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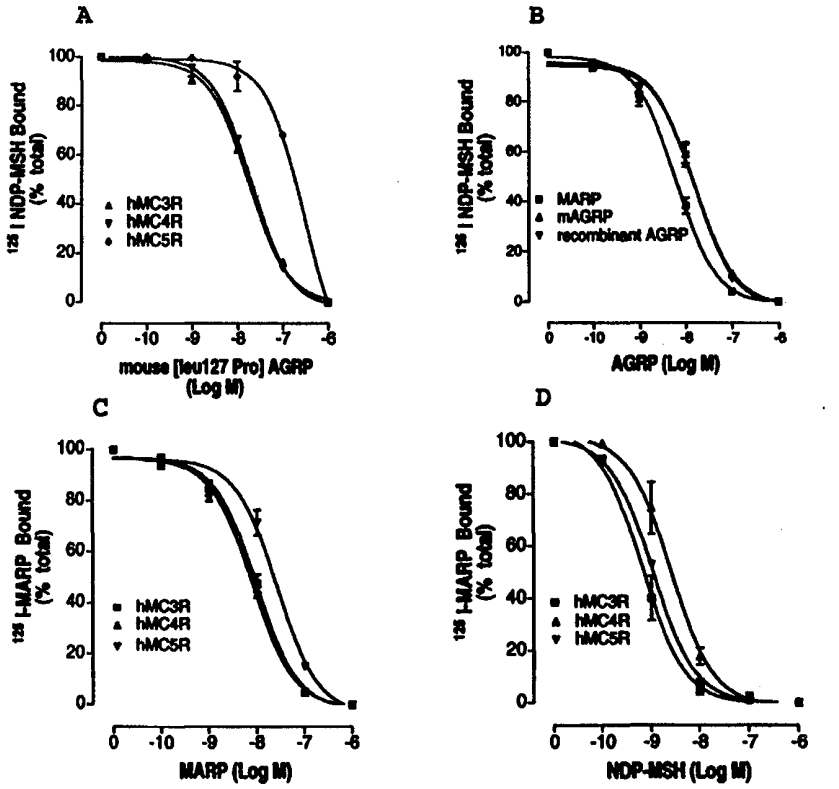
(54) Title: AGOUTI–RELATED PROTEIN ANALOGS AND METHODS OF USE

(57) Abstract

The present invention relates to Agouti–related protein (AGRP) analogues and methods of use. The invention is exemplified by chemical synthesis and detectable labeling of AGRP analogues, and their novel exploitation in a variety of different assays and screens. The inventions find use, for example, in drug discovery and diagnostics related to feeding disorders including wasting syndromes, obesity, and other disorders related to hypothalamic control of feeding.



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AGOUTI-RELATED PROTEIN ANALOGS AND METHODS OF USE

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INTRODUCTION

Background

Agouti-related protein (AGRP) is a recently discovered neuropeptide that has generated intense interest because a growing body of evidence indicates it has a major role in the regulation of mammalian feeding behavior (1,2). AGRP was identified by virtue of its sequence similarity to the product of the *Agouti* coat color gene, a paracrine signaling molecule normally expressed in skin whose transient expression during hair growth leads to the barring of coat fur in rodents (e.g. dark hair with a subapical yellow band) (3).

Ubiquitous expression of Agouti, which occurs in mice that carry mutations in the 5' flanking region of the *Agouti* gene, gives rise to pleiotropic effects including a yellow coat, obesity, insulin resistance, increased body length, and premature infertility (4). The recent identification of AGRP indicates that the obesity and diabetes caused by ectopic *Agouti* expression are likely explained by its ability to mimic AGRP.

AGRP is a naturally occurring antagonist of melanocortin action that is thought to play an important role in the hypothalamic control of feeding behavior.

In order to better understand the role of AGRP in control of feeding behavior, it would be helpful to have analogs of AGRP and methods of assaying for binding of AGRP and analogs to melanocortin receptors.

SUMMARY OF THE INVENTION

The present invention relates to antagonism of melanocortin receptors by analogues of agouti-related protein. Agouti-related protein (AGRP) is a natural antagonist of melanocortin receptors. The cysteine-rich carboxyl-terminal region of AGRP folds independently and retains the activity of the full-length protein. This region has been shown to be a minimized agouti-related protein (MARP). Both AGRP and MARP prove to bind to melanocortin receptors and inhibit binding of

melanocortin agonists to these receptors and thus inhibit the ability of these receptors to participate in the production of the satiety signal.

In one embodiment, the invention includes a group of polypeptides or peptide-related compounds comprised of sequences from the minimized agouti-related protein (MARP). Members of this group may be made synthetically and can be modified in myriad ways. In a preferred embodiment, the polypeptide may be labeled with biotin or with a fluorescent compound or with a radioactive isotope such as ^{125}I. Further, the polypeptide can be composed of D-amino acids either in part or as a whole.

In another embodiment, the invention includes a method of treating a disease state in mammals by treatment with MARP or a polypeptide comprised of sequences from MARP. A preferred embodiment would be a method of treating a wasting syndrome, such as HIV wasting syndrome, cachexia, or anorexia.

In yet another embodiment, the invention includes a method of screening for a compound that is capable of inhibiting binding of a melanocortin agonist or antagonist to a melanocortin receptor. This method can be used to screen for compounds that inhibit binding at specific melanocortin receptors such as MC3R, MC4R, or MC5R. Further, this method can be used to identify compounds that interfere with either agonist or antagonist binding at the melanocortin receptor. Examples of antagonists that interfere with agonist binding include, but are not limited to, MARP and AGRP.

In a fourth embodiment, the invention includes methods of screening for binding of compound to a melanocortin receptor. Such methods can be used to screen for compounds that bind at specific melanocortin receptors such as MC3R, MC4R, or MC5R, and can be used for high throughput screening.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows amino acid sequence alignments of mouse and human AGRP and mouse and human agouti protein. Conserved C-terminal cysteine residues are enclosed in boxes. The presumed signal sequence cleavage position is denoted by ▼. (m=mouse, h=human). SEQ. ID. NO:1 is provided, representing human AGRP(87-132). Mouse AGRP(88-131) is listed as SEQ. ID. NO. 2.

FIG. 2 shows a schematic representation of the synthesis strategy used to make MARP and mouse [Leu127Pro]AGRP. Chemical protein synthesis consisting of native chemical ligation followed by folding and disulfide formation, was used to make MARP. Native chemical ligation of unprotected peptide segments was used to join human AGRP (87-132) to the N-terminal portion of mouse AGRP, followed by folding and disulfide formation to make mouse [Leu127Pro]AGRP.

FIG. 3A shows that chemically synthesized mouse [Leu127Pro]AGRP does not affect the α-MSH-stimulated cAMP generation of cells expressing MC1R.

FIG. 3B shows that chemically synthesized mouse [Leu127Pro]AGRP does not affect the α-MSH-stimulated cAMP generation of cells expressing MC2R.

FIG. 3C shows the inhibition by chemically synthesized mouse [Leu127Pro]AGRP of α-MSH-stimulated cAMP generation of cells expressing MC5R.

