Isolation and partial purification of a melanocyte-stimulating hormone receptor from B16 murine melanoma cells

A novel approach using a cleavable biotinylated photoactivated ligand and streptavidin-coated magnetic beads

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The α -melanocyte-stimulating hormone (α -MSH) receptor of B16 mouse melanoma cells was characterized by photoaffinity labelling using radiolabelled photoactive derivatives of α -MSH. A doublet band of 43–46 kDa representing a ligand–receptor complex was identified. A novel adaptation of the streptavidin/biotin-based affinity system was used to isolate the α -MSH receptor. A probe was synthesized which contained biotin connected to a photolabelled α -MSH analogue via a cleavable disulphide linker and which displayed high affinity for the α -MSH receptor. Streptavidin-coated magnetic beads were used as a solid support instead of an affinity column. Covalently linked probe–receptor complexes solubilized in Triton X-100 were equilibrated with the beads, and after magnetic separation and washing, specifically bound complexes were treated with dithiothreitol to cleave the disulphide bridge in the biotin–peptide spacer arm and so release the receptor–ligand complex. The identity of the isolated protein was established by SDS/PAGE analysis. Methods to achieve purification to homogeneity and to allow quantitative isolation of the receptor are discussed.

INTRODUCTION

α -Melanocyte stimulating hormone (α -MSH; Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH₂) is a 13-amino-acid-peptide produced in the pituitary by post-translational cleavage of pro-opiomelanocortin. It is known to be a potent stimulator of pigment dispersion in lower vertebrates and to induce melanogenesis in various mammalian cells. It is also recognized as having a role as a neuropeptide, exhibiting a wide range of behavioural and electrophysiological effects in mammals. The mechanism of action of α -MSH is thought to be receptor-mediated. It binds to the cell surface of cultured melanoma cells, raising the intracellular concentration of cyclic AMP. This in turn produces an increase in tyrosinase activity, leading to an increase in melanin content (Eberle, 1988).

Structure–activity relationships for a number of analogues and conjugates of α -MSH have been established (Eberle, 1988). The analogue [Nle⁴,D-Phe⁷] α -MSH, first described by Sawyer *et al.* (1980) has been shown to have binding affinity and biological activity of the order of 10 $\times$ that of α -MSH (Eberle, 1988), and is routinely used in our laboratory for investigations into the binding efficiency of various analogues and derivatives of the native hormone. Structure–activity relationships have established that alterations to the N-terminus of the peptide do not abolish activity (Eberle, 1988). The relative ease of chemical substitution to the N-terminus makes it an ideal site for the attachment of non-amino acid groups such as biotin. N^α-Biotin-[Nle⁴,D-Phe⁷] α -MSH was first prepared by Chaturvedi *et al.* (1984) and was shown to retain a high specificity for the receptor.

The technique of photoaffinity labelling has become an important tool for the identification and characterization of hor-

mone receptor proteins (Hazum, 1983; Eberle, 1989). The photoaffinity label we chose for this study was 4-(1-azi-2,2,2-trifluoroethyl)benzoic acid (ATB), a diazirine derivative first introduced by Nassal (1983). Previously, the existence of α -MSH receptors on various mouse and human cell lines has been demonstrated by photoaffinity labelling using radiolabelled photoactive peptide analogues (Scimonelli & Eberle, 1987; Solca *et al.*, 1989). Full characterization of the interaction of the peptide with its receptor, however, will only be possible if the receptor proteins can be isolated and purified. This has yet to be achieved for the receptor to α -MSH.

In this paper, we describe the development of a new technique for the isolation and purification of α -MSH receptors from cultured melanoma cells. The two main features of the method are as follows: (a) The application of the streptavidin/biotin affinity system using a functionally unimpaired cleavable biotinyl derivative of α -MSH as the affinity ligand. (b) The use of magnetizable beads as a solid support instead of the classical affinity chromatography column. These beads are non-porous, uniform, supermagnetic polystyrene particles. Originally developed for immunoassays, the use of magnetic beads in combination with the biotin/streptavidin system has more recently found wider application in molecular biology (Uhlán, 1989).

