Vol. 116, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 31, 1983 Pages 527-533

ELUCIDATION OF THE ANTIGENIC DETERMINANT IN LIPOTROPIN FOR TWO MONOCLONAL ANTIBODIES

B. M. Austen, R. Lorenz, M. Spitz and R. Thorpe

Department of Surgery, St. George's Hospital Medical School, Cranmer Terrace, London, SW17 ORE and National Institute for Biological Standards and Control, Holly Hill, London, England NW3 6RB

Received August 8, 1983

Two monoclonal antibodies were found to give enhanced affinity for β-lipotropin when mixed, as evidenced by competitive radioimmunoassay. Both monoclonals were found to react with a pentapeptide Ala-Glu-Leu-Glu-Tyr, which is a sequence of high local hydrophilicity within the N-terminal section of β-lipotropin.

Immunoassay of naturally occurring opiate-active peptides and their precursors is complicated by the presence in the same tissue of several, related, modified forms, each with distinct biological activity, which may share common antigenic determinants, and therefore cross-react when using conventional antisera (1, 2). The use of monoclonal antibodies (Mc/Abs) available through the development of hybridoma technology (3) overcomes some of these disadvantages. They are highly specific, and specificity of assay can be increased by using two antibodies raised against different sites in the same antigen in sandwich assays (4). We have recently described the production of a monoclonal antibody to β-lipotropin (5). In order to develop multi-site assays for β-lipotropin, or peptides derived from it, it is necessary first to define the precise loci of the antigenic determinants in β-lipotropin.

MATERIALS AND METHODS

Mc/Ab NIB$_n$ 63/17-2 was produced as described previously (5) NIB$_n$ 63/17-6 and other clones were produced from the same fusion by identical methods. Ascitic fluid containing Mc/Ab to β-lipotropin was produced by injecting twice cloned hybridomas into the peritoneal cavity of pristane primed Balb/c mice (10^7 cells/mouse).

0006-291X/83 $1.50

β-Lipotropin was purified from porcine pituitaries (5). N-Fragment (lipotropin residues 1 to 38) was purified by chromatography of acid-acetone extracts of porcine pituitaries on Sephadex G-50 in 30% (v/v) acetic acid, and then by immune affinity chromatography on immobilised IgG from Mc/Ab NIB_n 63/17-6 (5). Porcine β-melanocyte stimulating hormone and β-endorphin were obtained from Peninsula Laboratories. β-Lipotropin was iodinated with Na[^{125}I] (1 mCi) in 0.1M potassium phosphate (pH 7.0) (0.04 ml) with chloramine T (20 μg). After 15 sec, sodium metabisulphate (40 μg) was added, and after 10 min ^{125}I-lipotropin was desalted on a column (0.5 x 5 cm) of Sephadex G-10 in 50% acetic acid (500 cpm/fmole). Samples containing extracts of pituitaries or known amounts of peptides were incubated overnight in 0.2 ml of 0.05M sodium phosphate (pH 7.6), 0.25% (w/v) bovine serum albumin, 0.5% (v/v) 2-mercaptoethanol with dilutions of ascitic fluid at +4°C. ^{125}I-Lipotropin (10,000 cpm) in 0.1 ml of the same buffer was added, incubation continued for 1h, then 0.3 ml of buffer followed by 0.4 ml of 0.05M sodium phosphate (pH 7.6) containing activated, acid-washed charcoal (3% w/v) (Sigma), 70,000 $\bar{M}r$ dextran (0.75% w/v) (Sigma), and horse serum (60% v/v). After 10 min at 4°C, mixtures were centrifuged at 2,400 x g_{av} for 15 min, the supernatant removed by aspiration, and unbound ^{125}I-lipotropin determined on a γ-counter. ^{125}I-lipotropin non-specifically bound was determined as cpm pelleted in the absence of antibody, and subtracted.