FIG. 3D shows the inhibition by chemically synthesized mouse [Leu127Pro]AGRP of α-MSH-stimulated cAMP generation of cells expressing MC3R. The insert graph represents a Schild analysis linear regression plot. (■ no AGRP, ▲ AGRP 10^{-9} M, ▼ AGRP 10^{-8} M, ◆ AGRP 10^{-7} M, ●AGRP 10^{-6} M).

FIG. 3E shows the inhibition by chemically synthesized mouse [Leu127Pro]AGRP of α-MSH-stimulated cAMP generation of cells expressing MC4R. The insert graph represents a Schild analysis linear regression plot. (■ no AGRP, ▲ AGRP 10^{-9} M, ▼ AGRP 10^{-8} M, ◆ AGRP 10^{-7} M, ●AGRP 10^{-6} M).

FIG. 3F shows recombinant AGRP Form A + B inhibition of α-MSH stimulated cAMP generation at the hMC4R. The insert graph represents a Schild analysis linear regression plot. (■ no AGRP, ▲ AGRP 10^{-9} M, ▼ AGRP 10^{-8} M, ◆ AGRP 10^{-7} M, ●AGRP 10^{-6} M).

FIG. 4A shows the displacement of ^{125}I-NDP-MSH binding from the hMCR 3, 4, and 5 stably expressed in HEK-293 cells by chemically synthesized mouse [Leu127Pro]AGRP.

FIG. 4B Compares of the displacement of ^{125}I-NDP-MSH binding from the hMC4R by recombinant human AGRP Form A + B, chemically synthesized mouse [Leu127Pro]AGRP, and MARP.

FIG. 4C shows the displacement of ^{125}I-MARP binding from the hMCR 3, 4, and 5 by MARP.

FIG. 4D shows the displacement of ^{125}I-MARP binding from the hMCR 3, 4, and 5 by NDP-MSH. ^{125}I-MARP does not bind the hMC1R or hMC2R.

FIG. 5 shows representative photomicrographs of ^{125}I-MARP binding to HEK 293 cells transfected with hMCR 3, 4, and 5. Under bright field illumination (right) cells are seen as outlines on a light background. Under dark field illumination (left) the identical cells are seen. Under dark field, cells binding ^{125}I-MARP are seen as white elements in a surrounding dark background. Because of the absence of binding wild-type cells only appear as faint outlines. No binding was observed in similar experiments with cells expressing the hMC 1R and hMC2R.

FIG. 6 is a table disclosing the nanomolar range inhibition constants (Ki) of the chemically synthesized mouse[Leu127Pro]AGRP, MARP and recombinant AGRP. The data indicate that both the chemically synthesized and minimized AGRP polypeptides have inhibition constants comparable to that of the recombinant protein for both MC3R and MC4R. Further, the table goes on to show IC50 of NDP-MSH and MARP at the MC3R and MC4R.

FIG. 7 shows the solid phase chemical synthesis of the mouse AGRP (21-85)-thioester and the human AGRP 87-132 polypeptide. The figure also shows the amino acid sequence of these two polypeptides.

FIG. 8 shows a schematic representation of the synthesis strategy used to make MARP and mouse [Leu127Pro]AGRP. Native chemical ligation of unprotected peptide segments was used to join human AGRP (87-132) to the N-terminal portion of mouse AGRP, followed by folding and disulfide formation to make mouse [Leu127Pro]AGRP. The amino acid sequence of mouse {leu127Pro]AGRP is also shown.

FIG. 9A shows the absorbance profile of the fully reduced and folded mouse [Leu127Pro]AGRP being purified using reversed phase HPLC on C4 columns with water (0.1% TFA)/acetonitfile (0.1% TFA) gradients. The peptides were fully reduced by incubating 1 hr with 20% β-mercaptoethanol, purified by HPLC, and lyophylized prior to protein folding. The folded proteins were then purified by HPLC and lyophilized.

FIG. 9B shows the absorbance profile of the purified folded mouse [Leu127Pro]AGRP as seen by reversed phase HPLC on a C4 comlumn with water(0.1% TFA)/acetonitrile)0.1% TFA) gradient.

FIG. 9C shows the loss of 10 mass units by ESI-MS, which is consistent with the formation of five disulfides in the oxidized form. Folded mouse [Leu127Pro]AGRP is calculated to have a moelcular weight of 12384.5 daltons whereas the reduced mouse [Leu127Pro]AGRP should have a molecular weight of 12395 daltons.

FIG. 10A shows the absorbance profile of the fully reduced and folded human MARP being purified using reversed phase HPLC on C4 columns with water (0.1% TFA)/acetonitfile (0.1% TFA) gradients. The peptides were fully reduced by incubating 1 hr with 20% β-mercaptoethanol, purified by HPLC, and lyophylized prior to protein folding. The folded proteins were then purified by HPLC and lyophilized.

FIG. 10B shows the absorbance profile of the purified folded human MARP as seen by reversed phase HPLC on a C4 column with water(0.1% TFA)/acetonitrile)0.1% TFA) gradient.

FIG. 10C shows the loss of 10 mass units by ESI-MS, which is consistent with the formation of five disulfides in the oxidized form. Folded human MARP is calculated to have a molecular weight of 5192 daltons whereas the reduced human NARP should have a molecular weight of 5202 daltons.

FIG. 11 shows the combination of two fragments of MARP by native chemical ligation. Prior to incubation, the two peptides are not covalently attached as indicated by the absorbance profile of the peptides during reversed phase HPLC on a C4 column with water(0.1% TFA)/acetonitrile)0.1% TFA) gradient. After incubation, the absorbance profile clearly shows that the peptides have been covalently combined.

FIG. 12 shows HPLC absorbance profile of the purified MARP after reverse phase purification.

FIG. 13A shows the absorbance profile of the reduced N-acetylated-MARP-Lys(Biotin)133 as seen by reversed phase HPLC on a C4 column with water(0.1% TFA)/acetonitrile)0.1% TFA) gradient.

FIG. 13B shows the absorbance profile of the purified partially folded N-acetylated-MARP-Lys(Biotin)133 as seen by reversed phase HPLC on a C4 column with water(0.1% TFA)/acetonitrile)0.1% TFA) gradient.

FIG. 13C shows the absorbance profile of the fully folded N-acetylated-MARP-Lys(Biotin)133 as seen by reversed phase HPLC on a C4 column with water(0.1% TFA)/acetonitrile)0.1% TFA) gradient.

FIG. 14 shows the absorbance profile of the purified folded N-acetylated-MARP-Lys(Biotin)133 as seen by reversed phase HPLC on a C4 column with water(0.1% TFA)/acetonitrile)0.1% TFA) gradient.

