH 7.4)/1 mM PMSF) followed by filtration through 4 layers of cheesecloth and centrifugation at 1700 × *g* for 10 min. The pellet was homogenized in 100 ml 0.05 M Tris (pH 7.9)/1 mM dithiothreitol/1 mM PMSF/0.1 mM leupeptin and centrifuged at 12 000 × *g* for 30 min. The super-

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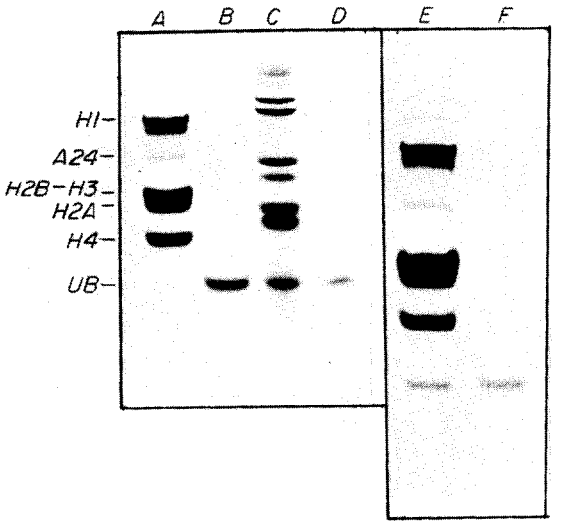

Fig.1. Polyacrylamide gel electrophoresis of ubiquitin. One-dimensional 12% SDS–polyacrylamide gel electrophoresis was performed as in section 2: (A) rat liver histone markers; (B) calf thymus ubiquitin marker (UB); (C) heat-stable proteins of rat erythrocytes; (D) purified red cell ubiquitin; (E) histone plus ubiquitin markers; (F) ubiquitin isolated from the protein A24 lyase reaction; (A–D and E,F) are taken from two separate runs.

natant was heated to 90°C for 15 min, cooled on ice for 30 min and centrifuged at 12 000 × *g* for 20 min. The supernatant was filtered through Whatman no. 1 paper and brought to 100% of saturation with ammonium sulfate. The precipitate was dissolved in 0.05 M Tris (pH 7.9)/0.5 mM PMSF/0.5 mM dithiothreitol, dialyzed against the same buffer at 4°C and centrifuged at 10 000 × *g* for 15 min. The supernatant was heat treated and centrifuged as above. The supernatant (fig.1C) was passed at room temperature through a combined column consisting of a 4 ml layer of DEAE-Sephadex upon which was placed a 4 ml layer of phosphocellulose, and which was equilibrated with 0.05 M Tris (pH 7.5)/1 mM PMSF/1 mM dithiothreitol. The flow-through fraction contained electrophoretically pure ubiquitin (fig.1D).

