Detection and affinity purification of β-endorphin precursors using a monoclonal antibody

Robin Thorpe, Lidia Spitz, Moises Spitz and Brian M. Austen

National Institute for Biological Standards and Control, Holly Hill, Hampstead, London NW3 6RB, and *Department of Surgery, St George's Hospital Medical School, Tooting, London SW17 0RE, England*

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A monoclonal antibody to porcine β-lipotropin has been produced which binds to the N-terminal (γ-lipotropin) portion of the molecule. The antibody can be used to detect β-lipotropin as well as other β-endorphin precursors (predominantly a M_r 38000 polypeptide) using radiobinding assay or the immunoblotting technique. Purification of the peptides can be readily achieved by affinity chromatography using the monoclonal antibody covalently bound to Sepharose 4B. As the antibody recognises the N-terminal part of β-lipotropin, it can be used to detect and purify β-lipotropin and other β-endorphin precursors in the presence of β-endorphin.

β-Endorphin precursor *β-Lipotrorin* *Monoclonal antibody* *β-Immunoblotting* *Radiobinding assay* *Affinity chromatography*

1. INTRODUCTION

In cultured mouse pituitary cells, the lipolytic peptide hormone β-lipotropin, and corticotropin (ACTH) are synthesised by the processing of a common precursor, M_r 32000, called pro-opiomelanocortin [1]. Analysis of corresponding mRNA from human, bovine and rat pituitaries has allowed the sequence of an even larger precursor, containing an additional signal sequence at its amino terminus, to be deduced [2–4]. β-Lipotropin is itself cleaved to give rise to γ-lipotropin, and the powerful opiate peptide β-endorphin, or modified forms of β-endorphin which are physiologically less active [5–7]. The study of these peptides would be facilitated by the development of a quick and efficient purification procedure and a sensitive specific method for their detection. We have employed immunochemical methods in order to achieve these objectives and have used a monoclonal antibody, as the exquisite specificity of such reagents as well as their inexhaustible supply, makes them superior to conventional polyclonal antisera [8]. The monoclonal antibody can be used for rapid and efficient affinity purification of porcine β-lipotropin and related peptides and for detection of these molecules using radiobinding assay or the immunoblotting technique [9]. The antibody binds to the N-terminal (γ-lipotropin) portion of β-lipotropin, and thus can be used to specifically detect β-lipotropin and precursor peptides in the presence of β-endorphin.

2. MATERIALS AND METHODS

2.1. *Preparation of β-lipotropin*

Porcine β-lipotropin used for the immunization of Balb/c mice and for screening hybridoma supernatants, was purified as in [10] and shown to be homogeneous by amino acid analysis. The peptide ran as a major band on polyacrylamide gel electrophoresis performed in SDS and urea, after visualization with Coomassie blue.

2.2. *Monoclonal antibody production*

For monoclonal antibody production, Balb/c mice were injected subcutaneously in 3 sites with β-lipotropin (100 μg), emulsified with Freund's complete adjuvant and boosted by intraperitoneal

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injection after 15 days with 50 μg antigen in phosphate-buffered saline. Animals were boosted a second time after a further 15 days, and cell fusion carried out 4 days later. Fusion of spleen cells from the immune donor with NSO/1 myeloma cells was carried out as in [11]. After fusion, the cells were cultured in five 24-well tissue culture plates, and hybridomas was selected in HAT medium. After culture for 1 week, 60% of the wells contained hybridomas and 75% of these grew to confluence. Wells containing hybridomas were assayed when confluent for antibodies directed against β-lipotropin using a solid phase radiobinding assay (see section 2.3). About 40% of the wells were positive and these were cloned in soft agar. Positive clones were recloned and then cryopreserved, grown in culture to produce antibody, containing tissue culture fluid, and injected intraperitoneally into Balb/c mice to produce ascitic fluid containing a high concentration of antibody. Monoclonal antibody heavy chain class was determined by biosynthetic radiolabelling of the hybridoma secretion products with tritiated leucine followed by analysis of the material by SDS–polyacrylamide gel electrophoresis and fluorography.

2.3. *Preparation of acid/acetone extract of pituitary*

Acid/acetone extraction of pig pituitary [6] was carried out by homogenising 5 pituitary glands in 4 ml of ice-cold acetone–water–concentrated HCl (40:5:1), centrifuging for 15 min at $1000 \times g_{av.}$ and precipitating proteins from the supernatant by the addition of 19 vol. ice-cold acetone. The precipitate was collected by centrifugation for 10 min at $1000 \times g_{av.}$ and the precipitate dissolved in PBS. The pH was then adjusted to 6.5 using 2 M NaOH and unwanted insoluble material removed by centrifugation as above.

