

PREPARATION AND CHARACTERISATION OF A SPECIFIC ANTISERUM TO THE C-FRAGMENT OF LIPOTROPIN

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1. Introduction

The recent discovery that a series of lipotropin fragments possess opiate activity [1–3] has led to extensive investigation of the central properties of these peptides and discussion of their possible physiological roles. It has been suggested that each of the peptides is elaborated to perform a distinct function, methionine enkephalin (lipotropin 61–65) as a neurotransmitter [4], γ -endorphin and α -endorphin (61–77 and 61–76) in the maintenance of behavioural states [5] and C-Fragment (61–91) in pain perception [6]. However, the unique potency exhibited by C-Fragment in its central activities [7] would seem to imply that this is the physiologically significant peptide.

A recent immunofluorescent study provided evidence that the enkephalins occur in the synaptic regions of nerve tracts in brain [8] but cross reactivity of the antisera with the longer peptides was not examined and the evidence could indicate that C-Fragment or γ -endorphin is present at the nerve junctions. Furthermore it has been shown that the enkephalins and endorphins can be formed by extracellular proteolysis of C-Fragment [9] and it is possible that the occurrence of the shorter peptides may reflect the occurrence of C-Fragment.

This communication describes the preparation and study of an antiserum which has a high specificity for human lipotropin and for C-Fragment. It does not react significantly with γ -endorphin or methionine enkephalin. The availability of this antiserum will open the way to study of the distribution of C-Fragment in specific regions of brain.

2. Materials and methods

2.1. Preparation of antigen and peptides used in specificity study

Human lipotropin was isolated from pooled pituitary glands [10]. Porcine lipotropin (1–91), γ -lipotropin (1–58), C-Fragment (61–91) and C'-Fragment (61–87) were isolated from porcine pituitary as described previously [2]. Human C-Fragment was prepared by Dr R. C. Sheppard and Dr E. Atherton by a modified solid phase synthesis [11]; its *in vitro* binding properties to brain opiate receptors and its analgesic properties in the rat were similar to those of natural porcine C-Fragment. Methionine enkephalin and the hexapeptide 86–91 were prepared by solid phase synthesis [12] and purified by ion-exchange chromatography on CM-Sephadex C25 and DEAE-Sephadex A-25 and by gel filtration on Sephadex G-15 in 50% (v/v) acetic acid. γ -Endorphin and a tetradecapeptide (lipotropin 78–91) were obtained by rennin digestion of porcine C-Fragment [13]; the peptides were purified by gel filtration on Sephadex G-50 in 50% acetic acid and by high voltage electrophoresis on paper, at pH 1.9. [125 I]C-Fragment (approx. 400 μ Ci/ μ g) was prepared by chloramine-T oxidation using a procedure similar to that reported for the labelling of ACTH [14]; the peptide was purified on a column of Sephadex G-10.

2.2. Immunization

Antisera to human lipotropin were raised by irregular multisite injections in random-bred New Zealand white rabbits using an emulsion of purified lipotropin in 10 mM sodium phosphate with

complete Freund's adjuvant (1:1). Injections were given over a period of 3 months, a total of 120 µg of human lipotropin being administered to each animal. Blood was collected at intervals after the last injection.

2.3. Brain extracts

Rats were stunned and decapitated; the brains were rapidly removed. After excision of pituitary, cerebellum and brain stem, the brain tissue was homogenized at 4°C in 10 ml acid acetone (acetone/H₂O/HCl, 5:40:1). The homogenate was centrifuged at 20 000 × *g* for 10 min and the supernatant evaporated in vacuo. The residue was examined by radioimmunoassay.

2.4. Radioimmunoassay procedure

Incubations were performed in 0.05 M sodium phosphate (final vol. 300 µl) at pH 7.6, containing 0.25% human serum albumin (A. B. Kabi, Stockholm, Sweden), 0.5% mercaptoethanol and [¹²⁵I]C-Fragment (20 000 cpm, approx. 20 pg). Antibody was used at a dilution of 1:1000, giving 50–60% saturation of binding. The concentrations of the peptide solutions were determined by amino acid analysis. Serial dilutions of each peptide were performed and incubations carried out for 24 h at 4°C in the presence of labelled C-Fragment and antiserum. Unbound peptide was adsorbed on charcoal by addition of 200 µl of a suspension of activated charcoal (3 g Sigma Chemical Co., St Louis, Mo.) and dextran, mol. wt 73 000 (0.75 g, Sigma Chemical Co., St Louis, Mo.) in 10 ml 0.5 M phosphate, at pH 7.6, and 60 ml horse serum (Difco Labs., West Molesey, Surrey) diluted with H₂O to 100 ml. The charcoal was separated by centrifugation at 4°C for 15 min at 3000 × *g*, the supernatant was removed by aspiration and the radioactive peptide assayed with the aid of a γ-ray spectrometer.

3. Results and discussion

Porcine C-Fragment and human C-Fragment exhibited a high affinity for the antiserum (table 1). Porcine lipotropin (1–91), the COOH-terminal tridecapeptide of porcine lipotropin (79–91), and porcine C'-Fragment (61–87) displayed significant binding properties but the affinities were lower than that of

Table 1
Cross reactivity of antiserum to fragments of porcine lipotropin

Peptide	Lipotropin residue (No.)	Relative affinity (%)
C-Fragment	61–91	100
C'-Fragment	61–87	9.8
Tetradecapeptide	78–91	3.0
γ-Endorphin	61–77	<0.1
Methionine enkephalin	61–65	<0.1
Lipotropin	1–91	71.4
γ-Lipotropin	1–58	<0.1

Peptides were tested in doses of less than 24 pmol. Antiserum was used at a dilution of 1:1000, with displacement of ¹²⁵I-labelled porcine C-Fragment.