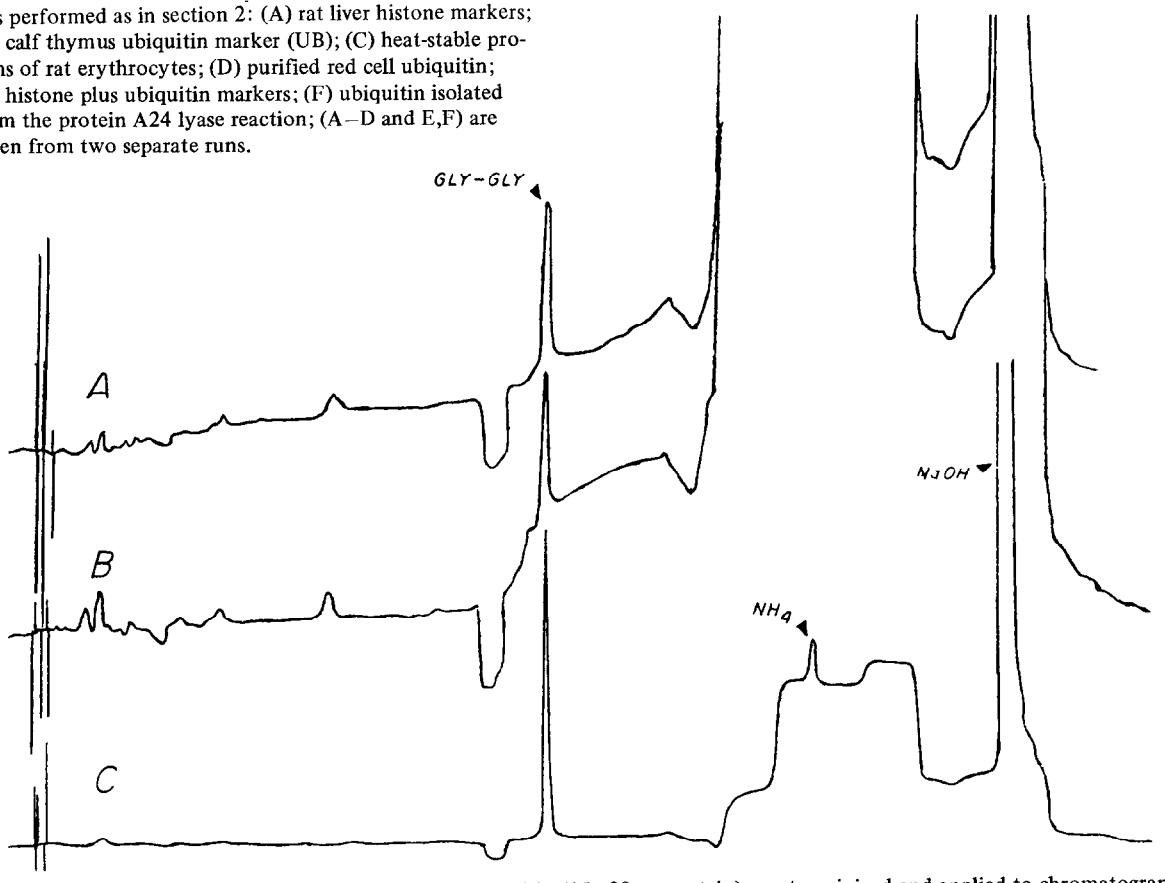

Fig.2. Analysis of trypsin-treated ubiquitin. Isolated ubiquitin (15–20 μg protein) was trypsinized and applied to chromatography on a Beckman 121 MB amino acid analyzer as in section 2. Tracings of the elution profile were superimposed for comparison: (A) protein A24 lyase generated ubiquitin; (B) rat erythrocyte ubiquitin; (C) 3 nmol commercial glycylglycine (Sigma) applied directly to the amino acid analyzer; (A,B) measured on the 0.1 absorbance range; (C) on the 0.5 absorbance range.

2.4. *Isolation of protein A24 lyase released ubiquitin*

Purified protein A24 (1 mg) prepared as in [24] was incubated at 37°C for 2 h with 1 mg protein A24 lyase in 1 ml 0.05 M Tris (pH 7.5)/1 mM PMSF/1 mM dithiothreitol (TPD) followed by heating at 100°C for 5 min [20]. After centrifugation at 15 000 × *g* for 3 min, the supernatant was made 2% with trichloroacetic acid followed by centrifugation as above and dialysis against 1 liter of TPD. Chromatography through a combined phosphocellulose–DEAE-Sephadex column was as described above (fig.1F).

2.5. *Trypsin treatment of isolated ubiquitin*

Ubiquitin (15–30 μg protein) isolated from rat erythrocytes or released from protein A24 by the lyase was digested with 2 μg trypsin for 2 h at 37°C in 0.1 ml 0.1 M *N*-ethyl morpholine acetate (pH 7.5) followed by heating at 100°C for 5 min. The sample was lyophilized, dissolved in pH 2.2 sodium citrate (Beckman Instruments), and analyzed on a Beckman 121 MB amino acid analyzer.

2.6. *Carboxyl-terminal analysis of ubiquitin*

In addition, ubiquitin (10 μg) was digested with 0.005 units carboxypeptidase A or 0.04 units carboxypeptidase B and the digests subjected to amino acid analysis [4,25]. Hydrazinolysis was performed on 40–50 μg ubiquitin as in [6,26].