Until now, attempts to purify hormone receptors have been greatly hindered on the one hand by their paucity, and on the other by the relatively low yield obtained using classical affinity chromatography. The method we have developed for the isolation of the α -MSH receptor is powerful and highly reproducible. It could also be easily scaled up to provide sufficient material for sequence determination. The isolation method is presented diagrammatically in Fig. 1.

Abbreviations used: α -MSH, α -melanocyte-stimulating hormone; ATB, 4-(1-azi-2,2,2-trifluoroethyl)benzoic acid; HOBT, hydroxybenzotriazole; TFA, trifluoroacetic acid; DIC, di-isopropylcarbodi-imide; EMS, ethylmethyl sulphide; EDT, ethanedithiol; NHS-Biotin, N-hydroxysuccinimidyl biotin; NHS-SS-Biotin, sulphosuccinimidyl 2-(biotinamido)ethyl-1,3-dithiopropionate; PBS, phosphate-buffered saline; PMSF, phenylmethane-sulphonyl fluoride; NEM, N-ethylmaleimide; DTT, dithiothreitol.

Fig. 1. Diagrammatic representation of the isolation of α-MSH receptors using N^2 -Biotin-SS-[Nle⁴, D-Phe⁷, ATB-Lys¹¹]α-MSH

EXPERIMENTAL

Peptide synthesis

Amino acid derivatives, polydimethylacrylamide-Kieselguhr resin (Pepsyn K) and *p*-[*R*,*S*-α-1(9H-fluoren-9-yl)methoxyformamido-2,4-dimethoxybenzyl]phenoxyacetic acid (AM-linker) were purchased from MilliGen/Bioscience, Watford, Herts., U.K. Hydroxybenzotriazole (HOBT), trifluoroacetic acid (TFA), diisopropylcarbodi-imide (DIC), ethylmethyl sulphide (EMS), ethanedithiol (EDT), anisole and phenol were bought from Aldrich, Gillingham, Dorset, U.K. *N*-Hydroxysuccinimidyl biotin (NHS-Biotin) and sulphosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate (NHS-SS-Biotin) were supplied by Pierce, Chester, U.K. ATB came from Bachem Feinchemikalein, Bubendorf, Switzerland.

Peptides were assembled on either a CRB Pepsynthesiser II semi-automatic machine or a Milligen 9050 fully automatic synthesizer. H.p.l.c. columns were prepared by packing stainless steel columns with Techsphere 10 μm ODSWP300 (PTS 031WP300) (HPLC Technology, Macclesfield, Cheshire, U.K.)

as the stationary phase. A 4.6 mm (i.d.) × 25 cm column was used for analytical work and a 10 mm (i.d.) × 25 cm column was used for semi-preparative work.

All the peptides used in this study were prepared by solid-phase synthesis using the Fmoc strategy as developed by Atherton & Sheppard (1989). The peptides were prepared as their carboxamide forms using the AM-linker on Pepsyn K resin. All of the amino acid reagents were used as their pentafluorophenyl (Pfp) esters with the exception of serine, for which the 3,4-dihydro-4-oxobenzotriazine-3-yl (Dhbt) ester was employed, and Fmoc-D-Phe-OH, which was treated with DIC and HOBT to form its HOBT active ester *in situ*. Side chains were protected as follows: arginine, methoxytrimethylbenzenesulphonyl (Mtr); glutamic acid, *t*-butoxy (OBu^t); histidine, *t*-butoxycarbonyl (Boc); lysine, Boc; serine, *t*-butyl (But); Tyr, But. A 4-fold molar excess of reagents was used in all instances.