β-Lipotropin (2.4 mg) was incubated with cyanogen bromide (500 fold excess) in 70% formic acid (0.5 ml) at room temperature overnight, diluted with 0.2M acetic acid, and lyophilised. Cyanogen bromide fragments were fractionated on a column (15 x 1 cm) of carboxymethyl-cellulose (CM 52; Whatman) in 0.02M sodium phosphate, eluting with a gradient up to 0.3M NaCl (total vol. 250 ml). One half of the unretarded peptide was concentrated to 1 ml, adjusted to pH 7.5 with 0.1M NaOH, digested with TPCK-trypsin (Millipore Corp.) (20 μg) at 30°C for 2h, and lyophilised. The digest was then subjected to HPLC on Aquapore RP-300 (25 x 4.6 mm) (Brownlee Labs.) in 0.1% trifluoroacetic acid, eluting with a gradient of acetonitrile. Amino acid analyses of peptides were performed on hydrolysates (6M HCl; 110°C; 20h) on an LKB 4400 amino acid analyser.

RESULTS

The ascitic fluids produced by two cloned hybridoma lines NIB_n 63/17-2 and 63/17-6 grown intraperitoneally in Balb-c mice were found to be satisfactory for radioimmunoassay, using dextran-coated charcoal to separate bound from free β-lipotropin. At 1:15,000 dilutions, both fluids either separately or mixed bound 30% of added ^{125}I-lipotropin. The ability of β-lipotropin to displace ^{125}I-lipotropin from the antibodies are compared in Fig. 1. By themselves, NIB_n 63/17-2 and 63/17-6 showed ED_{50} values of 21 nM and 32 nM respectively, but when

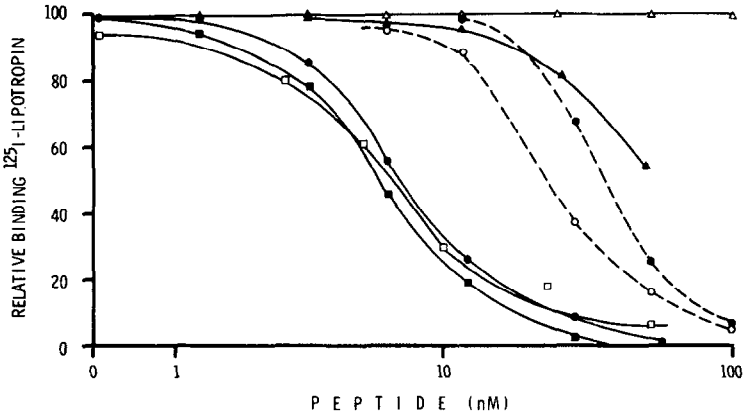

Fig. 1 Radioimmunoassay with monoclonal antibodies

Continuous lines show displacement of ^{125}I-lipotropin by lipotropin (●——●), cyanogen bromide fragment lipotropin residues 1 to 47 (■——■), N-fragment residues 1 to 38 (□——□), pentapeptide Ala-Glu-Leu-Glu-Tyr (▲——▲), and β-endorphin, β-melanocyte stimulating hormone and cyanogen bromide fragment (lipotropin residues 48 - 91) (△——△), using a mixture of Mc/Abs NIB$_n$ 63/17-2 and 63/17-6, and broken lines show displacement with lipotropin using 63/17-2 (○— —○) alone, and 63/17-6 (●— —●) alone.

mixed gave enhanced affinity (ED_{50} of 5.6 nM). Other combinations of antibodies from other clones did not yield enhancement.

Cyanogen bromide treatment of porcine β-lipotropin gave two fragments, which were isolated by chromatography on CM-cellulose. Amino acid analyses of the peptide that was not retarded by this ion-exchange resin (Table 1), and comparison with the known sequence of β-lipotropin (6), demonstrated that it was the N-terminal fragment (residues 1 to 47), while the C-terminal fragment (residues 48 - 91) eluted from the column with a molarity of 0.22M NaCl. Radioimmunoassay of fractions obtained from the column with a mixture of Mc/Abs NIB$_n$ 63/17-2 and 63/17-6 showed that only the N-terminal fragment was immunoreactive.