2.4. *Solid-phase radiobinding assay*

Solid-phase radiobinding assay for β-lipotropin was carried out as follows. 50 μl of β-lipotropin dissolved in PBS (100 μg/ml) was pipetted into each well of a 96-well flexible tissue culture plate and the plate incubated for 18 h at room temperature. The wells were then washed 3-times with phosphate-buffered saline containing 3% bovine haemoglobin (Hb–PBS) and incubated for 30 min at room temperature with 50 μl of this solution to block unoccupied protein-binding sites on the well surfaces. After a further wash with Hb–PBS, dilutions of monoclonal antibody made in Hb–PBS were added to the wells (45 μl/well) and incubated for 1 h. After 3 washes with Hb–PBS, 45 μl of ^{125}I-labelled goat anti-mouse F(ab')$_2$ diluted in Hb–PBS were added to each well (10^5 cpm/well), and the plate incubated for a further h. After washing 4-times with Hb–PBS, individual wells were cut out using a hot-nichrome-wire plate cutter, and then counted in a gamma counter. All incubations were carried out at room temperature, and counts obtained with dilutions of normal mouse serum instead of monoclonal antibody were used as a negative control.

Radiobinding assay for β-endorphin, or β-lipotropin and precursor peptides present in acid/acetone extract of pituitary gland [6] was carried out by substituting these materials for β-lipotropin at the coating stage.

2.5. *Affinity chromatography*

Monoclonal antibody was prepared from ascitic fluid by isolation of the precipitate produced by 45% saturation with ammonium sulphate. This precipitation was repeated and the precipitate dissolved on 0.1 M solium citrate buffer (pH 6.5). The protein concentration was adjusted to 1 mg/ml and 10 ml of this solution exhaustibly dialysed against the same buffer. This protein was coupled to 1 g of Sepharose 4B as in [13] except that the coupling buffer was 0.1 M sodium citrate buffer (pH 6.5). This material was packed in a column, equilibrated with PBS at 4°C and β-lipotropin and precursor peptides were purified by affinity chromatography. For this, 4 ml of 20% (w/v) pituitary homogenate prepared in PBS or acid/acetone extract of pituitary [6] adjusted to pH 7, was allowed to slowly pass through the column and 2 ml-fractions were collected. The column was then washed with cold PBS containing 1 mM phenylmethylsulphonyl fluoride (PMSF) until the A_{280} fell to 0.001. Bound peptides were then eluted from the column using 0.1 M glycine–HCl (pH 2.0) containing 1 mM PMSF at room temperature, again collecting 2 ml-fractions, and the pH of the fractions were immediately adjusted to pH 7.0 using 1 M Tris. When the A_{280} fell to near zero, the column was equilibrated with cold PBS containing 0.05% sodium azide, and stored at 2–4°C.

2.6. *Polyacrylamide gel electrophoresis (SDS–PAGE)*

This was carried out using 12.5% (w/v) polyacrylamide slab gels and a buffer system as in [12].

2.7. *Immunoblotting*

This was carried out essentially as in [9]. Proteins were transferred from SDS polyacrylamide gels to nitrocellulose sheets using an Electroblot EC 215 transfer system (EC Apparatus Corporation, St Petersburg, FL). A transfer time of one h was used at full power, and after transfer, remaining protein-binding sites on the sheet were blocked by incubation for 30 min with 3% bovine haemoglobin, dissolved in PBS (Hb–PBS). The blot was then incubated overnight with 30 μl of ascitic fluid containing monoclonal antibody, diluted in 30 ml Hb–PBS, then washed for 30 min with 5 changes (100 μl each) of Hb–PBS and incubated for 3 h with ^{125}I-labelled goat anti-mouse F(ab')$_2$ (10^6 cpm) diluted in 30 ml Hb–PBS. After washing for 30 min with 5 changes of PBS the blot was dried and exposed to X-ray film for two days in a cassette equipped with a fast tungstate intensifying screen.

2.8. *Cyanogen bromide cleavage of β-lipotropin*

This was carried out as in [14].

3. RESULTS AND DISCUSSION

One monoclonal antibody (NIBn 63/17-2) which reacted strongly with β-lipotropin was selected for characterization. It was of the IgG class, and it was found that passage of the hybridoma cells in the peritoneal cavity of Balb/c mice produced a cell-line which grew faster in culture.

Radiobinding assays showed that the monoclonal antibody reacted with purified porcine β-lipotropin and with material isolated from pig pituitary enriched in lipotropin by extraction into acid–acetone [6] (fig.1). Using this technique the antibody failed to react with porcine β-endorphin (fig.1), suggesting that the determinant recognised was in the N-terminal (γ-lipotropin) part of the β-lipotropin molecule.