1. N^2 -Fmoc[Nle⁴, D-Phe⁷]α-MSH. This peptide was assembled on resin (2 g, 0.1 mEq/g substitution) using standard solid-phase synthesis techniques. After assembly, the side-chain-protected peptide on the resin was washed down and dried, with the serine¹- N^2 amino group still Fmoc-protected.

2. [Nle⁴, D-Phe⁷]α-MSH. A 1 g portion of the deprotected peptidyl resin (1) was acetylated with acetic anhydride in HOBT/DMF solution. Deprotection and cleavage was carried out using 2% EDT, 2% anisole and 1% water in TFA for 8 h at room temperature. Identity was confirmed by co-chromatography with authentic [Nle⁴, D-Phe⁷]α-MSH (Sigma Chemical Co.) and by fast-atom bombardment (f.a.b.)-m.s. (M + H calc. 1646.8; found 1647).

3. N^2 -Biotin-[Nle⁴, D-Phe⁷]α-MSH. This was prepared by addition of NHS-Biotin in DMF to 300 mg of the resin (1) after deprotection of the N -terminus. The target peptide was obtained after deprotection and cleavage of the peptidyl resin and h.p.l.c. purification using the same conditions as for peptide 2. Identity was confirmed by f.a.b.-m.s. (M + H calc. 1831.1; found 1831).

4. N^2 -ATB-[Nle⁴, D-Phe⁷]α-MSH. An active ester of the photoaffinity label ATB was preformed with DIC and HOBT in DMF (activation time of 45 min to 1 h) and then reacted with the deprotected N^2 -terminus of peptide 1. The target peptide was obtained after deprotection and cleavage and the identity was confirmed by FAB-MS (M + H calc. 1816.8; found 1817). All procedures involving the ATB photoaffinity label were carried out either in a dark room or in a container protected from the light.

5. N^2 -Biotin-SS-[Nle⁴, D-Phe⁷]α-MSH. This peptide was prepared by reacting NHS-SS-Biotin in the presence of HOBT with the deprotected N^2 -terminus of peptide 1. A mixture of 2.5% phenol, 2.5% EMS and 2.5% anisole in TFA was employed for deprotection and cleavage for 8 h contact at room temperature. The target peptide (f.a.b.-m.s. M + H calc. 1994.4; found 1994) was obtained as the major product, but in poor yield.

[Nle⁴, D-Phe⁷, ATB-Lys¹¹]α-MSH (6), N^2 -biotin-[Nle⁴, D-Phe⁷, ATB-Lys¹¹]α-MSH (7) and N^2 -Biotin-SS-[Nle⁴, D-Phe⁷, ATB-Lys¹¹]α-MSH (8). These were prepared from peptides 2, 3 and 5 respectively. An HOBT active ester of ATB was preformed as for peptide 4 above and allowed to react with the previously purified peptide (5 mg) in solution in DMF (100 μl) for 1 h. The reaction solution was then diluted 50-fold with water/acetonitrile (19:1, v/v) and purified directly by semi-preparative h.p.l.c. without u.v. monitoring. Fractions were subsequently analysed for purity by h.p.l.c. The entire procedure was carried out in a dark room, under red safety light.

All peptides were purified by semi-preparative h.p.l.c. employing a gradient elution of 0.1% TFA in water and 0.1% TFA in acetonitrile/water (7:3, v/v) at a flow rate of 3 ml/min. The eluent was monitored continuously by u.v. spectro-

photometry at 217 nm. Fractions were collected in 1.5 ml aliquots and each fraction was tested by analytical-scale h.p.l.c. Fractions were pooled according to their chromatographic profile and non-photoaffinity-labelled products were freeze-dried.

Iodination of [Nle⁴,D-Phe⁷] α -MSH derivatives

Peptides were iodinated at the tyrosine in position 2 on the peptide using the method of Eberle (1988). The mono-iodinated product was used in all experiments.