C-Fragment. Methionine enkephalin, γ-endorphin and lipotropin did not bind to a significant extent (fig. 1). The results show that the antigenic site in C-Fragment must be located in the COOH-terminal region of the peptide and it appears that the COOH-terminal tetrapeptide represents a portion of the antigenic site since C'-Fragment was less potent than C-Fragment. That human C-Fragment exhibited binding properties equivalent to those of the porcine peptide indicates that the two replacements (isoleucine for valine at position 82 and tyrosine for histidine at position 87) are not involved in the interaction with antiserum, either directly or by influencing the disposition of neighbouring residues.

When ¹²⁵I-labelled human or porcine C-Fragment was used with antiserum at a dilution of 1:1000, it was possible to detect less than 0.02 pmol C-Fragment (as C-Fragment or lipotropin). When ¹²⁵I-labelled human lipotropin was used in place of the labelled C-Fragment, at an antiserum dilution of 1:22 000, there was no cross-reaction with human or porcine C-Fragment and less than 0.03 pmol human lipotropin could be detected. Thus this single antiserum provides a sensitive and specific assay for both human lipotropin and human C-Fragment. Clearly the lipotropin antibody has a greater affinity for the NH₂-terminal region of human lipotropin than for the COOH-terminal region and at low antibody concentration the NH₂-terminus is the sole determinant. It is interesting to note that when labelled porcine lipotropin

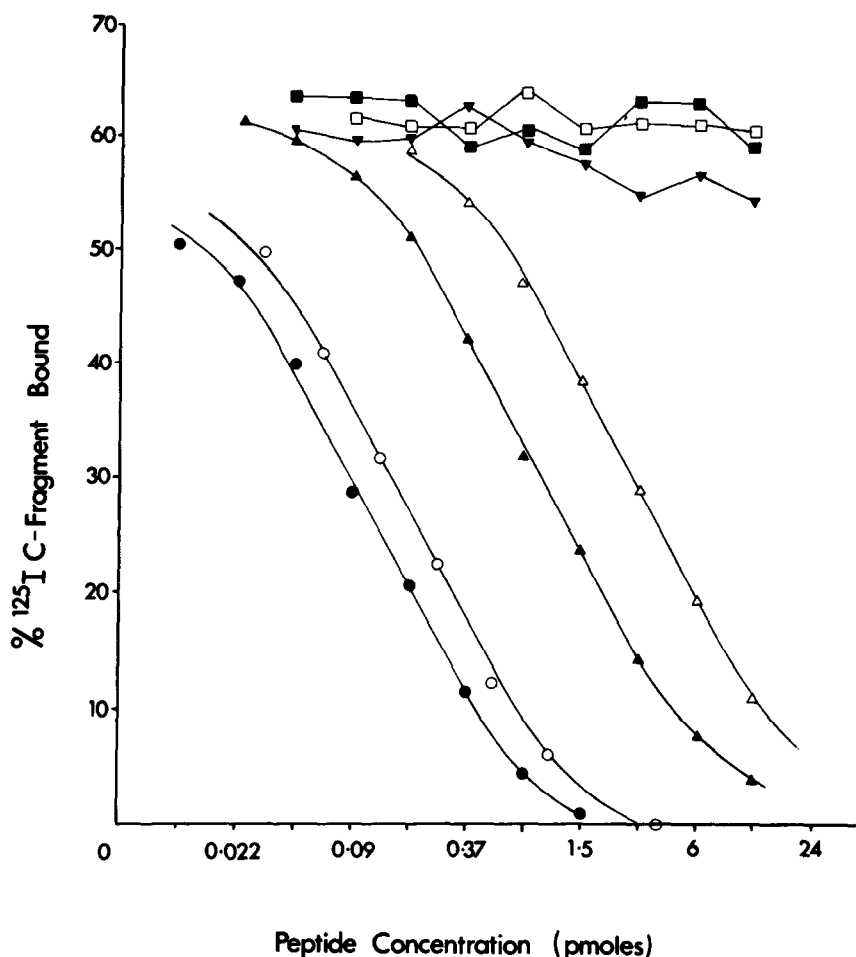


Fig.1. Inhibition of binding of ^{125}I -labelled porcine C-Fragment to antiserum by lipotropin peptides. Antiserum was used at a dilution of 1:1000. Porcine C-Fragment (●—●), porcine lipotropin (○—○), C'-Fragment (▲—▲), lipotropin 78-91 (△—△), methionine enkephalin (▼—▼), γ -endorphin (□—□) and γ -lipotropin (■—■).

was used, the NH_2 -terminal specificity was not observed. This can be attributed to the large number of differences in sequence which exist in the NH_2 -terminal region of human and porcine lipotropins.

Evidence was obtained that C-Fragment or lipotropin is present in rat brain. When brain or cortex homogenates were examined by radioimmunoassay with [^{125}I]C-Fragment, it was found that the displacement curves were similar to that of authentic C-Fragment. Further study will reveal the concentrations of C-Fragment in tissue, plasma and cerebrospinal fluid.

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