In order to define the antigenic determinant more precisely, the N-terminal cyanogen bromide fragment was digested with trypsin, and the resulting peptides were resolved by HPLC on an Aquapore RP 300 column by elution with a gradient of

TABLE 1

Amino acid analyses of fragments derived from lipotropin

	CNBr1	T2	T3	T4	T5	T6	N-Frag
Asp	1.98(2)	0.96(1)	0.87(1)		0.01(0)	0.01(0)	1.09 (1)
Glu	10.81(10)	1.12(1)	1.97(2)	2.10(2)	2.18(2)	3.21(3)	8.20(8)
Pro	5.51(6)	1.09(1)	1.96(2)	3.01(3)			4.99(5)
Gly	4.12(4)	1.00(1)	1.01(1)	1.24(1)		1.21(1)	3.45(3)
Ala	13.44(13)	0.01(0)	4.24(5)	2.89(3)	1.24(1)	3.94(4)	12.40(13)
Val	0.82(1)					0.97(1)	0.95(1)
Leu	2.88(3)		0.01(0)	0.92(1)	0.89(1)	1.02(1)	3.13(3)
Tyr	1.71(2)	0.49(1)			0.83(1)		0.71(1)
Lys	3.21(3)	1.81(2)	0.02(0)		0.02(0)	1.97(1)	0.01(0)
Arg	0.77(1)		0.78(1)				1.64(2)
Met	0.73(1)						

acetonitrile. The elution profile and corresponding radioimmunoassay are shown in Fig. 2. Only peptide T5 cross-reacted with Mc/Abs NIB_n 63/17-2 and 63/17-6, both when assayed separately, and together. Amino acid analyses showed that T2 to T6 accounted for all residues of the N-terminal cyanogen bromide fragment (Fig. 3), excluding a C-terminal homoserine residue, and that the immunoreactive T5 was the peptide Ala-Glu-Leu-Glu-Tyr corresponding to residues 24 to 28 in β-lipotropin. Cleavage between residues 28 (Tyr) and 29 (Gly) was presumed due to chymotryptic impurity in the trypsin preparation.

Relative affinities of peptides related to β-lipotropin for mixed Mc/Abs NIB_n 63/17-2 and 63/17-6 are expressed in Fig. 1. Both N-fragment (residues 1 to 38) and cyanogen bromide fragment (residues 1 to 47) have similar affinities to the parent molecule β-lipotropin, while the pentapeptide Ala-Glu-Leu-Glu-Tyr has reduced affinity (ED_{50} ∿80 nm). The C-terminal fragments, including β-endorphin, do not displace at 100 nM concentration.

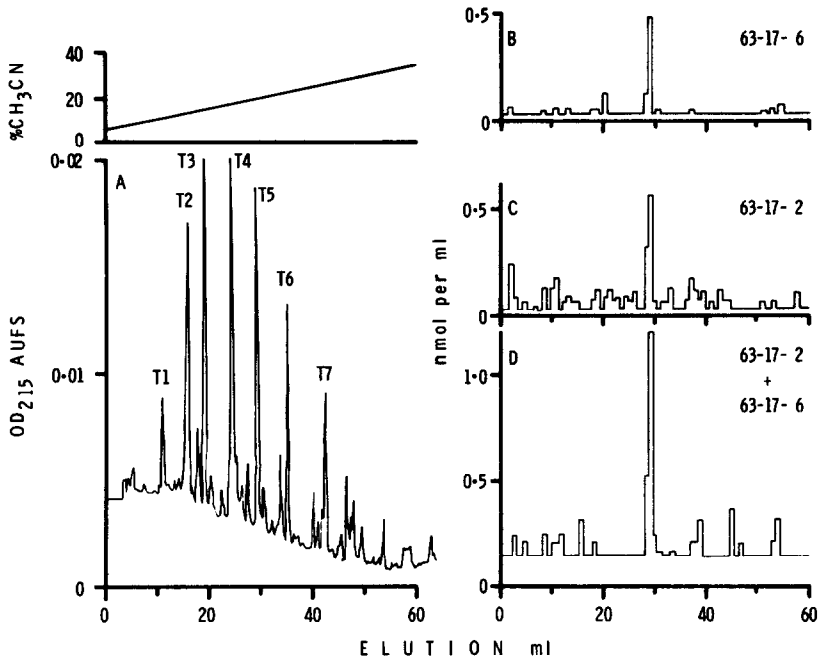

Fig. 2 Elution profile of tryptic digest of N-terminal cyanogen bromide peptide from lipotropin from HPLC on Aquapore RP-300, eluted with acetonitrile gradient (A), and corresponding radioimmunoassay with antibodies NIB$_n$ 63/17-6 (B), 63/17-2 (C) and a mixture of 63/17-6 and 63/17-2 (D).

