Chemical synthesis of a hexadecapeptide segment of ubiquitin that activates adenylate cyclase and induces lymphocytes to differentiate

D.H. Schlesinger[1,2], G. Goldstein, Margrit P. Scheid and M. Bitensky

Endocrine Unit, Massachusetts General Hospital and Harvard Medical School, Boston (Massachusetts 02114, USA), Memorial Sloan-Kettering Cancer Center, New York (New York 10021, USA) and Department of Pathology, Yale University School of Medicine, New Haven (Connecticut 06510, USA), 31 October 1977

Summary. A hexadecapeptide corresponding to positions 59–74 of ubiquitin was synthesized and purified. The peptide was characterized by its mobility in TLC and electrophoresis, amino acid sequence and composition, and molar rotation. The peptide possessed approximately 40% activity compared with native ubiquitin in each of 3 biological assays in vitro: a) thymocyte induction, b) B cell induction and c) elevation of intracellular cyclic AMP levels in sarcoma 180 cells.

Ubiquitin, a 74 amino acid polypeptide chain[3], is probably represented universally in living cells since it has been found in animal tissues, yeasts, bacteria, and higher plants[4]. The physiological role of ubiquitin is unknown, although it has recently been reported as a component of the nuclear protein A-24 in covalent association with histone 2A[5,6]. Ubiquitin appears to contain a β-adrenomimetic active site in that it can induce intracellular cyclic AMP elevations in certain cell types, an effect blocked by the β-adrenergic blocking drug propranolol. Examples of these actions are in vitro induction of T cell (thymus-derived lymphocyte) and B cell (bone marrow derived lymphocyte) differentiation and elevation of adenylate cyclase and intracellular cyclic AMP in sarcoma 180 cells.

Materials and methods. The following materials were purchased: α-Boc-amino acids (Beckman Instruments and Bachem, Inc.), dicyclohexylcarbodiimide (Schwartz-Mann), fluorescamine (Roche Diagnostics), 200–400 mesh polystyrene resin containing 0.75 mmoles chloride/g resin (lab Systems, Inc.), RPMI 1640 medium and fetal calf serum (immunoprecipitin tested) (Grand Island Biological Co.), bovine serum albumin (35% pathocyte 5 lot 20 and 21) (Pentex, Inc.).

Healthy 5–6 weeks old athymic mice (nu/nu) of both sexes (Sloan-Kettering breeding colonies) (MSKCC) were bred on a BALC/c background (thymocytes expressing Thy-1.2 surface antigen) and maintained under conventional conditions. Anti-Thy-1.2 sera were prepared in Thy-1 congenic mice. Ubiquitin was isolated as described[4].

Synthetic method. Synthesis of the hexadecapeptide was performed according to the method of Merrifield[7]. The protected peptide was cleaved from the resin and side chain protecting groups were removed with anhydrous HF in anisole of 0 °C for 60 min. The peptide was purified by gel filtration on Bio Gel P6 and ion exchange chromatography on CM cellulose.

Analysis of the hexadecapeptide. Amino acid analysis following acid hydrolysis was performed as described previously[3]. Total enzymic hydrolysis using papain and leucine aminopeptidase M was performed according to the method of Keutman et al.[8]. The entire sequence of the hexadecapeptide was confirmed by manual Edman degradations[3]. 3 TLC systems were used to further evaluate the purity of the intact, deblocked synthetic hexadecapeptide: system I, 1-butanol (15), acetic acid (3), H_2O (12), pyridine (10); system II, 2-butanone (2), acetic acid (6), H_2O (5); system III, 1-propanol (7), 37% ammonium hydroxide (3). Thin layer electrophoresis was carried out on silica gel using pyridine acetate pH 3.5 buffer.

Molar rotation (Perkin Elmer Model 141 spectropolarimeter), $[M]_{689}^{24°}$, was -1110° (0.89 mM, 1.0 M AcOH). Molar extinction coefficient (Cary, Model 15 spectrometer) was 1690 with an absorption maximum at 277 nm.

Induction of thymocyte differentiation from prothymocytes in vitro was performed as described by Komuro and Boyse[9] and induction of the differentiation of B cells bearing receptors for complement (CR^+) from CR^- B cell precursors was performed as previously described. Determination of intracellular cyclic AMP levels in sarcoma 180 cells was performed as follows: Cells were incubated at 30 °C in vitro in aerated Tyrode's solution with or without polypeptide at various concentrations for 10 min. Incubations were terminated by freezing and then adding ethanol-hydrochloric acid (60:1). Intracellular cyclic AMP levels were measured by the method of Gilman[10] (figure 2).