Tissue culture

A B16 mouse melanoma cell line (donated by Dr. L. R. Kelland, Institute of Cancer Research, Sutton, Surrey, U.K.) was grown in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum (Flow), 2 mM-L-glutamine, 1% MEM non-essential amino acids (Flow), penicillin (50 units/ml) and streptomycin (50 mg/ml) at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Confluent cells were detached weekly with an incubation of 0.02% EDTA in phosphate-buffered saline (PBS; Dulbecco A), pH 7.4, and counted on a haemocytometer.

Photoaffinity labelling

B16 melanoma cell monolayers were grown in Ezin tissue culture flasks (175 cm²) (Flow) to a density of approx. 2×10^7 cells per flask. They were then washed twice with cold PBS and once with 10 mM-Tris/HCl, pH 7.4. The cells were then incubated with the appropriate radioiodinated photoactive probe (0.1 nM) in binding buffer (50 mM-Tris/HCl, pH 7.4, 1 mM-CaCl₂, 10 mM-MgCl₂, 100 mM-NaCl, 5 mM-KCl and 0.3 mM-1,10-phenanthroline) in the presence or absence of a 1000-fold excess (1 μ M) of unlabelled [Nle⁴,D-Phe⁷] α -MSH. After 2 h incubation at 22 °C in the dark, the upper surface of the flask was peeled off and the cells were irradiated on ice with u.v. light of 320 nm (2×15 W fluorescent tubes; FL15E UV-320) at a distance of about 10 cm. After irradiation, the cells were washed and then scraped from the flask, resuspended in 10 mM-Tris/HCl, pH 7.4, and washed by low-speed centrifugation (700 g) three times to remove further unbound ligand. The cells were then resuspended in homogenization buffer [10 mM-Tris/HCl, pH 7.4, 0.3 mM-phenanthroline, 1 mM-phenylmethanesulphonyl fluoride (PMSF)]. They were kept on ice for 30 min and then homogenized by five passages through a 21-gauge needle and five passages through a 25-gauge needle. The homogenate was then centrifuged at 20000 g for 30 min and the pellet (crude membrane fraction) was resuspended in solubilization buffer (10 mM-Tris/HCl, pH 7.4, 1.5% Triton X-100, 1 mM-PMSF, 1 mM-EDTA and 1 μ g of leupeptin/ml) (0.5 ml/100 mg wet weight). This suspension was kept on ice for 60 min and then centrifuged at 100000 g for 60 min to remove non-solubilized material. *N*-Ethylmaleimide (NEM) was included in the buffers at 0.5 mM to protect the disulphide bridge when *N*^α-Biotin-SS-[Nle⁴,D-Phe⁷,ATB-Lys¹¹] α -MSH was used as the ligand.

Magnetic streptavidin affinity purification of α -MSH receptor complexes

Prewashed Dynabeads M-280 (100 μ l; 100 mg/ml) streptavidin (Dyna, New Ferry, Merseyside) was added to 500 μ l of Triton X-100-solubilized biotinylated probe-receptor covalent complexes in a 1.5 ml Eppendorf tube. The mixture was incubated with gentle mixing at 4 °C for 30 min. The tube was then placed in a strong magnetic field (Magnetic Particle Concentrator; Dynal) for a few minutes. During this time the magnetic beads with bound biotinylated complexes were attracted to the wall of

the tube by the magnetic field, forming a firm pellet. The supernatant containing the unbound material was removed using a pipette while the magnet remained in place. The beads were then released from the magnet and subjected to several cycles of washing using the following buffers: 10 mM-Tris/HCl, pH 7.4, 0.5% Triton X-100, 5 mM-EDTA and 1 mM-PMSF (buffer A); buffer A + 0.1% SDS; buffer A + 1 M-NaCl. Magnetic separation was performed between each washing. Elution of the complexes was achieved by gentle agitation of the beads in a 10 mM-Tris/HCl, pH 7.4, buffer containing 50 mM-dithiothreitol (DTT) and 1% SDS at 30 °C for 15 min. The supernatant was removed by magnetic separation of the beads and concentrated in Filtron Microsep10 microconcentrators.