3. Results

Trypsin treatment of ubiquitin should cleave at arginine residue 74 to release any peptide on its C-terminal side because the remainder of the ubiquitin (residues 1–74) is trypsin resistant [5,7]. When rat red-cell ubiquitin and the protein A24 lyase generated ubiquitin were trypsinized and amino acid analysis was done on the products, the peaks from both sources of ubiquitin (fig.2A,B) comigrated with commercial glycylglycine (fig.2C).

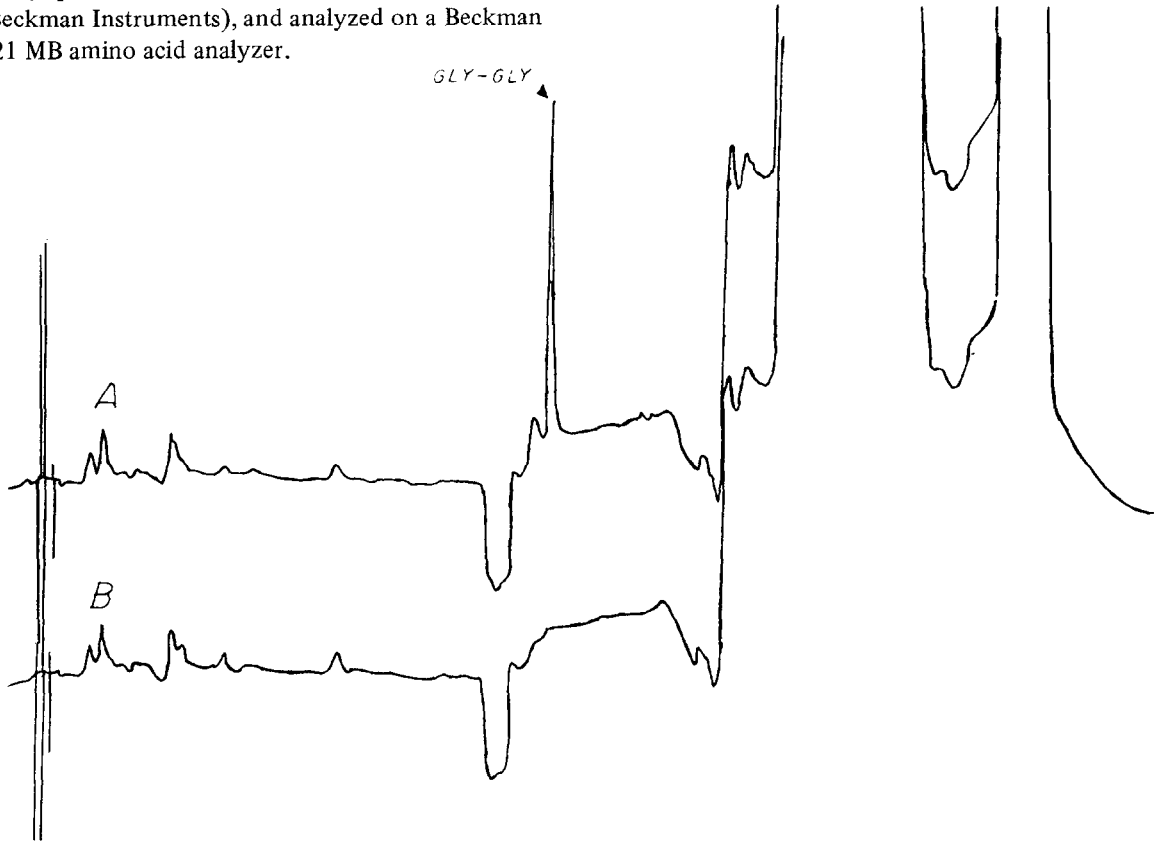

Fig.3. Trypsin treatment of protein A24 lyase-released ubiquitin. The protein A24 lyase reaction was performed as in section 2, both in the absence and presence of 1 mg protein A24. The trichloroacetic acid step was omited from the ubiquitin isolation procedure and the tandem DEAE-Sephadex and phosphocellulose flow-through fractions were trypsin treated prior to analysis as in fig.2. Tracings of the analyzer profiles were superimposed for comparison: (A) protein A24 lyase reaction in the presence of protein A24; (B) without protein A24; (A,B) were measured on the 0.1 absorbance range.