Affinity purification of β-lipotropin and precursor peptides was readily achieved using a column of immobilised NIBn 63/17-2. Important modifications to the conventional affinity chromatography procedures were that the antibody was coupled to activated sepharose at pH 6.5 (rather than pH 8.5), and that the material bound to the affinity column was eluted with buffer pH 2.0 (rather than pH 2.8). These modifications were found to be essential for successful affinity chromatography. Initially β-lipotropin was affinity-purified by applying to the immobilised antibody column, homogenates of pituitaries made in phosphate-buffered saline, but the large amounts of protein and lipid present in this material clogged the column. Consequently, it was found that rapid and more efficient purification was possible if the acetone-precipitated, acid–acetone extract [6], dissolved in PBS and adjusted to pH 7, was used for affinity chromatography, and under these latter conditions the column could be re-used many times (fig.2a). Analysis of the material eluted at pH 2.0 using SDS–polyacrylamide gel electrophoresis, and staining with Coomassie blue, generally showed one band running in an identical position to conventionally purified physiologically active β-lipotropin (fig.2b). Occasionally, other products of lower M_r, possibly N-terminal fragments of β-lipotropin, were also eluted from the column.

The monoclonal antibody was used to detect β-lipotropin and other related polypeptides by the

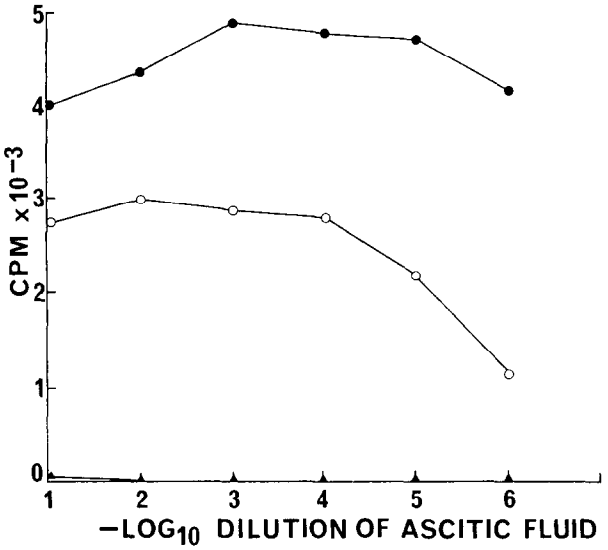

Fig.1. Solid-phase radiobinding assay using monoclonal antibody NIBn 63/17-2. β-Lipotropin (●), β-endorphin (▲), or acid–acetone extract of pituitary gland (○) were used to coat the wells of the tissue culture plate (see text).

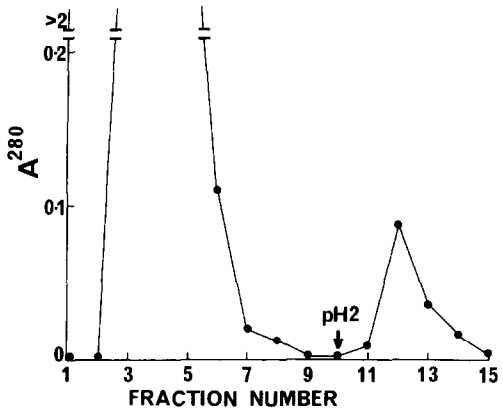

Fig.2(a). Affinity chromatography of porcine pituitary acid–acetone extract [6] on a column of Sepharose 4B to which monoclonal antibody NIBn 63/17-2 was covalently attached (see text).

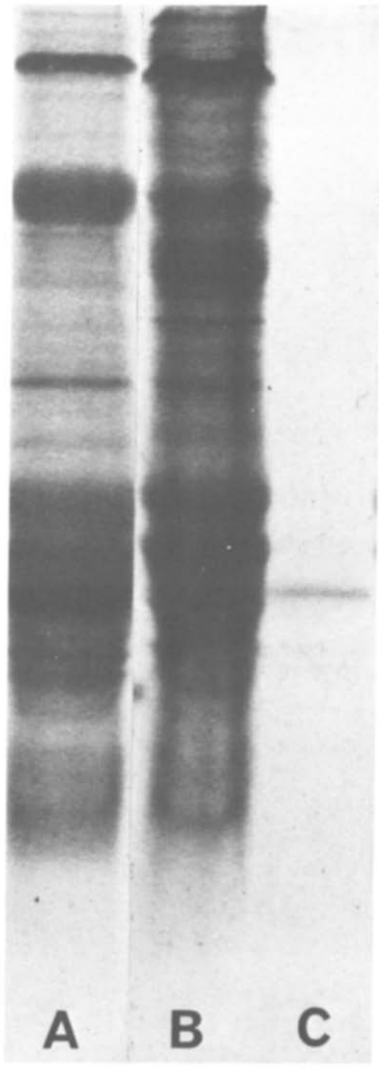

Fig.2(b). SDS-polyacrylamide gel electrophoresis of: (A) acid/acetone extract of pig pituitary; (B) whole pig pituitary; and (C) affinity purified peptides (see fig.2(a)).