SDS/polyacrylamide-gel electrophoresis

SDS/PAGE was performed using the discontinuous buffer system of Laemmli (1970), with homogeneous 0.75 mm thick slab gels containing 10% acrylamide monomer in the separating gel and 4% acrylamide in the stacking gel. Sample buffer was 62.5 mM-Tris/HCl (pH 6.8)/2% (w/v) SDS/10% (v/v) glycerol/0.001% Bromophenol Blue/5% (w/v) 2-mercaptoethanol. 2-Mercaptoethanol was omitted from samples electrophoresed under non-reducing conditions. Following electrophoresis (200 V, 45 min, Bio-Rad mini-ProteanII apparatus), the gels were stained with Coomassie Brilliant Blue or silver nitrate (Bio-Rad) according to the manufacturer's instructions. For autoradiography, stained gels were dried in a gel drier (Bio-Rad) and exposed on pre-flashed X-ray film (Hyperfilm MP; Amersham) with an intensifying screen (Hyperscreen; Amersham) for 1–3 days at –70 °C. The apparent molecular mass of the labelled band was determined from a standard curve based on the mobility of ¹⁴C-labelled molecular mass markers co-electrophoresed with the samples. The markers used were myosin (200 kDa), phosphorylase *b* (97.4 kDa), BSA (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa).

RESULTS

Photoaffinity labelling

The strategy we have developed requires the covalent labelling of the receptor prior to solubilization and magnetic separation. Two probes, [¹²⁵I-Tyr²,Nle⁴,D-Phe⁷,ATB-Lys¹¹] α -MSH and *N*^α-ATB-[¹²⁵I-Tyr²,Nle⁴,D-Phe⁷] α -MSH, were initially used to identify the receptor in B16 melanoma cells. The latter probe did not photoaffinity label the receptor, and ATB-Lys¹¹ derivatives of the hormone were developed for further investigations. Photoaffinity labelling was performed in the presence and absence of a large excess of unlabelled peptide to demonstrate the specificity of the binding. The association constant, *K*_a, for this probe, obtained from replicate binding isotherms, is 2.76 nm^{–1}, compared with 2.42 nm^{–1} for [Nle⁴,D-Phe⁷] α -MSH and 0.291 nm^{–1} for the native peptide, α -MSH. Incubation of B16 cells with the probe followed by u.v. irradiation, SDS/PAGE and autoradiography demonstrated that most of the radioactivity was localized in an apparent doublet band corresponding to a molecular mass of 43–46 kDa (Fig. 2). A small amount of radioactivity was also observed at a higher molecular mass of 57 kDa. The presence of an excess of unlabelled [Nle⁴,D-Phe⁷] α -MSH during incubation and irradiation completely inhibited the 43–46 kDa doublet, but did not affect the minor 57 kDa band, indicating that the former represented specific labelling of the α -MSH receptor (or subunit). Boiling the sample before electrophoresis (which is normally performed with SDS/PAGE samples to ensure denaturation of the proteins) resulted in the radio-

Fig. 2. Photoaffinity labelling of B16 cells with iodinated $[Nle^4,D-Phe^7,ATB-Lys^{11}]\alpha$ -MSH

Autoradiography of labelled membrane proteins separated by SDS/PAGE under reducing conditions: lane 1, ^{14}C -labelled molecular mass standards; lane 2, labelled protein boiled in SDS sample buffer prior to loading; lanes 3 and 4, cells labelled with radioactive probe only; lane 5, cells labelled in the presence of excess non-radioactive probe.

Fig. 3. Photoaffinity labelling of B16 cells with iodinated N^{α} -Biotin-SS- $[Nle^4,D-Phe^7,ATB-Lys^{11}]\alpha$ -MSH

Lane 1, ^{14}C -labelled molecular mass standards; lanes 2 and 3, labelled membrane proteins separated under reducing and non-reducing conditions respectively; lane 4, cells labelled in the presence of excess non-radiolabelled probe; lane 5, cells labelled in the presence of excess ATB; lane 6, cells labelled in the presence of pre-irradiated probe.

activity remaining at the origin of the gels, suggesting aggregation of the proteins. As an alternative procedure, samples were incubated at 37 °C for 30 min prior to loading on the gel.