Table 1
Carboxyl-terminal sequence analysis of free and lyase-released ubiquitin

Treatment	Red cell ubiquitin	Lyase ubiquitin
Carboxypeptidase B	Neg.	Neg.
Hydrazinolysis	Gly	Gly
Trypsin	Gly–Gly	Gly–Gly

Carboxypeptidase treatments were performed on 10 μg ubiquitin as in section 2. Hydrazinolysis was performed on 25–50 μg ubiquitin as described. *Abbreviation:* Neg., negative release of any amino acid

Hydrazinolysis showed the presence of a terminal glycine in both red-cell and lyase-cleaved ubiquitin, while carboxypeptidase B treatment released no amino acids. Accordingly, all of the ubiquitin from the protein A24 lyase reaction had a C-terminal glycine which is resistant to carboxypeptidase B and none had an arginine C-terminal (table 1).

To determine whether the protein A24 lyase derived ubiquitin was contaminated with endogenous ubiquitin the lyase reaction was carried out both in the absence and the presence of exogenous protein A24 as substrate. Analysis of trypsin treated flow-through fractions from the tandem DEAE-Sephadex and phosphocellulose columns showed that the putative glycylglycine cleaved from ubiquitin, was not found in the absence of substrate (fig.3).

4. Discussion

Previous reports indicated that arginine residue 74 of the ubiquitin portion of protein A24 was directly linked to a glycylglycine 'bridge' between the ubiquitin and histone 2A portions [6–8]. Arginine was reported to be the COOH-terminal amino acid of free ubiquitin from calf thymus [5,27]. Ubiquitin from trout testis was reported to have heterogeneity at the COOH-terminus, 1/2 of the population terminating with arginine and 1/2 with glycine [16]. In the rabbit reticulocyte lysate system, ubiquitin (APF-I) conjugation [28,29] has been identified as a necessary factor involved in ATP-dependent proteolysis [22,23]. The form of ubiquitin active in ATP-dependent proteolysis terminates in glycine [30]. This study demonstrates that rat erythrocyte ubiquitin contained a COOH-terminal glycylglycine which confirms previous results with human ubiquitin (Wilkinson, K. D. and Audhya, T. K., personal communication). The reported heterogeneity of the carboxyl-terminus of free ubiquitin [5,16,27] may possibly have resulted from proteolysis during the isolation procedures [31,32].

Here, trypsinization, carboxypeptidase B treatment, and hydrazinolysis of ubiquitin released from purified protein A24 by the lyase reaction indicated that it had a carboxyl-terminal glycylglycine. Accordingly, rat liver nuclear protein A24 lyase cleaved at the isopeptide linkage between the carboxyl-group of glycine residue 76 of ubiquitin and the ε-amino group of lysine residue 119 of histone 2A [6–8]. Therefore, protein A24 lyase is an isopeptidase (fig.4).

Protein A24 turnover throughout interphase [18] and loss of protein A24 in metaphase chromosomes [33] and in nuclei during chicken erythropoiesis [34] suggests that the conjugate may be associated with potentially active chromatin structures [34,35]. Moreover, the ability of protein A24 lyase to release ubiquitin with the glycylglycine COOH-terminus as shown here implies that lyase-relased ubiquitin may be reutilized.

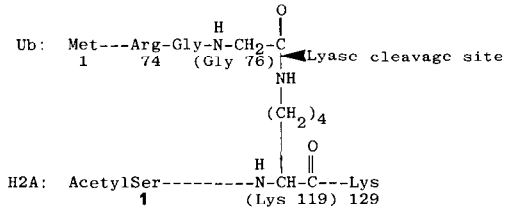

Fig.4. The site of protein A24 lyase cleavage. The lyase cleavage site is shown to be the isopeptide linkage between the COOH-terminus of ubiquitin and the ε-NH_2 group of lysine 119 of histone 2A.

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