Results and discussion. The synthetic hexadecapeptide was purified by chromatography on Bio-Gel P6 ion exchange chromatography on carboxy-methyl cellulose in 8 M urea and gel filtration on Bio-Gel P-2. A single major spot was found for the purified product in each thin layer system with the following values: system I, Rf = 0.57; system II, Rf = 0.71; system III, Rf – 0.42. Thin-layer electrophoresis

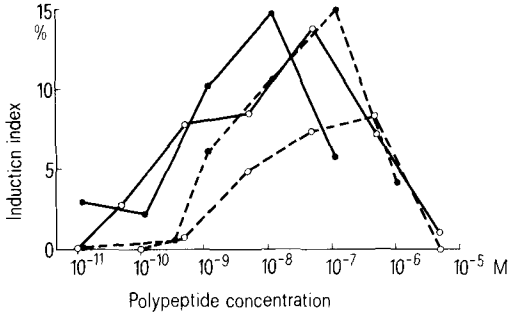

Fig. 1. Dose-response relationship of synthetic hexadecapeptide (open circles) and ubiquitin (solid circles) in the induction in vitro of prothymocyte-to-thymocyte differentiation (solid lines); dose-response relationship of synthetic hexadecapeptide (open circles) and ubiquitin (closed circles) in the induction in vitro of CR^- B cell-to-CR^+ B cell differentiation (dotted lines).

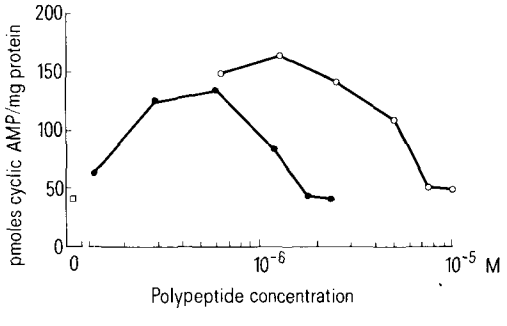

Fig. 2. Dose-response relationship of synthetic hexadecapeptide (open circles) and ubiquitin (closed circles) and the levels of intracellular cyclic AMP in sarcoma 180 cells.

at pH 3.5 revealed a single spot with an Rf of 0.80 compared to lysine.

Amino acid composition of the hexadecapeptide following acid hydrolysis was Asp(1.1), Thr(0.9), Ser(1.0), Glu(2.0), Val(1.0), Ile(1.0), Leu(4.2), Tyr(0.9), Lys(1.0), His(0.9), Arg(1.9). Complete removal of protecting groups was assessed by total enzymatic hydrolysis[8] revealing no unnatural amino acids and peptide extinction coefficient of 1690 with maximal absorbance at 277 nm.

The quantitative amount of truncated or failure sequences in the purified hexadecapeptide was determined by complete sequence analysis, a stringent technique of assessing purity[11,12]. The presence of deletion peptides for truncated sequences never exceeded 6% (cycle 8 of the degradation) on a molar basis, as evaluated by gas-liquid chromatography[13].

The dose-response relationships of the synthetic hexadecapeptide and ubiquitin are presented for prothymocyte-to-thymocyte differentiation (figure 1), CR^- B cell-to-CR^+ B cell differentiation (figure 1) and determination of intracellular cyclic AMP levels in sarcoma 180 cells (figure 2). The activity of the synthetic hexadecapeptide paralleled that of ubiquitin in each assay, showing approximately 40% activity by comparison with ubiquitin in each assay. For each assay the synthetic hexadecapeptide showed, like ubiquitin, a concentration range with maximal activity and, at higher concentrations, inhibition. The activity of the synthetic hexadecapeptide fragment of ubiquitin paralleled that of the parent molecule in all 3 assays. By contrast thymopoietin induces selective T cell differentiation in vitro[4] and a synthetic tridecapeptide fragment of thymopoientin (residues 24–41) also induced selective T cell differentiation in vitro, with no induction of CR^- to CR^+ B cell differentiation[15,16]. Additionally, the synthetic tridecapeptide segment of thymopoietin, like thymopoietin itself, produced impaired neuromuscular transmission in mice[17], an effect not caused by ubiquitin or the synthetic hexadecapeptide fragment of ubiquitin (data not shown).

Thus the synthetic peptide fragments of ubiquitin and thymopoietin showed contrasting biological activities, each having the biological characteristics of the parent molecules. We infer that each contains the amino acid sequence of the parent molecule involved in biological activity and that the putative epinephrin-mimetic active site of ubiquitin is present within the COOH-terminal 16 amino acids of the 74 amino acid sequence of ubiquitin and does not require the full tertiary structure of ubiquitin for biological activity in the assays studied. Yet there is rigorous evolutionary conservation of the entire amino acid sequence of ubiquitin, with complete identity between cattle and man[18] and close similarity of the NH_2-terminal sequence between these mammalian ubiquitins and ubiquitin isolated from the higher plant celery[4]. This suggests that there are constraints on amino acid substitutions in ubiquitin that are unrelated to the formation of an active site comprising an amino acid sequence capable of stimulating adenylate cyclase through β-adrenergic receptors. Thus, ubiquitin almost certainly has other vital function(s) (possibly nuclear[5,6]) which have contributed to its extraordinary evolutionary conservation in living cells, but which have not yet been detected in our biological assays.