FIG. 15 shows that N-acetylated MARP-Lys(Biotin)133 is a potent antagonist of MC4R. Whole cells are used for the Ac-MARP Lys(Biotin)133 binding assays. Experiments are performed in 12 well culture plates with 0.4 million cell per plate. Maintenance Media is removed and the cells are washed twice with 0.5 ml OPTI-medium (Life technologies, Grand Island, NY). Cells are then incubated with OPTI-medium containing 10e-10 M of Ac-MARP Lys(Biotin)133 for 30 minutes at 37°C. Binding reactions were terminated by removing the medium and washing the cells twice with OPTI-medium. 0.5 ml of Extravidin-Peroxidase (20 mg.ml) (Sigma Chemical Co., St. Louis, MO) are added for another 30 minute incubation at 37°C. The cells are then washed twice with OPTI-medium and 0.5 ml of peroxidase substrate is added (3,3'-diamino benzidine, Sigma) and incubated for 30 minutes. The supernatant is taken and absorbancy of the supernatant is measured at 405 nm. **FIG. 15** shows the inhibition of α-MSH-stimulated cAMP generation of cells expressing MC4R by the biotinylated MARP. (■ no AGRP, ● AGRP 10^{-9} M, ◆ AGRP 10^{-8} M, ▼ AGRP 10^{-7} M, ▲ AGRP 10^{-6} M).>>>

FIG. 16 shows that MARP forms a single, stable and homogeneous folded state as shown by 2-D NMR. Fingerprint region of the 50ms TOCSY spectrum of Ac-MARP. This data set was acquired on a Varian Unity Plus 500 MHz instrument at 25°C with a protein concentration of 2 mM. Some HN/HA assignments are shown.

DESCRIPTION OF SPECIFIC EMBODIMENTS :

Structurally, the similarity between Agouti and AGRP is confined almost entirely to their 40 residue carboxyl termini where a total of 20 residues, including 10 cysteine residues, are identical (Figure 1).

Both Agouti and AGRP have been shown to antagonize the action of melanocortin peptides such as alpha-melanocyte stimulating hormone (α-MSH) and adrenocorticotrophic hormone (ACTH) at specific melanocortin receptor subtypes. Agouti potently antagonizes the action of melanocortins at the melanocyte melanocortin receptor (MC1R), adrenocortical ACTH receptor (MC2R), and the MC4R (5,6). In contrast, we recently demonstrated that AGRP primarily antagonizes the MC3R and MC4R (2). Importantly, both the MC3R and MC4R are melanocortin receptor subtypes expressed in hypothalamic centers involved in the regulation of feeding behavior (7,8).

The pathophysiology of mice carrying mutant *Agouti* genes suggests that one or more of the melanocortin peptides conveys an important satiety message. The recent demonstration that mice with targeted disruption of the MC4R display a pattern of obesity and increased growth similar to that caused by overexpression of Agouti or AGRP indicates that this satiety signal is mediated at least in part through that melanocortin receptor subtype (9). Recent pharmacological and anatomical data have further strengthened the link between melanocortins and weight control and indicate that melanocortins act down-stream of the fat hormone leptin (10-13). In this scheme AGRP presumably acts as a naturally occurring orexigenic agent that antagonizes melanocortins.

Previous biochemical studies of Agouti and AGRP have been complicated by the need to purify recombinant protein from the conditioned media of insect cells infected with a recombinant baculovirus (2,7). The estimated purity of the recombinant protein was 60-70%. In contrast to recombinant techniques, chemical protein synthesis can be used to make a defined homogeneous preparation that can be chemically labeled, derivatized, and which can serve as a substrate for structure-function analyses. As demonstrated in the present studies this technical capability can greatly accelerate research. Herein we describe the synthesis and biological activities of two novel AGRP variants. The first MARP, or Minimized Agouti-Related Protein, is a small protein containing five disulfide bonds formed by folding the carboxyl-terminal (C-terminal) portion of human AGRP, residues (87-132). The second, mouse [Leu127Pro]AGRP represents a 111 amino acid AGRP molecule (mature AGRP minus its 20 amino acid signal sequence) made by joining the amino-terminal (N-terminal) residues 21-85 of

mouse AGRP to human AGRP (87-132) by native chemical ligation (14). Mouse [Leu 127Pro]AGRP also contains five disulfide bonds formed by folding the purified ligation product. These variants of AGRP are used to more fully address the pharmacological mechanisms of AGRP action. In addition, these reagents are used to develop the novel radioligand ^{125}I-MARP. Our studies with ^{125}I-MARP provide the first data on the binding characteristics of this important regulatory protein.

The inventions described herein can find use in discovering drugs for use in treating feeding disorders, including wasting syndromes, obesity, and other disorders related to hypothalamic control of feeding. A wasting syndrome is an illness characterized by significant weight loss accompanied by other indicia of poor health, including poor appetite, gut disorder, or increased metabolic rate. Wasting syndromes include, but are not limited to, the wasting syndrome afflicting some patients diagnosed with Acquired Immune Deficiency Syndrome (AIDS) and various cancers. As methods of treating other symptoms of diseases such as AIDS progress, the incidence of wasting syndrome as the cause of death increases. Improved prophylaxis and treatment for HIV wasting syndrome is required (Kravick et al., Arch Intern Med 157:2069-2073, 1997). Anorexia and cachexia are well-known results of cancer that contribute to morbidity and mortality (Simons et al, Cancer 82:553-560, 1998; Andrassy & Chwals Nutrition 14:124-129, 1998). The reasons for the significant weight loss are multiple and may be directly related to the tumor, such as increased metabolic rate, but also include decreased intake due to poor appetite or gut involvement. Further, excessive leptin-like signaling may contribute to the pathogenesis of wasting illness (Schwartz et al. Pro Nutr Soc 56:785-791, 1997).

The inventions described herein include methods for screening for agonists and antagonists of MCR subtypes, particularly 3, 4 and 5. As one embodiment, synthetic D-MARP, which can be folded into the mirror-image of L-MARP, can be used as a target for screening a library of small molecules. The positive results of this screen represent molecules that bind to D-MARP. Then using standard techniques of synthetic chemistry, the mirror-images of the molecules that bind to D-MARP can be synthesized. This group of enantiomers represents molecules that are expected to bind selectively to L-MARP. Molecules that bind to L-MARP can be assayed for interference with binding to melanocortin receptors, and help identify lead molecules for therapeutic application in feeding disorders.

Moreover, the cellular assays described herein can be used in high throughput screening for drug discovery.

EXAMPLES

Example 1

Mouse [Leu127Pro]AGRP and MARP Synthesis.

Peptides were synthesized by Boc chemistry using manual stepwise solid phase peptide synthesis as previously described (15). The 46 amino acid polypeptide corresponding to the C-terminal module, human AGRP (87-132), was assembled on Thr-OCH_2-Pam-resin (PE Applied Biosystems, Foster City, CA). The N-terminal basic segment, mouse AGRP 21-85, was assembled on a thioester resin (16). Peptides were cleaved from the resin with hydrogen fluoride containing 5-10% p-cresol (Fluka, Buchs, Switzerland) for 1 hr at 0°C, lyophilized, and then purified using reversed phase HPLC on C4 columns (Vydac, Murrieta, CA) with water (0.1% TFA)/acetonitfile (0.1% TFA) gradients. The molecular weights of these peptides were confirmed by electrospray ionization mass spectrometry (ESI-MS) (PE SCIEX, Foster City, CA). To generate the full length construct, purified mouse AGRP (21-85) thioester and human AGRP (87-132) were dissolved in 6 M guanidine hydrochloride and 200 mM phosphate (pH 7.0) containing 1% thiophenol at a concentration of 2-4 mM and stirred overnight. Under these conditions native chemical ligation joined the two peptides to form full length mouse [Leu127Pro]AGRP (21-131), appearing as a new peak on analytical HPLC with molecular weight indicative of segment condensation by peptide bond formation (*observed*: 12,397.4 ± 1.50 *Da calculated*: 12,394.5 *Da (av. isotopes)*). Ligated peptides were then fully reduced by incubating 1 hr with 20% β-mercaptoethanol, purified by HPLC, and lyophylized (24 mg mouse AGRP (21-85) thioester ± 16.9 mg human AGRP (87-132) yielded 14.3 mg mouse [Leu127Pro]AGRP. Protein folding of human AGRP (87-132) and mouse [Leu 127Pro]AGRP was initiated by dissolving the lyophilized peptide in a solution of 2 M guanidine hydrochloride and 100 mM Tris (pH 8.0) containing 8 mM cysteine and 1 mM cystine (Fluka), and stirring overnight. The folded proteins were then purified by HPLC and lyophilized. 138.2 mg human AGRP (87-132) (reduced) yielded 52.5 mg MARP (oxidized). 14.3 mg [Leul27Pro]AGRP (reduced) yielded 4.7 mg [Leu127Pro]AGRP (oxidized). Two dimensional NMR studies of MARP confirmed the existence of a single homogeneous folded state (K. Bolin, J. Trulson, G. L.