Photoaffinity labelling with iodinated N^{α} -Biotin-SS- $[Nle^4,D-Phe^7,ATB-Lys^{11}]\alpha$ -MSH showed identical results, i.e. a major specific doublet corresponding to molecular mass of 43–46 kDa (Fig. 3). In other control experiments, in which the probe was u.v.-irradiated prior to incubation, no bands were apparent on the gel, indicating the specificity of the labelling (Fig. 3, lane 6). The presence or absence of the reducing agent β -mercaptoethanol did not change the electrophoretic mobility of the complex, suggesting that the labelled protein is unlikely to be a disulphide-linked oligomer (Fig. 3, lanes 2 and 3). Experiments carried out using the probe N^{α} -Biotin- $[Nle^4,D-Phe^7,ATB-Lys^{11}]\alpha$ -MSH gave identical results.

Magnetic separation of covalent receptor complexes

* Routinely, 10^7 B16 melanoma cells harvested from a single 150 mm² tissue culture flask were used as the starting material for

receptor isolation. The yield of receptor–ligand complex at each stage was estimated, and typical values are given below in parentheses as c.p.m. and as % of total radioactivity. B16 cells were labelled with ^{125}I -Biotin-SS- $[Nle^4,D-Phe^7,ATB-Lys^{11}]\alpha$ -MSH. Under the conditions used (2 h at 22 °C; see above for details), the bound ligand (2.0×10^6 c.p.m.; 100 %) represented largely specific binding to the α -MSH receptor, as demonstrated by parallel experiments performed in the presence of excess non-radioactivity labelled $[Nle^4,D-Phe^7]\alpha$ -MSH. At the concentration of labelled ligand used, the non-specific activity was estimated to be less than 10 %. The membranes were exposed to u.v. light and harvested as described above. The crude membrane fraction contained more than half of the initial radioactivity (1.2×10^6 c.p.m.; 60 %). After solubilization, approx. half of the remaining activity was associated with the solubilized membrane preparation (6.8×10^5 c.p.m.; 34 % of total). The loss at this stage is most probably associated with the incomplete solubilization of the membranes by Triton X-100, and possibly the presence of some receptor–ligand complexes in organelles as a result of internalization during the initial 2 h incubation period.

An estimation of the covalent receptor–ligand complexes present in the solubilized membrane fraction was obtained by SDS/PAGE analysis of an aliquot containing 10^4 c.p.m. The band taken to represent the receptor–ligand complex (43–46 kDa) contained 8×10^2 c.p.m. From this analysis, it is estimated that the covalent receptor–ligand complex in the solubilized membrane fraction represents approx. 3 % of the radioactivity initially bound to the cells (5.4×10^4 c.p.m.; 2.7 %). An estimate of the efficiency of photoaffinity labelling may be made by using the data above obtained from SDS/PAGE analysis of the solubilized membrane. If it is assumed that all the radioactivity associated with the solubilized membrane is receptor–ligand complex, and that denaturation during SDS/PAGE releases uncovalently bound ligand, then the ratio $8 \times 10^2/10^4$ gives the proportion of covalent receptor–ligand complex (8 %). This is the worst-case estimate, as some of the radioactivity in the solubilized membrane fraction will be due to the small proportion of non-specifically bound ligand and, in addition, some of the covalent receptor–ligand complex in the SDS/PAGE analysis may be seen at the top of the gel as receptor aggregates.

