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BIOSYNTHESIS OF A UBIQUITIN-RELATED PEPTIDE IN RAT BRAIN AND IN HUMAN AND MOUSE PITUITARY TUMORS

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SUMMARY. A biosynthetic labeled peptide structurally related to the thymic peptide ubiquitin was first identified fortuitously in bovine pars intermedia cells in regard to its partial NH_2 terminal amino acid sequence (Met 1, Leu 8, 15 and Lys 6, 11, 27, 29, 33) after a protein segment data bank search. A peptide with the same behavior on carboxymethylcellulose chromatography and polyacrylamide gel electrophoresis has been purified after labeling experiments in two areas of rat brain, hypothalamus and striatum, and in a mouse and a human ACTH-secreting pituitary tumors. It represents about 1 to 10% of the total labeled proteins in the various experiments. Its identity with the above mentioned bovine pituitary peptide was confirmed by microsequence analysis with respect to Met 1, Lys 6, 11 in hypothalamus, Met 1 in striatum, and Lys 6, 11, 27, 29, 33 in the two pituitary tumors. The availability of standard purified ubiquitin allowed us to show that labeled and cold peptides have the same electrophoretic mobility and elution volume on Sephadex G-50 chromatograph this further confirms their identity. Possible interests of such a biosynthetic characterization of a ubiquitin-related peptide are discussed, particularly in view of the structural relationship of ubiquitin to the non histone component of nuclear protein A-24, and as a test of tissue viability and biosynthetic efficiency in our in vitro biosynthetic systems.

INTRODUCTION

Ubiquitin is a polypeptide of 74 amino acids, first identified in bovine thymus (1,2) during isolation of the thymic polypeptide hormone

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thymopoietin. It has been subsequently detected in a large variety of animal cells in vertebrates and invertebrates, in yeasts, bacteria and higher plants (1). The sequence of ubiquitin, which is identical in human and bovine species (3) appears to have been highly conserved throughout evolution. Both the constancy of its structure and its wide distribution, likely in all living cells, suggest that ubiquitin has an important cellular function, still unknown. In vitro it induces non-selective lymphocyte differentiation and activation of adenylate cyclase in lymphocyte precursors and other tissues, presumably acting via a beta-adrenergic receptor (1). Recently (4), the amino-terminal sequence of ubiquitin has been discovered to be identical to that of the non-histone component of bovine nuclear protein A-24 (5); these two peptides are most likely products of the same gene (4). Ubiquitin could then be involved in the structural or functional properties of the eukaryotic genome as it has been suggested for non-histone chromosomal proteins (for review, see ref. 6-8).

The first in vitro biosynthesis of ubiquitin was demonstrated in bovine pituitary pars intermedia in this laboratory (9). This was a fortuitous discovery during the course of biosynthetic studies of beta-lipotropin, beta-endorphin, ACTH and related peptides: the partial sequence of a pure isolated radiolabeled peptide unrelated either to beta-LPH or ACTH peptides was found to match with the amino-terminal sequence of ubiquitin by a protein segment data bank search (10). In this report, a similar biosynthesized peptide has been characterized in two areas of rat brain, hypothalamus and striatum, and in two ACTH-secreting pituitary tumors; the first one was an experimental mouse pituitary tumor (AtT-20), the second one was a human pituitary tumor. The identity of this peptide with native purified ubiquitin has been further established on the basis of their electrophoretic and chromatographic properties. The biosynthetic characterization of ubiquitin

could provide a useful tool to study the kinetics and the regulation of its biosynthesis, and its relationship to the nuclear protein A-24 in the field of non histone-chromosomal proteins.

MATERIALS AND METHODS

Preparation of tissues and cells: Brains were removed from Sprague-Dawley male rats (Canadian Breeding Laboratory, St. Constant, Que.) (150-200 g) killed by decapitation. Hypothalamus or striatum were quickly dissected according to Glowinski and Iversen (11), cut into small cubes (about 1-2 mm square) and immediately immersed in Krebs Ringer buffer containing bicarbonate 0.025M, 0.2% glucose, 0.1% bovine serum Albumin (Fraction V, Sigma) (KRBGA)*, and 0.018% soybean trypsin inhibitor (Sigma) previously gassed with 95% O_2 / 5% CO_2, at room temperature. Pooled hypothalamus or striatum fragments were preincubated for 1 hour at 37° in fresh gassed buffer in a Dubnoff metabolic shaker with a gentle agitation.