DISCUSSION

Mixing Mc/Ab NIB$_n$ 63/17-2 and 63/17-6 gives increased binding to β-lipotropin over that given by each antibody singly. Enhancement has been observed previously between antibodies against different antigenic sites in the same antigen (7, 8). Although both Mc/Abs bind to the pentapeptide Ala-Glu-Leu-Glu-Tyr, it is possible that the actual determinant recognised in the

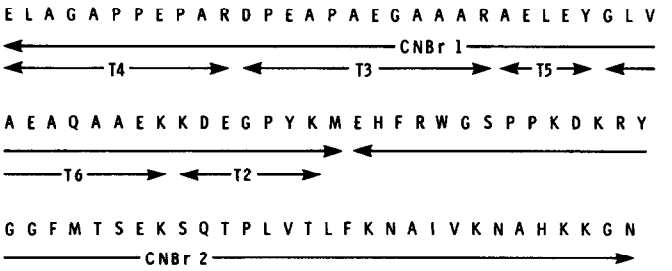

Fig. 3 Amino acid sequence, and arrangement of peptide fragments in lipotropin. Immunoreactive peptide is T5.

intact β-lipotropin molecule (and the 38K precursor) is a larger sequence involving flanking residues to the pentapeptide. It is conceivable that the two antibodies may bind this determinant in such a way that each can bind independently of the other, and give rise to enhancement by formation of tetramolecular complexes (7). Alternatively, an entirely different mechanism may operate to give enhancement.

X-ray diffraction studies (9) have shown that the antibody binding site is a pocket or cleft able to accommodate a peptide of five residues in β-sheet structure or ten residues as an α-helix. It is possible that each antibody might bind on opposite sides of an α-helical segment of polypeptide.

It has been suggested (10) that antigenic determinants are almost invariably found in hexapeptide sequences of the highest average hydrophilicity within a protein, and occasionally at regions of greatest local hydrophilicity within a restricted section of sequence. Calculations have shown that the hexapeptide of highest hydrophilicity in β-lipotropin comprises residues 38 to 43, which is C-terminal of the antigenic determinant. Ala-Glu-Leu-Glu-Tyr is, however, located at a region of high local hydrophilicity between residues 14 and 38. Secondary structural predictions (11) have shown that Ala-Glu-Leu-Glu-Tyr is located in a short sequence lying between residues predicted to adapt α-helical folding (residues 14 - 26 and 30 - 40). Development of sandwich assays using Mc/Abs NIB_n 63/17-2 and 63/17-6 in combination with other antibodies are in progress.

ACKNOWLEDGMENT

We gratefully thank Dr. J. A. Barchas for the support of one of us (R. Lorenz).

REFERENCES

1. Mains, R.E., Eipper, B.A. and Ling, N. (1977) Proc. Natl. Acad. Sci. USA 74, 3014-3018.

2. Zakarian, S. and Smyth, D.G. (1982) Nature 296, 250-252.

3. Köhler, G. and Milstein, C. (1975) Nature 256, 495-497.

4. Votila, M., Ruoslahti, E. and Engrevall, E. (1981) J. Immunol. Methods 42, 11-15.

5. Thorpe, R., Spitz, L., Spitz, M. and Austen, B.M. (1983) Febs. Lett. 151, 105-110.

6. Gráf, L., Barát, E., Cseh, G. and Sajgó, M. (1971) Biochim. Biophys. Acta. 229, 276-278.

7. Holmes, N.J. and Parham, P. (1983) J. Biol. Chem. 258, 1580-1586.

8. Ehrlich, P.H., Moyle, W.R., Moustafa, Z.A. and Canfield, R.E. (1982) J. Immunol. 128, 2709-2713.

9. Poljak, R.J., Amzel, L.M., Chen, Y.Y., Chiu, R.P., Phizackerley, R.P., Saul, F. and Ysern, X. (1976) Cold Spring Harbor Symp. Quant. Biol. 41, 639-645.

10. Hopp, T.P. and Woods, K.R. (1981) Proc. Natl. Acad. Sci. 78, 3824-3828.

11. Geisow, M.J. (1978) Febs. Lett. 87, 111-114.