In a separate experiment to estimate the efficiency of the binding of the receptor–ligand complexes to the streptavidin beads, an aliquot of solubilized membrane fraction containing approx. 10^6 c.p.m. was incubated with the beads as described, and after magnetic separation 5×10^5 c.p.m. (50 %) was found associated with the beads. Subsequent incubation of the solubilized membrane fraction with a second sample of beads resulted in no detectable radioactivity associated with the beads. This suggests that, within detectable limits, 100 % of the biotinylated receptor–ligand complexes are separated on to the beads. The most likely cause of the 50 % of radioactivity not separated on to the beads is cleavage of the biotin from the ligand by reduction of the disulphide bridge due to incomplete protection, particularly during the initial incubation in the presence of the cells. In this context, it is worthy of note that a more stable but eventually cleavable link to replace the disulphide bridge would significantly increase yields. The specificity of the binding to the beads was tested by a parallel experiment using an aliquot incubated in the presence of excess biotin, which completely inhibits binding of radioactivity to the beads.

Following extensive washing of the beads with buffers to remove non-specifically adsorbed probes, the receptors–ligand complexes were eluted from the beads with 10 mM-Tris/HCl, pH 7.4, containing 50 mM-DTT and 1 % SDS. After elution, less than 10 % of the radioactivity (7×10^4 c.p.m.) remained associated with the beads.

Fig. 4. Magnetic separation of photolabelled proteins

(a) Coomassie Blue-stained gel of solubilized membrane proteins labelled with iodinated N^{α} -Biotin-SS-[Nle⁴,D-Phe⁷,ATB-Lys¹¹] α -MSH. (b) Autoradiography of gel in (a). Lane 1, Molecular mass standards [visible in (a) only]; lane 2, ¹⁴C-labelled molecular mass standards [visible in (b) only]; lane 3, blank; lane 4, cells labelled with ¹²⁵I-Biotin-SS-[Nle⁴,D-Phe⁷,ATB-Lys¹¹] α -MSH; lane 4, cells labelled in the presence of excess unlabelled uniodinated [Nle⁴,D-Phe⁷] α -MSH; lanes 6 and 7, cells labelled with ¹²⁵I-Biotin-SS-[Nle⁴,D-Phe⁷,ATB-Lys¹¹] α -MSH after magnetic separation and elution from streptavidin beads with DTT buffer.

The eluate, initially containing 4.3×10^5 c.p.m., after concentration using a Filtron Microsep 10 microconcentrator was found to retain about 37% of the radioactivity (1.6×10^5 c.p.m.). The lost counts are likely to be associated with ¹²⁵I-Biotin-SS-[Nle⁴,D-Phe⁷,ATB-Lys¹¹] α -MSH, which was not covalently bound to the receptor during photoaffinity labelling. This provides an alternative estimate of the efficiency of photoaffinity labelling (37%) compared with the worst-case estimate (8%) described above.

The concentrated eluate was analysed by SDS/PAGE. The electrophoretic pattern produced on the autoradiogram (Fig. 4b) was identical to the one obtained from the non-purified sample, showing a major doublet band corresponding to molecular mass of 43–46 kDa and a minor band of 57 kDa representing the non-specific labelling. In the corresponding photograph of the gel stained with Coomassie Blue (Fig. 4a), it can be seen that after elution from the beads the concentration of non-specifically bound proteins has been decreased to below that detectable by this staining procedure (Fig. 4a, lanes 6 and 7).

DISCUSSION

In this report we have described a novel approach to the isolation and purification of the α -MSH receptor involving

photoaffinity labelling and the use of streptavidin-coated magnetic beads as the solid support.

Photoaffinity labelling of B16 mouse melanoma cells with mono-iodinated [Nle⁴,D-Phe⁷,ATB-Lys¹¹] α -MSH followed by SDS/PAGE and autoradiography revealed the presence of an apparent doublet with an apparent molecular mass of 43–46 kDa. The complete abolition of these bands by the presence of excess non-radiolabelled probe indicated that the labelling was specific. SDS/PAGE analysis under reducing or non-reducing conditions has demonstrated that the labelled proteins do not contain external disulphide bridges. Whether the band is genuinely a doublet, due to either a difference in the primary structure or the result of a post-translational change such as glycosylation, or an artefact of the solubilization and analysis has not been established. The results of binding experiments do, however, suggest that the two labelled proteins represent one class of receptor. Preliminary experiments in which the labelled protein was treated with glycosidases have confirmed the glycoprotein nature of the receptor. This is not unexpected, since most membrane receptor proteins are known to be glycosylated. More detailed analysis of the receptor is required in order to elucidate the exact nature and extent of glycosylation.