Millhauser, University of California at Santa Cruz). This observation was supported by the formation of a sharp peak on analytical reverse phase HPLC eluting earlier than the reduced state, and the loss of 10 mass units by ESI-MS, which is consistent with the formation of five disulfides in the oxidized form (MARP observed: 5,191.1 ± 1.05 *Da calculated*: 5,191.2 *Da (av. isotopes)*; mouse [Leu127Pro]AGRP *observed*: 12,384.9 ± 1.11 *Da calculated*: 12,383.5 *Da (av. isotopes)*.

Baculovirus produced recombinant human AGRP Form A + B was produced and partially purified as previously described (2,17). Form A + B refers to inhomogeneous fractions of recombinant AGRP that run closely together on Western blot (2). Form A consists of mature AGRP minus its signal sequence of 20 amino acids and Form B contains several fragments cleaved after residues 46, 48, or 50.

Example 2.

cAMP Assays.

cAMP generation was measured using a competitive binding assay kit (TRK 432, Amersham, Arlington Heights, IL) according to a standardized protocol (6). Heterologous cell lines stably expressing the human (h) melanocortin receptors that have been previously described were used in these assays (6). For assays, culture media was removed and cells were incubated with 0.5 ml Earle's Balanced Salt Solution (EBSS) that contained AGRP and melanocortin agonist for 30 min at 37 C in the presence of 10^{-3} M isobutylmethylxanthine. The reaction was stopped by adding ice cold 100% ethanol (500 µl/well). The cells in each well were scraped and transferred to a 1.5 ml tube and centrifuged for 10 min at 1900 x g and the supernatant was evaporated in a 55°C water bath with prepurified nitrogen gas. cAMP content was measured according to instructions accompanying the assay kit. a-MSH and human adrenocorticotropic hormone (ACTH 1-39) were obtained from Peninsula Laboratories, Inc. (Belmont, CA). Each experiment was performed a minimum of three times with duplicate wells. The mean value of the dose-response data were fit to a sigmoid curve with a variable slope factor using the non-linear squares regression in Graphpad Prism (Graphpad Software, San Diego, CA). EC_{50} values determined from these fits were used for plotting Schild analysis linear regressions. pA_2 values were derived from the y = 0-intercept of the Schild plot of the log of dose ratio minus one (log DR-1) as previously described (6,18). Ki values were determined as the negative log of the pA_2. All statistical analyses represent the mean of the data ± standard error.

Example 3.

Radioiodination.

[Nle^4, D-Phe^7] α-MSH (NDP-MSH), a long acting superpotent melanocortin agonist, was obtained from Peninsula Laboratories,Inc. (19). ^{125}I-NDP-MSH and ^{125}I-MARP were prepared by a modification of chloramine-T method previously described (20). 0.5 mCi of Na ^{125}I (Amersham) was added to 20 μg of eigher NDP-MSH or MARP in 5 μl of 50 mM sodium phosphate buffer (pH 7.4). 10 μl of a 2.4 mg/ml solution of chloramine T (Sigma) in 50 mM sodium phosphate (pH 7.4) was added for 15 seconds and the reaction stopped with 50 μl of a 4.8 mg/ml solution of sodium metabisulfite (Sigma, St. Louis, MO). The reaction mixture was then diluted in 800 μl of 50 mM ammonium acetate (pH 5.8) and purified by reverse phase chromatography. 100 μl of a 2% solution of bovine serum albumin were added to all fractions containing radioactivity.

Example 4.

Binding experiments.

After removal of media, the cells were washed twice with MEM then preincubated with AGRP in 0.5 ml MEM (Life Technologies) containing 0.2% BSA for 30 rain prior to incubation with radioligand. Binding experiments were performed using conditions previously described (6). 3 x 10^5 cpm of ^{125}I-NDP-MSH (approximately 61 fmol) or 3 x 10^5 cpm ^{125}I-MARP (approximately 55 fmol) were used. Binding reactions were terminated by removing the media and washing the cells twice with MEM containing 0.2% BSA. The cells were lysed with 0.1 N NaOH 1% Triton X-100 and the radioactivity in the lysate was quantified in an analytical gamma counter. Nonspecific binding was determined by measuring the amount of ^{125}I-label remaining bound in the presence of 10^5 M unlabeled ligand and specific binding was calculated by subtracting nonspecifically bound radioactivity from total bound radioactivity. For photoemulsion studies, the binding assays were performed directly on a chambered microscope slides (SlideFlask, NUNC, Roskilde, Denmark). Approximately 10^5 cells were placed on each slide and allowed to grow for 12 hours. After binding experiments were performed slides were fixed with gluteraldehyde and dried. Slides were then dipped in Kodak NTB2 photoemulsion (Eastman Kodak Co., New Haven, CT) and exposed for 3 days prior to being developed, examined, and photographed using a Leica DMRB microscope.

Example 5.

Effect of mouse [Leu127Pro]AGRP and MARP on α-MSH stimulated cAMP generation in cell lines transfected with melanocortin receptors.

To help verify the biologic activity of the chemically synthesized proteins we examined their ability to inhibit α-MSH-stimulated cAMP generation mediated by each of the various human melanocortin receptor subtypes. Figures 3A through 3E demonstrate that chemically synthesized mouse [Leu127Pro]AGRP potently inhibits the action of α-MSH at the hMC3R and hMC4R. With increasing concentrations of mouse [Leu127Pro]AGRP a progressive rightward shift of the α-MSH dose response curves is observed. Mouse [Leu127Pro]AGRP was completely devoid of activity at the hMC1R and hMC2R. However, at higher concentrations mouse [Leu127Pro]AGRP had a modest inhibitory effect on α-MSH action at the hMC5R. Schild analysis performed by plotting a linear regression of the log concentration of AGRP (X-axis) and log (DR-1) (Y-axis) revealed a slope of 0.94 and 0.96 for mouse [Leu127Pro]AGRP at the hMC3R and hMC4R, respectively (Figures 3D and 3E inserts) (18). Slopes approaching unity indicate that mouse [Leu127Pro]AGRP has the characteristics of a competitive antagonist of α-MSH action at the hMC3R and hMC4R. Inhibitory constants (Ki) for mouse [Leu127Pro]AGRP derived from this Schild analysis revealed a Ki of 4.3±0.6 nM at the hMC3R and a Ki of 2.5±0.25 nM at the hMC4R (Table 1).