The AtT-20 tumor (12,13) obtained from Dr A.E. Bogden (Mason Research Institute, Worcester, Massachusetts) was periodically retransplanted in adrenalectomized LAF_1 mice. Three tumors (total wet weight 7.1 g) were dissected, washed with saline buffer, and cross-cut at 250 um intervals using a McIlwain tissue chopper. An ACTH secreting human pituitary tumor following adrenalectomy in a patient having a Cushing's disease, was excised by a transphenoidal approach; a fragment of the tumor was collected in physiological saline at 4°C. Fragments of the mouse or human tumor were then immediately immersed in KRBGA buffer, incubated at 37°C under 95% O_2 / 5% CO_2 in KRBGA containing collagenase (6 g/l) for 15 minutes followed by KRBGA containing DNase (8 mg/l) for 15 minutes in the same conditions. After washing in fresh gassed KRBGA buffer, the cells were isolated by sucking several times the fragments through a plastic tubing (3 mm inside diameter) attached to a disposable plastic syringe, and filtered through a fine nylon net (100 mesh). The isolated cells were harvested by low speed centrifugation and preincubated for 1 hour at 37° as described before. Viability of the cells was determined with the trypan blue exclusion technique and found to be higher than 90%.

Incorporation of labeled amino acids in vitro: After preincubation, brain fragments or cells harvested by low speed centrifugation wer resuspended in fresh KRBGA buffer with trypsin inhibitor and a labeled amino acid. The incubation was carried out at 37° under 95% O_2 / 5% CO_2 atmosphere in a Dubnoff metabolic shaker for 2 to 3 hours. The radioactive amino acids incorporated were ^{35}S-methionine (1 to 1.5 mCi, 800-1100 Ci/mmol, Amersham), ^{3}H-lysine (1 to 2 mCi, 60-80 Ci/mmol, New England Nuclear (NEN), and ^{3}H-leucine (5 mCi, 115 Ci/mmol, NEN).

Protein Extraction: After the incubation, tissue fragments or cells were diluted in cold KRBGA containing 10 mM of unlabeled amino acid and immediately centrifuged at low speed. Brain fragments were homogenized using a Teflon^{R} glass homogenizer in 1 N acetic acid containing 0.3 mg/ml phenylmethylsulfonyl fluoride, 0.3 mg/ml iodoacetamide, 5 x 10^{-4}M bacitracin (Sigma), 0.5 mg/ml BSA and 10 mM of the corresponding unlabeled amino acid and centrifuges (15,000 rpm, 30 min, 4°C). Cell pellets were extracted in 5 N acetic acid containing the same enzymatic

* Krebs Ringer Buffer Glucose Albumin

inhibitors, except bacitracin. The supernatants were desalted on Biogel P2 or Sephadex G-25 columns equilibrated and eluted with 1N acetic acid.

Carboxymethyl cellulose chromatography: After desalting the fraction excluded from the gel was lyophylized, dissolved in 0.01 M NH_4OAc buffer (pH 4.6) with 50 mg of sheep or human (for the human tumor extract) fraction D prepared as earlier described (14). The mixture was chromatographed on a carboxymethyl cellulose column (0.7 x 26 cm) at 4°C using the same NH_4OAc gradients with a mixing flask of 100 ml (14). The protein content was determined by absorbance at 230 nm and an aliquot of 80 ul/tube was taken for determination of radioactivity by liquid scintillation counting.

Disc electrophoresis: Polyacrylamide gel electrophoresis of carboxymethyl cellulose fractions were performed at pH 4.5 or pH 8.3 according to Reisfield et al. (15) and Davis et al. (16) respectively. Gels containing radioactive proteins were cut into 2 mm slices immediately after the electrophoresis with a Gilson Aliquogel fractionator; gel fragments were then digested by incubation overnight at 50°C in a mixture of 0.2 ml of 60% (vol/vol) perchloric acid and 0.4 ml of 30% (vol/vol) H_2O_2.