The MSH receptor has been identified in previous studies using photoaffinity labelling. Using α -MSH derivative [Nle⁴,D-Phe⁷,1'-(2-nitro-4-azido-phenylsulphenyl)Trp⁹] α -MSH, Solca *et al.* (1989) reported a hormone receptor complex with an apparent molecular mass of approx. 45 kDa on mouse B16-F1, B16-F10 and Cloudman S91 melanoma cells, as well as on human D10 and 205 melanoma cells. However, Gerst *et al.* (1988) have reported the identification of two receptor proteins of approx. 43–46 kDa on B16-M2R mouse melanoma cell lines, using an iodinated azidophenyl derivative of β -MSH. The apparent discrepancy in the number of labelled proteins reported here and in the other studies may reflect the presence of different subtypes of the MSH receptor. This is further supported by the recent work of Solca *et al.* (1991), who tested various B16 murine melanoma subclones and reported the identification of three different subtypes of the α -MSH receptor, which they suggested represent very similar proteins.

The main objective of this investigation was to develop a method for the isolation of the α -MSH receptor, a prerequisite for the elucidation of its structure and function. To our knowledge, there has been no published report on this objective to date. The extreme paucity of the receptor, particularly in cultured cells, dictates that a highly efficient isolation method be employed. The application of (strept)avidin/biotin technology in receptor isolation was first proposed by Hoffmann & Kiso (1976). It was prompted by the exceptional affinity ($K_d = 10^{15} \text{ M}^{-1}$) of the biotin/avidin binding. More recently, Hoffmann *et al.* (1988) described a method for the isolation of a corticotropin-binding protein from adrenal particulates using a desthiobiotinyl derivative as a probe and affinity chromatography on a succinylavidin-Sepharose column. Streptavidin, which has an equal affinity for biotin, is considered to be superior to avidin because it shows relatively low non-specific binding.

There are several strategies for the use of streptavidin/biotin systems. In our first attempt to isolate the α -MSH receptor, we used the probe N^{α} -Biotin-[Nle⁴,D-Phe⁷] α -MSH as an affinity ligand. This ligand was found to retain its high affinity to the receptor and the ability to interact with streptavidin. Using this probe, B16 cells were cross-linked by u.v. irradiation and the solubilized probe-receptor complexes were incubated with the beads. After binding of the biotinylated complexes the beads were washed with buffer to remove non-specifically bound proteins. Attempts were made to dislodge the receptor complexes from the beads using excess biotin, 6 M-guanidinium chloride

and 70 % formamide. The recovery in all cases was poor. This led us to the development of the *N*²-Biotin-SS-[Nle⁴,D-Phe⁷,ATB-Lys¹¹]- α -MSH probe, having a cleavable disulphide bridge in the spacer unit between the biotin and the peptidyl ligand. Although the synthesis gave low yields of the target ligand, sufficient material was obtained to provide receptor complexes using different experimental conditions. NEM was included in the purification steps to minimize disulphide bridge cleavage due to the presence of endogenous thiol groups. Elution of the receptor complexes was achieved by disrupting the disulphide linkage in the biotin ligand chain using DTT. The efficiency of this step in the recovery of receptor complexes was in excess of 90 %, with little contamination by released streptavidin. SDS/PAGE and autoradiographic analysis of the purified receptor complex revealed that it contains a major 43–46 kDa doublet band which was identical to that obtained for the crude solubilized membrane preparation.

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