Chemically synthesized MARP displayed identical potency, efficacy, and selectivity to mouse [Leu127Pro]AGRP at the various hMCR subtypes. MARP had no effect at hMC1R or hMC2R and only a minimal effect at the hMC5R. Schild analysis revealed that MARP, like mouse [Leu127Pro]AGRP, is a competitive antagonist of α-MSH at the hMC3R and hMC4R. Ki values for MARP inhibition of α-MSH at the hMC3R and hMC4R were 3.3±0.28 nM and 2.6±0.21 nM, respectively. For comparison we examined the effect of recombinant human AGRP Form A + B on α-MSH stimulated cAMP generation at the hMC4R (Figure 3F). Although the dose-response curves for recombinant human AGRP Form A + B were not parallel, a linear regression of the data revealed a slope of 0.94 and Ki of 1.2+0.17 nM (Figure 3F insert). In contrast to chemically synthesized mouse [Leu127Pro]AGRP, the E_{max} of α-MSH in the presence of recombinant human AGRP Form A + B was about 10% below that observed in the

absence of this antagonist. Neither chemically synthesized nor recombinant AGRP had an effect on cAMP accumulation when applied to cells in the absence of agonist.

Example 6

^{125}I-NDP-MSH binding to cell lines transfected with melanocortin receptors.

In addition to using cAMP generation as a measure of antagonism, we measured the ability of chemically synthesized or recombinant AGRP to inhibit the binding of ^{125}I-NDP-MSH to cells expressing the melanocortin receptor subtypes. Figure 4A reveals that chemically synthesized mouse [Leu127Pro]AGRP dose dependently displaces ^{125}I-NDP-MSH from the hMC3R, hMC4R, and hMC5R. The displacement curve of ^{125}I-NDP-MSH from the hMC5R was shifted to the right as compared to the hMC3R and hMC4R. No significant displacement was observed at the hMC1R (data not shown). These binding studies are consistent with the actions of mouse [Leu127Pro]AGRP and MARP in the cAMP assays. IC_{50} values for ^{125}I-NDP-MSH displacement are hMC1R>10^{-6} M, hMC3R=17.4±3.7 nM, hMC4R=15.7±4.1 nM, hMC5R=310.6±18.7 nM. Figure 4B compares the ability of mouse [Leu127Pro]AGRP, MARP, and recombinant human AGRP Form A + B to displace ^{125}I-NDP-MSH from the hMC4R. The displacement curves of baculovirus produced human AGRP Form A + B and chemically synthesized mouse [Leu127Pro]AGRP are identical while the curve of chemically synthesized MARP is slightly shifted to the left (3 times more potent). The IC_{50} for recombinant human AGRP Form A + B was 13.4±2.9 nM.

Example 7.

^{125}I-MARP binding to cell lines transfected with melanocortin receptors.

Although competition with ^{125}I-NDP-MSH binding provided an indirect means of evaluating AGRP binding we sought to more directly examine its binding using an AGRP radioligand. We radiolabeled MARP (human AGRP 87-132) which contains two tyrosine residues which could serve as sites for iodination (Figure 1). Figure 4C and 4D shows that the radioligand ^{125}I-MARP was dose-dependently displaced from cells expressing the hMC3R, hMC4R, and hMC5R by either unlabeled MARP (Figure 4C) or NDP-MSH (Figure 4D). Typically, total binding of ^{125}I-MARP was about 13.5 ± 1.3 x 10^4 cpm and nonspecific binding was 3.0 ± 0.4 x 10^3 cpm. No specific binding was observed at wild-type HEK 293 cells, or cells expressing the hMC1R or hMC2R. Notably, the displacement curve of ^{125}I-MARP from the hMC3R and hMC4R are overlapping in these binding studies. These data are consistent with the cAMP data and indicate that MARP is essentially equipotent at these two receptor subtypes. The IC_{50} values of ^{125}I-MARP

displacement by MARP at the hMC3R=11.2±3.1 nM, hMC4R=9.0±1.7 nM, and hMC5R=25.6±4.3 nM. The IC_{50} values of ^{125}I-MARP displacement by NDP-MSH at the hMC3R=1.9±0.15 nM, hMC4R=3.75±0.1 nM, and hMC5R=11.2±2.1 nM. The ^{125}I-NDP-MSH and ^{125}I-MARP displacement data and the cAMP data reveal a hierarchy of melanocortin receptor subtype sensitivity to AGRP such that hMC3R=hMC4R>hMC5R.

Example 8.

Photoemulsion studies using ^{125}I-MARP.

We also sought to examine the site(s) of ^{125}I-MARP binding using a second technique. Representative photomicrographs of ^{125}I-MARP binding to heterologous cell lines expressing the various melanocortin receptor subtypes are shown in Figure 5. Using a photoemulsion technique we found that ^{125}I-MARP only binds to cells expressing the hMC3R, hMC4R, and hMC5R. No radioligand binding was observed at wild-type cells or at the hMC1R or hMC2R. Since no binding was observed at the hMC1R and hMC2R photographs of these cell lines are not shown. The absence of ^{125}I-MARP binding to wild-type HEK 293 cells, or cells expressing the hMC1R and hMC2R in these photoemulsion studies is consistent with results obtained in the conventional binding studies and the cAMP assays. Although only semi-quantitative, the intensity of binding observed in the photoemulsion studies appear to be consistent with the rank order of AGRP binding and inhibition noted in the other studies (hMC4R=hMC3R >hMC5R). Since both the hMC3R and hMC5R expressing HEK 293 cells have roughly equal numbers of melanocortin receptors (approximately 2.5 million/cell) the difference in intensity observed under dark field microscopy should not be due to this variable.

Example 9

MARP and AGRP Display the Same Receptor Binding Specificity

Inhibitory constants (Ki) for mouse [Leu127Pro]AGRP and MARP for human Melanocortin Receptors 3 and 4. Inhibitory constants were derived from Schild analysis performed by plotting a linear regression of the log concentration of antagonist versus the log (DR-1). Within experimental error the inhibitory constants for mouse [Leu127Pro]AGRP and MARP are identical. See **FIG. 6.** IC50 values for mouse [Leu127Pro]AGRP displacing 125I NDP-MSH and MARP displacing 125I MARP are also shown in **FIG. 6.** In assays with Melanocortin Receptors 1 and 2, MARP had no effect on cAMP production, and could not displace 125I NDP-MSH.

Example 10.

Competitive Binding Studies With Chemically Synthesized AGRP and 125I-NDP-MSH and with 125I-MARP.

125I-NDP-MSH and 125I-MARP were prepared by jodification of the chloramine-T method as described herein. See Tatro, J.B. et al., Endocrinology 121:1900-1907 (1987). As shown in **Figure 4A**, chemically synthesized AGRP displaces bound 125I-NDP-MSH from human Melanocortin receptors 3, 4, and 5. **Figure 4B** shows that recombinant AGRP and chemically synthesized AGRP displace bound 125I-NDP-MSH from the Melanocortin 4 receptor with identical efficiency, and that MARP is slightly more potent. As shown in **Figures 4C and 4D**, cold MARP and cold NDP-MSH displace 125I-MARP. Binding studies were performed according to the method previously described by Yang et al., Mol. Endocrin. 1997.