Ubiquitin: Ubiquitin purified from bovine thymus, kindly supplied by Dr G. Goldstein, was taken as a standard for comparison on polyacrylamide gel electrophoresis and molecular sieving.

Sequencing of labeled peptides: Automatic Edman degradation of the purified peptides was performed on a Beckman 890B sequencer using 150 nmoles of sperm whale apomyoglobin as carrier and 0.1 M quadrol buffer (17). The thiazolinone collected in butylchloride were counted directly in toluene-base scintillation mixture (4 g omnifluor (New England Nuclear)/1 toluene).

RESULTS

1. Rat hypothalamus.

Fig. 1A shows the carboxymethylcellulose chromatography of the desalted supernatant of hypothalamus homogenate after a 3 h incorporation of ^{3}H-lysine. Comparable profiles were obtained after ^{35}S-methionine and ^{3}H-leucine incorporation. The fraction corresponding to tubes 106-133 was lyophilized and characterized by polyacrylamide gel electrophoresis at pH 4.5 and 8.3; it is seen that it contains a labeled peptide which migrates as a single band with Rf 0.57 and 0.21 respectively (Fig. 2A, 2B); this electrophoretic mobility was comparable with that of the homologous biosynthetic peptide previously isolated from beef pituitary pars intermedia (9). The availability of purified bovine ubiquitin later allowed us to establish the identity of its mobilities with those of this

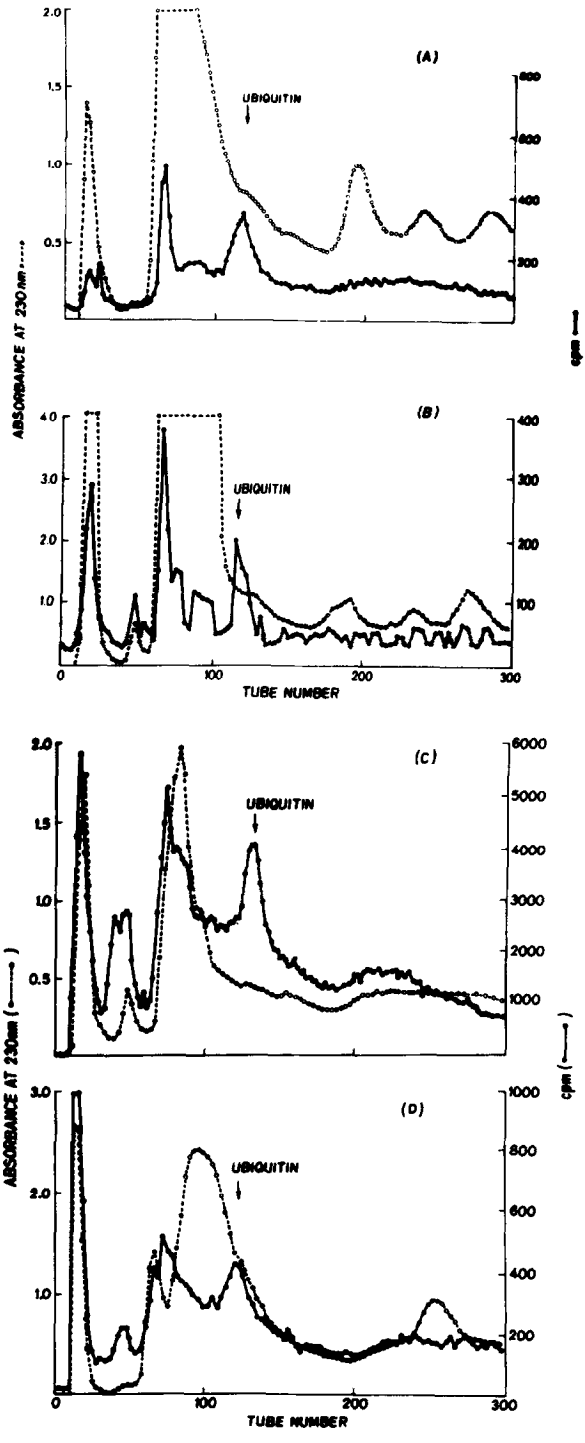

Figure 1: Carboxymethyl cellulose chromatography of labeled proteins extracted from (A) rat hypothalamus, (B) rat striatum, (C) mouse AtT-20 tumor, (D) human pituitary tumor. A concave gradient was started at