1 Address for reprint requests: Department of Physiology and Biophysics, University of Illinois Medical Center, Post Office Box 6998, Chicago (Illinois 60680).

2 Acknowledgments. We thank Dr Geoffrey Tregear for helpful suggestions for the synthesis strategy, Dr Jim Burton for guidance with the methods for establishing peptide purity, and Ronald King, Miriam Miller, Jeanette Dilley and Linda Townley for excellent technical assistance. This work was supported by U.S. Public Health Service Grants CA-08748, CA-16889, CA-08415, CA-17085 and AI-12487, and by NCI Contract CB-53868.

3 D.H. Schlesinger, G. Goldstein and H.D. Niall, Biochemistry *14*, 2214 (1975).
4 G. Goldstein, M.P. Scheid, U. Hammerling, E.A. Boyse, D.H. Schlesinger and H.D. Niall, Proc. nat. Acad. Sci. USA *72*, 11 (1975).
5 L.T. Hunt and M.O. Dayhoff, BBRC *74*, 650 (1977).
6 Olson, Biol. Chem. *251*, 5901 (1976).
7 R.B. Merrifield, J. Am. chem. Soc. *85*, 2149 (1963).
8 H.T. Keutmann, J.A. Parsons and J.T. Potts, Jr, J. biol. Chem. *245*, 1491 (1970).
9 K. Komuro and E.A. Boyse, Lancet *1*, 740 (1973).
10 A.G. Gilman, Proc. nat. Acad. Sci. USA *67*, 305 (1970).
11 G.W. Tregear, J. van Rietschoten, E. Greene, N.D. Niall, H.T. Keutmann, J.A. Parsons, J.L.H. O'Riordan and T.J. Potts, Jr, Z. Physiol. Chem. *355*, 415 (1974).
12 G.W. Tregear, in: Peptides, p.177. Proc. 13th European Peptide Symposium, 1974.
13 J.J. Pisano and T.J. Bronzert, J. biol. Chem. *244*, 5597 (1969).
14 P. Edman, in: Protein Sequence Determination, p.211. Ed. S. Needleman. Springer, New York 1970.
15 D.H. Schlesinger and G. Goldstein, Cell *5*, 361 (1975).
16 D.H. Schlesinger, G. Goldstein, M.P. Scheid and E.A. Boyse, Cell *5*, 367 (1975).
17 G. Goldstein and D.H. Schlesinger, Lancet *2*, 256 (1975).
18 D.H. Schlesinger and G. Goldstein, Nature *255*, 423 (1975).

Inhibition of liver fructose 1,6-bisphosphatase activity by Zn^{2+}: Reversal by imidazole pyruvate[1]

P.F. Han, G.Y. Han[2], T.W. Cole, Jr, G.S. Owen and J. Johnson, Jr

Department of Chemistry, Atlanta University, Atlanta (Georgia 30314, USA), 27 December 1977

Summary. Imidazole pyruvate was found to be a very potent natural chelating agent in reversing the inhibition of liver fructose 1,6-bisphosphatase activity by Zn^{2+}. This metabolite may play a physiological role in gluconeogenesis.

The activity of fructose 1,6-bisphosphatase (Fru-P_2ase, EC 3.1.3.11) is markedly activated by a variety of chelating agents[3]. The basis of this activation is attributed to the chelation of the endogenous Zn^{2+}, a specific and very potent metal inhibitor of Fru-P_2ase[3,4]. Based on the observations that Zn^{2+} inhibition of Fru-P_2ase activity can be reversed by histidine at concentrations found in rabbit liver under gluconeogenic conditions[3,5], it was recently proposed that 'Zn^{2+} and histidine together may act to modulate the levels of Fru-P_2ase activity'[6]. We report here that imidazole pyruvate, which may exist in about the same concentration as histidine in liver cells[7], is significantly more potent than histidine in reversing the inhibitory effect of Zn^{2+}.

Materials and methods. Chelex 100 was obtained from Bio-Rad. Imidazole pyruvate, 1-methylhistidine, 3-methylhistidine were purchased from Calbiochem. Other chemicals were obtained from Sigma. Fru-P_2ase was purified from rabbit liver, and assayed at pH 7.5 in the presence of