Example 11.

Ac-MARP Lys(Biotin)133 Is a Potent Antagonist of MC4-R and is Active in an ExtrAvidin-Peroxidase Assay.

Whole cells are used for the Ac-MARP Lys(Biotin)133 binding assays. Experiments are performed in 12 well culture plates with 0.4 million cells per plate. Maintenance Media is removed and the cells are washed twice with 0.5 mL OPTI-medium (Life Technologies, Grand Island, NY). Cells are then incubated with OPTI-medium containing 10e-10M of Ac-MARP Lys(Biotin)133 for 30 min. at 37 °C. Binding reactions were terminated by removing the medium and washing the cells twice with OPTI-medium. 0.5 ml of Extravidin-Peroxidase (20 mg/ml) (Sigma Chemical Co., St. Louis, MO) are added for another 30 min. incubation at 37°C. The cells are then washed twice with OPTI-medium and 0.5 ml of peroxidase substrate is added (3,3'-diaminobenzidine, Sigma) and incubated for 30 min. Supernatant is taken and absorbency of the supernatant is measured at 405 nm. The results may be seen in **FIG. 15.**

Example 12

Ac-MARP as described above, is analyzed by 2-D NMR. **FIG. 16** shows that Ac-MARP forms a single, stable, and homogeneous folded state, as shown by 2-D NMR. The figure shows a fingerprint region of the 50 ms TOCSY spectrum of Ac-MARP. The

data set was acquired on a Varian Unity Plus 500 MHz instrument at 25°C with a protein concentration of 2 mM. Some HN/HA assignments are shown.

The identification of AGRP has added additional complexity to our attempts to understand weight homeostasis. In its role as an antagonist of the melanocortin satiety message, AGRP represents a unique target for anti-obesity drug development. While the use of baculovirus produced AGRP allowed us to determine some aspects of this protein's action, its laborious production and the inability to produce highly purified product created significant challenges. Chemical protein synthesis uses native chemical ligation of unprotected synthetic peptide segments in aqueous solution, followed by folding/disulfide formation to give the functional protein molecule (14). Our present experiments demonstrate that the techniques of chemical protein synthesis can be used to rapidly produce highly purified, biologically active AGRP molecules in amounts of tens of milligrams in a convenient and straightforward fashion.

Both chemically synthesized mouse [Leu127Pro]AGRP and MARP have similar inhibitory potency and efficacy as baculovirus produced human AGRP Form A + B. In cAMP assays [Leu127Pro]AGRP, MARP, and recombinant human AGRP Form A + B are essentially equipotent at inhibiting the hMC4R. Both chemically synthesized variant AGRP molecules were also found to display a similar nanomolar range of activity as previously observed for human recombinant Form A + B at the hMC3R (2). Like recombinant human Form A + B both chemically synthesized AGRP variants had only minimal activity at the hMC5R and neither displayed any inhibitory activity at the hMC 1R or the hMC2R. In contrast to their identical effects on α-MSH-induced cAMP generation, in a side-by-side radioligand displacement study (Figure 4B), MARP was slightly more potent than either longer synthetic or recombinant forms of AGRP in displacing ^{125}I-NDP-MSH from the hMC4R. Although the reason(s) for this is unclear, these observations suggest that the N-terminal domain of AGRP may play a role in regulating its activity. In regard to this point, it will be important to determine whether AGRP is proteolytically cleaved in mammals since the processing observed in the baculovirus expression system may not reflect the actual state of physiological events (2).

Having demonstrated the biological activity of the chemically synthesized AGRP variants we sought to use these unique reagents to further study the actions of this protein. Our successful radiolabeling of MARP with iodine 125 allowed us to directly study MARP binding. ^{125}I-MARP bound only to those heterologous cell lines expressing melanocortin receptor subtypes susceptible to AGRP inhibition in cAMP assays and at

which ^{125}I-NDP-MSH was displaced by mouse [Leu127Pro]AGRP (Figures 3-5). Typical displacement curves appear to indicate that the biological activity of MARP was not altered by the iodination process. The finding that the displacement of ^{125}I-MARP from the hMC5R was shifted to the right is consistent with the decreased potency of AGRP at this receptor subtype observed in cAMP assays.

A persistent controversy that has existed regarding the action of agouti protein is whether it has effects independent of its antagonism of α-MSH (21-23). Much of this speculation is based on the sequence similarity between agouti protein, and cone snail (conotoxins) and spider (plectoxins) toxins. These toxins, which affect calcium channels, contain a cysteine-rich motif that can be closely aligned against ten cysteine residues present in the carboxyl-terminus of both Agouti and AGRP (Figure 1). While some of the effects of Agouti in the absence of α-MSH may be explained by its ability to act an inverse agonist it has been suggested that a separate agouti receptor may exist (24,25). This controversy has been approached by examining the action of Agouti on melanoma cell lines lacking the MC1R and more recently using epitope tagged Agouti (26,27). However, this matter has been somewhat difficult to study since a radiolabeled Agouti has not been developed. Because of this controversy we used the novel radioligand ^{125}I-MARP to examine the binding sites of AGRP. Both conventional binding studies and photoemulsion studies indicate that $^{(125)}$I-MARP only binds to melanocortin receptors demonstrated to be susceptible to AGRP inhibition in cAMP assays. This does not, however, exclude the possibility that an endogenous cell type that expresses a native hMC3R, hMC4R, or hMC5R may also possess additional binding sites.

The competitive pattern of AGRP inhibition of melanocortins binding to the MC3R and MC4R observed in the present studies does not necessarily imply that AGRP and melanocortin agonist occupy the same site on the receptor. It is possible that the two ligands simply influence each other's binding through an allosteric mechanism. In fact, there is no significant sequence similarity between melanocortins and AGRP although this does not exclude some similarity on the basis of three dimensional structure. Future receptor mutagenesis studies using our novel radioligand ^{125}I-MARP and the radioligand ^{125}I-NDP-MSH should be helpful in this respect.

Importantly, our present data indicate that MARP is equipotent to the mature form of AGRP. As previously noted, both agouti protein and AGRP contain a cysteine-rich C-terminus (Figure 1). Studies by others have shown that a 48 amino acid C-terminal enzymatic digest that encompasses the cysteine-rich portion of agouti protein is equipotent to full length agouti protein in inhibiting α-MSH action at the mouse MC1R

(28). However, in the referenced work, the C-terminal fragment of agouti was only characterized on the endogenous MC1R of the mouse B 16 melanoma cell line. Therefore, conclusions about the potency and selectivity of that portion of agouti protein at other melanocortin subtypes could not be made. In the present experiments the truncated molecule MARP was examined on all five MCR subtypes. Our data indicates that the cysteine-rich C-terminal portion of AGRP not only contains information for full biological activity, but is also responsible for the receptor selectivity of the protein. Even lacking the N-terminal portion of AGRP, MARP maintains its selectively for the MCR subtypes 3, 4, and 5. Further truncation and other manipulations of human AGRP 87-132 will help identify its minimally active form and modification of residues within this fragment should provide insight into the determinants of receptor subtype selectivity. Whether the C-terminal portion of agouti protein retains the same spectrum of inhibition and selectivity for the MCR subtypes as full length agouti protein remains to be determined.