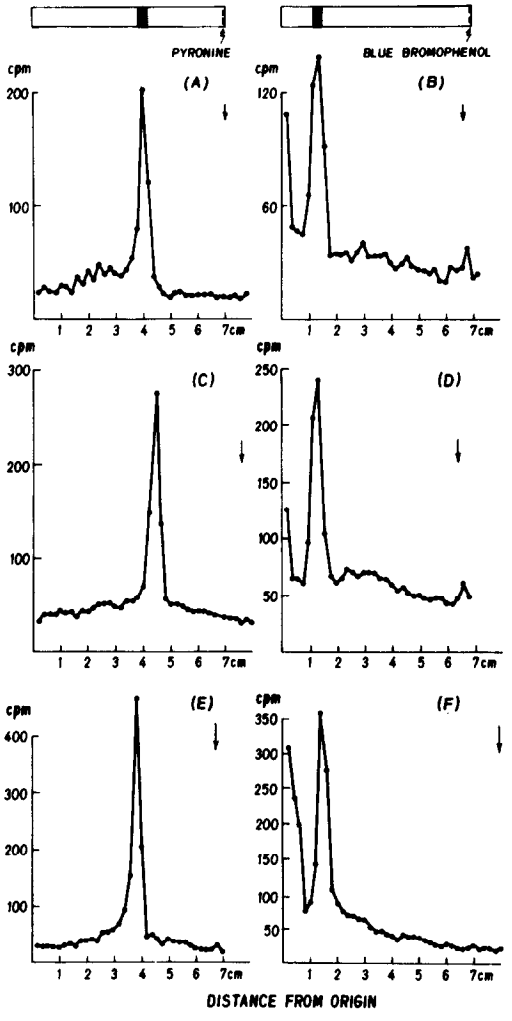

Figure 2: Polyacrylamide gel electrophoresis at pH 4.5 (A, C, E) and at pH 8.3 (B, D, F) of the radioactive material obtained after the carboxymethyl cellulose chromatography of labeled proteins from hypothalamus (A,B), mouse AtT-20 tumor (C,D) and human pituitary tumor (E,F). The arrow shows the position of the tracking dye at the end of the migration. The positions of cold purified ubiquitin run as a reference standard on identical gels are shown in A (pH 4.5) and B (pH 8.3). Although not shown, the same pattern was obtained with polyacrylamide gel electrophoresis at pH 4.5 of the corresponding rat striatal peptide.

Fig.1 cont.'d.

tube 20 by the addition of 0.1 M NH_4OAc buffer, pH 6.7 to a 100 ml mixing chamber containing 0.01 M NH_4OAc buffer pH 4.6. At tube 160 (180 for human tumor), a 0.2 M NH_4OAc buffer, pH 6.7 was used to increase the gradient. Fractions of 0.8 ml were collected at a rate of 0.6 ml/min.

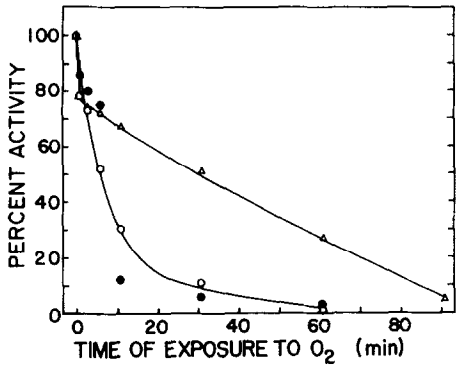

Fig. 3. Inactivation of the unidirectional and the bidirectional hydrogenases by oxygen. The unidirectional hydrogenase (in Tris.Cl, pH 8, with 0.58 M sucrose, Δ - Δ) was from the washings of the protoplast preparation. The bidirectional hydrogenase (in Tris.Cl, pH 8, with (0 - 0) or without (● - ●) 0.58 M sucrose) was partially purified through the Sephadex G-100 column step (1). Other details were described in Materials and Methods.