immunoblotting technique. Immunoblotting of an extract of pig pituitary, made directly in electrophoresis sample buffer containing SDS and mercaptoethanol, demonstrated the presence of β-lipotropin, and also a strongly reacting protein of app. M_r 38000 (fig.3). Often, small amounts of peptides migrating in between the positions of this protein and β-lipotropin and below the latter were also seen (fig.3). Immunoblotting of polypeptides eluted from the antibody-affinity column also showed the presence of β-lipotropin and a very small amount of the M_r 38000 immunoreactive protein (fig.4). This latter protein was not visualised by Coomassie blue, presumably because the small quantities present produced insufficient staining. The M_r 38000 protein was also detected by immunoblotting using a polyclonal anti-ACTH antibody, showing that it is a common precursor of ACTH and β-lipotropin, and likely to be analogous to the proopiomelanocortins found in other species. One or two proteins of intermediate M_r, which may represent intermediates in the processing of the M_r 38000 form into β-lipotropin, were also seen (fig.4).

Of the peptides released by cyanogen bromide cleavage of β-lipotropin, only the N-terminal fragment (β-lipotropin residues 1–47) was detected by immunoblotting with the monoclonal antibody, whilst the C-terminal (β-endorphin residues 61–91) failed to react. Moreover, no cross-reaction was found with rat β-lipotropin, which differs considerably from pig β-lipotropin in its N-terminal sequence [4] confirming that the antigenic determinant is located in the N-terminal region of the porcine β-lipotropin molecule. Thus, the antibody provides a useful and specific reagent for detection and quantification of peptides containing the N-terminal sequence of β-lipotropin, and can be used to specifically detect these molecules in the presence of β-endorphin. The antibody has been

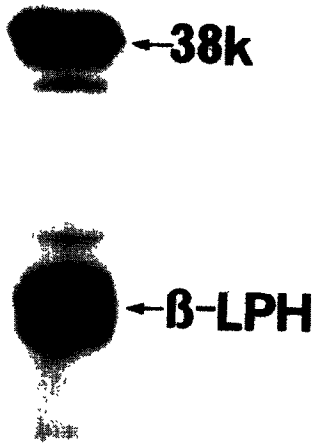

Fig.3. Immunoblot of whole pig pituitary extracted in electrophoresis sample buffer [12] using monoclonal antibody NIBn 63/17-2. β-LPH = β-lipotropin; 38k = M_r 38000 protein.

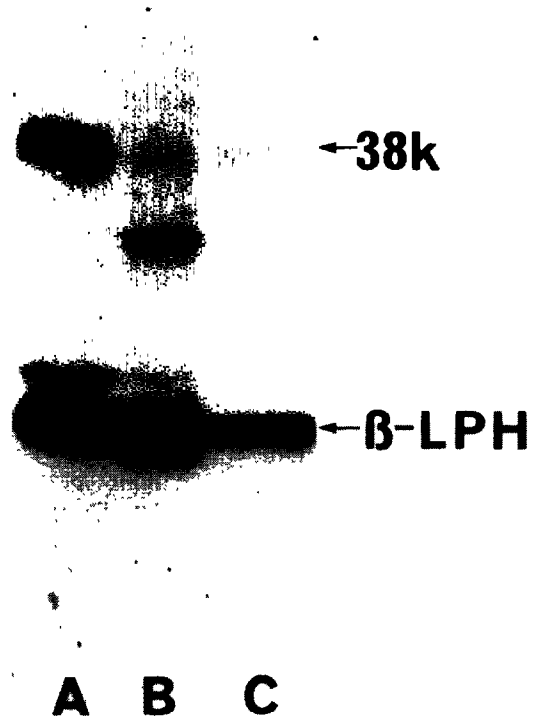

Fig.4. Immunoblot of: (A) porcine acid–acetone extract; (B) and (C) affinity-purified peptides (see fig.2 – two different preparations) using monoclonal antibody NIBn 63/17-2. β-LPH = β-lipotropin; 38k = M_r 38000 protein.

shown to be useful in achieving a rapid affinity purification of β-lipotropin and other precursors of β-endorphin, and the one-step method devised is preferable to the lengthy purification procedures which involve multiple chromatographic steps.

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