The extensively purified unidirectional H_2ase can use Fd, MB, MV, benzyl viologen or dichlorophenolindophenol as the sole electron acceptor, and the measured activity decreases when an electron acceptor with a more negative midpoint redox potential is used (data not shown). The H_2-oxidizing activity in the heterocysts of Anabaena 7120 was reported to use O_2 as the electron acceptor (10, 11), and the activity was not significantly enhanced when Fd or MV was added to the assay mixture (10); however, the tests were performed with intact heterocysts.

Significant difference in O_2-sensitivity was found between the unidirectional and the bidirectional H_2ases (Fig. 3). Fifty percent inactivation occurred in approximately 30 and 5 min., respectively, after the unidirectional and the bidirectional H_2ases were exposed to air.

Like the other classical H_2ases (12), the unidirectional H_2ase from C. pasteurianum is also inhibited by carbon monoxide (98% inhibition at 0.5 atm CO). This suggests that the new H_2ase is likely a metalloenzyme, perhaps an iron-sulfur protein, too. Direct metal analysis must await further purification of the enzyme.

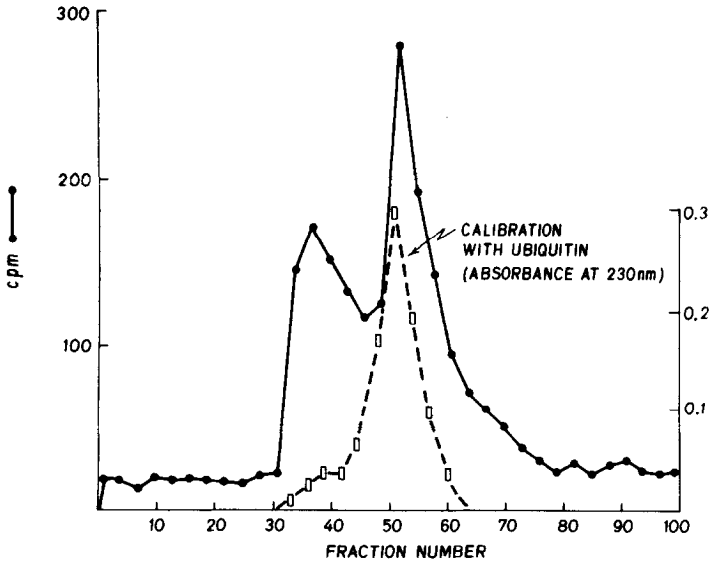

Figure 4: Sephadex G-50 superfine chromatography (column 1 x 55 cm) in 0.1 N acetic acid of fraction 113-130 of CM-cellulose chromatography of incubated human pituitary tumor cell extract. Calibration with purified ubiquitin is shown. 0.5 ml fractions were collected.

3. Pituitary tumors.

In the carboxymethyl cellulose chromatography of mouse AtT-20 tumor and human pituitary tumor radiolabeled extracts (Fig. 1C, 1D), a major peak of ^{3}H-lysine labeled proteins was found in a comparable region of the carboxymethyl cellulose elution profile: respectively fractions 120-140 and 113-130 were lyophilized. In AtT-20 tumor, this fraction was found to contain a pure ^{3}H-lysine labeled peptide with the same Rf as ubiquitin (Fig. 2C, 2D). Its sequence with respect to lysine residues is identical to that of NH_2-terminal portion of ubiquitin (Fig. 3D). With the human tumor fraction, an additional purification step was needed and was performed on a Sephadex G-50 superfine column (1 x 55 cm) eluted with 0.1 N acetic acid (Fig. 4). The fraction 48-65, coeluting with cold ubiquitin, was lyophilized and identified to be ubiquitin by polyacrylamide gel electrophoresis (Fig. 2E, 2F) and sequence analysis (Fig. 3E).

DISCUSSION

This paper reports the in vitro biosynthesis of an ubiquitin-related peptide in rat brain hypothalamus and striatum and in two pituitary tumors, an experimental AtT-20 mouse tumor and a human ACTH secreting tumor Such an actively biosynthesized peptide has first been identified, fortuitously, in the beef pituitary pars intermedia during the course of biosynthetic studies of beta-LPH, beta-endorphin and related peptides (9). Its partial sequence: Met 1, Lys 6, 11, 27, 29, 33 and Leu 8, 15 was found to fit completely the known sequence of ubiquitin (2,9,10). Furthermore, the availability of purified bovine ubiquitin allowed us to establish that the biosynthetic peptides have the same electrophoretic mobility as ubiquitin on polyacrylamide gels at both pH 4.5 and 8.3. Thus they appear to be newly biosynthesized ubiquitin. The possibility that they were produced by degradation of a larger precursor protein during the isolation process is unlikely because 1) all the experiments were performed at 4°C, 2) protease inhibitors were used for the extraction, and 3) cold carrier (pituitary fraction D) was added to the extract. The disparity between the apparent molecular weight of the biosynthetic peptide on sodium dodecyl sulfate/urea gel electrophoresis (3,500-4,000 daltons) (9) and the 8,451 daltons calculated from the known sequence of the 74 amino acids of ubiquitin seems to be related to an abnormal migration of ubiquitin on sodium dodecyl sulfate/urea polyacrylamide gel, as it has been reported for other peptides (18,19). A comparable apparent molecular weight has been found by Sephadex G-75 (9) or G-50 chromatography (Fig. 4) in 0.1 N acetic acid, both for biosynthetic peptides and ubiquitin, as they coelute with standard sheep beta-endorphin. The reasons for this abnormal behavior on sodium dodecyl sulfate/urea gel and on Sephadex gel chromatography remain to be clarified.

The biosynthesis of ubiquitin in these different tissues: brain, normal pituitary, pituitary tumors, in several mammalian species:

beef, rat, mouse and man and the constancy of its sequence are in agreement with immunological detection of ubiquitin in a large variety of cells (1). Newly biosynthesized ubiquitin has been found in each of our incubation experiments whenever we have systematically looked for it; it represents 1 to 10% of the totality of labeled proteins in the various incubation experiments. Its "ubiquitous" in vitro biosynthesis remains to be fully confirmed with other tissue studies. However, we suggest that it could represent a biosynthetic "marker", testifying that the tissue or cells used for an in vitro incubation are viable enough to synthesize this specific peptide. Accordingly, the absence of other sought biosynthetic peptides would not be attributable to impaired biosynthetic machinery in the in vitro incubation conditions. Indeed, in the experiments reported here with rat striatum and hypothalamus, we were unable to find significant amounts of beta-endorphin (manuscript in preparation) while ubiquitin was readily detectable. Both were present in bovine pars intermedia (20).

Regarding ubiquitin itself, this biosynthetic characterization could provide a useful tool to study the kinetics of its maturation and its regulation, and could help in the elucidation of its as yet unknown basic cellular function. It has been recently discovered (4) that the sequence of the amino terminal 37 residues of ubiquitin is identical to that of the non histone component of the nuclear protein A-24 complex, which contains a non histone polypeptide linked to a histone 2A molecule (5) and is present in nucleosomes (21). Such a large structural similarity implies that these two sequences share a common evolutionary origin or are the products of a single gene (4). The second alternative has been preferentially suggested (4) in view of the highly conserved structure of ubiquitin with a mutation acceptance rate as low as that of histone 2A: thus ubiquitin would be a cleaved metabolite of the nuclear non histone component of protein A24, which, after enzymatic cleavage,

would diffuse from the nucleus into the cytoplasm (4). The demonstration of active in vitro biosynthesis of ubiquitin could contribute to the understanding of its biosynthetic relationship to protein A24 and its biological function in regard to the possible roles of non histone chromosomal proteins, particularly in the control of expression of genetic information in eukaryotes (6-8).

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