The observation that AGRP is equipotent at the hMC3R and the hMC4R is potentially of great significance to present concepts regarding the role of melanocortin receptors in the regulation of feeding behavior. Previous *in situ* hybridization studies demonstrate that both MC3R and MC4R transcripts are present in hypothalamic feeding centers (7,8). Therefore, from an anatomical standpoint it is plausible to hypothesize that the activity of both receptors is regulated by AGRP in events surrounding feeding behavior and weight regulation. At present, the fact that targeted disruption of the MC4R leads to obesity provides quite compelling evidence that the MC4R is involved in this process (10). The participation of the MC3R in feeding behavior is at present more circumstantial. To date, no MC3R knock-out has been reported and subtype specific agonists and antagonists that could help clarify this issue are only now being developed. Nonetheless, the present data appears to further implicate the MC3R in events surrounding weight regulation.

In summary, these studies demonstrate the ability to chemically synthesize a biologically active AGRP and have defined a minimized form, MARP, consisting of the C-terminus cysteine-rich module, which retains the biological activity of AGRP. These studies also describe the first AGRP radioligand, ^{125}I-MARP and demonstrate the binding of this radioligand directly to melanocortin receptor protein. ^{125}I-MARP will be a helpful tool for anatomical studies of the natural sites of AGRP binding. It should also facilitate the development of a radioimmunoassay that can be used to

study AGRP release within the hypothalamus. Finally, ^{125}I-MARP may prove useful in identification of small molecule antagonists of AGRP interaction with the melanocortin receptors. Such compounds could have potential applications as regulators of human feeding behavior. These data have important implications for structural studies of AGRP, anti-obesity drug development and obesity research.

REFERENCES

1. Shutter, J. R., Graham, M., Kinsey, A. C., Scully, S., Luthy, R., Stark, K. L. (1997) *Gene Dev.* 11:593-602.

2. Ollmann, M. M., Wilson, B. D., Yang, Y.-K., Kerns, J. A., Chen Y., Gantz, I., Barsh, G. S. (1997) *Science* **278**, 135-138.

3. Bultman, S. J., Michaud, E. J., Woychik, R. P. (1992) Cell 71, 1195-1204.

4. Leibel, R. L., Chung, W. K., Chua, S.C. (1997) J. Biol. Chem. 272, 31937-31940.

5. Lu, D., Willard, D., Patel, I. R., Kadwell, S., Overton, L., Kost, T., Luther, M., Chen, W., Woychik, R. P., Wilkison, W. O., Cone, R. D. (1994) Nature 371,799-802.

6. Yang, Y.-K., Ollmann, M. M., Wilson, B. D., Dickinson, C., Yamada, T., Barsh, G. S., Gantz, I. (1997) Mol. Endocrinol. 11, 274-280.

7. Gantz, I., Miwa, H., Konda, Y., Shimoto, Y., Tashiro, T., Watson, S. J., DelValle, J., Yamada, T. (1993) J. Biol. Chem. 268, 15174-15179.

8. Mountjoy, K. G., Mortrud, M. T., Low, M. J., Simerly, R. B., Cone, R. D. (1994) Mol. Endocrinol. 8, 1298-1308.

9. Huszar, D., Lynch, C. A., Fairchild-Huntress, V., Dunmore, J. H., Fang, Q., Berkemeier, L. R., Gu, W., Kesterson, R. A., Boston, B. A., Cone, R. D., Smith, F. J., Campfield, L. A., Bum, P., Lee, F. (1997) Cell 88:131-141.

10. Fan, W., Boston, B. A., Kesterson, R. A., Hruby, V. J., Cone, R. D. (1997) Nature 385, 165-168.

11. Seeley, R. J., Yagaloff, K. A., Fisher, S. L., Burn, P., Thiele, T. E., Van Dijk, G., Baskin, D. G., Schwartz, M. W. (1997) Nature 390, 349.

12. Thronton, J. E., Cheung, C. C., Clifton, D. K., Steiner, R. A. (1997) Endocrinol. 138, 5063-5066.

13. Hakansson, M.-L., Brown, H., Ghilardi, N., Skoda, R. C., Meister, B. (1998) J. Neurosci. 18, 559-572.

14. Dawson, P. E., Muir, T. W., Clark-Lewis, I., Kent, S. B. H. (1994) Science 266 776-779.

15. Schnolzer, M., Alewood, P., Jones, A., Alewood, D., Kent, S. B. H. (1992) Int. J. Peptide Protein Res. 40, 180-193.

16. Canne, L. E., Walker, S. M., Kent, S. B. H. (1995) Tetrahedron Letters 36, 1217-1220.

17. Potenza, M. N., Lerner, M. R. (1992) Pigment Cell Res. 5, 372-378.

18. Kenakin T. P. (1987) in Pharmacologic Analysis of Drug-Receptor Interaction (Raven Press, New York), pp. 221-236.

19. Hadley, M. E., Anderson, B., Heward, C. B., Sawyer, T. K., Hruby, V. J. (1981) Science 213, 1025-102.

20. Tatro J. B., Reichlin S. (1987) Endocrinology 121, 1900-1907.

21. Manne, J., Argeson, A. C., Siracusa, L. D. (1995) Proc. Natl. Acad. Sci. USA 92 4721-4724.

22. Hunt, G., Thody, A. J. (1995) J. Endocrinol 147, R1-R4.

23. Siegrist, W., Drozdz, R., Cotti, R., Willard, D. H., Wilkison, W. O., Eberle, A. N. (1997) J. Recept. Signal Transduct. Res. 17, 75-98.

24. Sakai, C., Gilmann, M., Kobayashi, T., Abdel-Malek, Z., Muller, J., Vierira, W. D., Imokawa, G., Barsh, G. S., Hearing, V. J. (1997) Embo J. 16, 3544-3552.

25. Conklin, B. R., Bourne, H. R. (1993) Nature 364, 110.

26. Siegrist, W., Willard, D. H., Wilkison, W. O., Eberle, A. N. (1996) Biochem. Biophys. Res. Commun. 218, 171-175.

27. Gilmann, M. M., Lamoreux, M. L., Wilson, B. D., Barsh, G. S. (1998) Genes and Development 12, 316-330.

28. Willard, D. H., Bodnar, W., Harris, C., Kiefer, L., Nichols, J. S., Blanchard, S., Hoffman, C., Moyer, M., Burkhart, W., Weiel, J., Luther, M. A., Wilkison, W. O., Rocque, W. J. (1995) Biochemistry 34, 12341-12346.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

WE CLAIM:

1. A polypeptide comprising the amino acid sequence of Sequence I.D. No.1 or a modified form thereof.

2. The polypeptide of Claim 1 wherein the polypeptide is chemically synthesized.

3. The polypeptide of Claim 2, wherein the polypeptide is N-acetyl-MARP.

4. The polypeptide of Claim 1, wherein the polypeptide is labeled with Biotin.

5. The polypeptide of Claim 1, wherein the polypeptide is labeled with a radioactive isotope.

6. The polypeptide of Claim 5, wherein the radioactive isotope is ^{125}I.

7. The polypeptide of Claim 1, wherein the polypeptide is labeled with fluorescent compound.

8. The polypeptide of Claim 1, wherein the polypeptide contains one or more D-amino acids.

9. The polypeptide of Claim 1, wherein the polypeptide contains all D-amino acids.

10. A polypeptide comprising the amino acid sequence of Sequence I.D. No.2 or a modified form thereof.

11. The polypeptide of Claim 10 wherein the polypeptide is chemically synthesized.

12. The polypeptide of Claim 10, wherein the polypeptide is labeled with Biotin.

13. The polypeptide of Claim 10, wherein the polypeptide is labeled with a radioactive isotope.

14. The polypeptide of Claim 13, wherein the radioactive isotope is ^{125}I.

15. The polypeptide of Claim 10, wherein the polypeptide is labeled with fluorescent compound.

16. The polypeptide of Claim 10, wherein the polypeptide contains one or more D-amino acids.

17. The polypeptide of Claim 10, wherein the polypeptide contains all D-amino acids.

18. A polypeptide comprising the amino acid sequence of Sequence I.D. No.3 or a modified form thereof.

19. The polypeptide of Claim 18, wherein the polypeptide is chemically synthesized.

20. The polypeptide of Claim 18, wherein the polypeptide is labeled with Biotin.

21. The polypeptide of Claim 18, wherein the polypeptide is labeled with a radioactive isotope.

22. The polypeptide of Claim 21, wherein the radioactive isotope is ^{125}I.

23. The polypeptide of Claim 18, wherein the polypeptide is labeled with fluorescent compound.

24. The polypeptide of Claim 18, wherein the polypeptide contains one or more D-amino acids.

25. The polypeptide of Claim 18, wherein the polypeptide contains all D-amino acids.

26. A method of treating a disease state in mammals that is alleviated by treatment with a polypeptide an amino acid sequence of Sequence I.D. No. 1, which method comprises administering to a mammal in need of such a treatment a therapeutically effective amount of said polypeptide, or a pharmaceutically acceptable salt thereof.

27. The method of claim 26 wherein said disease state is a wasting syndrome.

28. A method of treating a disease state in mammals that is alleviated by treatment with a polypeptide an amino acid sequence of Sequence I.D. No. 2, which method comprises administering to a mammal in need of such a treatment a therapeutically effective amount of said polypeptide, or a pharmaceutically acceptable salt thereof.

29. The method of claim 28 wherein said disease state is a wasting syndrome.

30. A method of treating a disease state in mammals that is alleviated by treatment with a polypeptide an amino acid sequence of Sequence I.D. No. 3, which method comprises administering to a mammal in need of such a treatment a therapeutically effective amount of said polypeptide, or a pharmaceutically acceptable salt thereof.

31. The method of claim 30 wherein said disease state is a wasting syndrome.

32. A pharmaceutical composition comprising a therapeutically effective amount of a polypeptide of the sequence of Sequence I.D. No. 1 or a pharmaceutically acceptable salt thereof.

33. A pharmaceutical composition comprising a therapeutically effective amount of a polypeptide of the sequence of Sequence I.D. No. 2 or a pharmaceutically acceptable salt thereof.

34. A pharmaceutical composition comprising a therapeutically effective amount of a polypeptide of the sequence of Sequence I.D. No. 3 or a pharmaceutically acceptable salt thereof.

35. A method of determining whether a compound is capable of inhibiting binding of a melanocortin agonist or antagonist to a melanocortin receptor comprising:

combining a cell line expressing the melanocortin receptor with the melanocortin agonist or antagonist containing a detectable label and said compound under conditions suitable to permit binding of the melanocortin agonist or antagonist to said receptor and

detecting the decrease or lack of decrease in the binding of the melanocortin agonist or antagonist to the receptor in comparison to the binding of the melanocortin agonist or antagonist to the receptor in control that does not contain the compound.

36. The method of Claim 35, wherein the detectable label is a radioactive isotope.

37. The method of Claim 36, wherein the radioactive isotope is ^{125}I.

38. The method of Claim 35, wherein said melanocortin agonist is α-MSH.

39. The method of Claim 35, wherein said melanocortin antagonist is MARP.

40. The method of Claim 35, wherein the compound is MARP.

41. The method of Claim 35, wherein said melanocortin antagonist is AGRP or a derivative thereof.

42. The method of Claim 35, wherein said cell line is expressing MC3R.

43. The method of Claim 35, wherein said cell line is expressing MC4R.

44. The method of Claim 35, wherein said cell line is expressing MC5R.

45. The method of Claim 35, wherein the method is a High Throughput Assay.

46. A method of measuring binding of a compound to a melanocortin receptor comprising:

combining a cell line expressing a melanocortin receptor with said compound containing a detectable label and the unlabeled compound under conditions suitable to permit binding of the compound to said receptor and

detecting the decrease or lack of decrease in the binding of the labeled compound to said receptor as a function of increasing concentrations of the unlabeled compound.

47. The method of Claim 46, wherein said detectable label is a radioactive isotope.

48. The method of Claim 47, wherein said radioactive isotope is ^{125}I.

49. The method of Claim 46, wherein said compound is MARP or a derivative thereof.

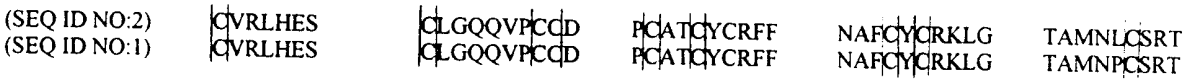
50. The method of Claim 46, wherein said cell line is expressing MC3R.

51. The method of Claim 46, wherein said cell line is expressing MC4R.

52. The method of Claim 46, wherein said cell line is expressing MC5R.

53. The method of Claim 46, wherein the method is a high throughput assay.

FIG. 1

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                                        ▼
  mAGRP       MLTAM LLSCVLLLAL PPTLGVQMGV APLKGIRRPD QALFPEFPGL 45
  hAGRP       MLTAA LLSCALLLAL PATRGAQMGL APMEGIRRPD QALLPELPGL 45
magouti MDVTRLLLAT LVGFLCFFTV HSHLALEETL GDDRSLRSNS SMNSLDFSSV 50
hagouti MDVTRLLLAT LLVFLCFFTA NSHLPPEEKL RDDRSLRSNS SVNLLDVPSV 50

  mAGRP SLNG.LKKTT ADRAEEVLLQ KAEALAEVLD PQNRESRSPR R..CVRLHES 94
  hAGRP GLRAPLKKTT AEQAEEDLLQ EAQALAEVLD LQDREPRSSR R..CVRLHES 95
magouti SIVALNKKSK KISRKEAEKR KRSSKKKASM KKVARP.PPP S.PCVATRDS 98
hagouti SIVALNKKSK QIGRKAAEK. KRSSKKEASM KKVVRPRTPL SAPCVATRNS 99

  mAGRP CLGQQVPCCD PCATCYCRFF NAFCYCRKLG TAMNLCSRT 131
  hAGRP CLGQQVPCCD PCATCYCRFF NAFCYCRKLG TAMNPCSRT 132
magouti CKPPAPACCD PCASCCCRFF GSACTCRVL. NP.N.C    131
hagouti CKPPAPACCD PCASCCCRFF RSACSCRVL. SL.N.C    132
```

FIG. 2

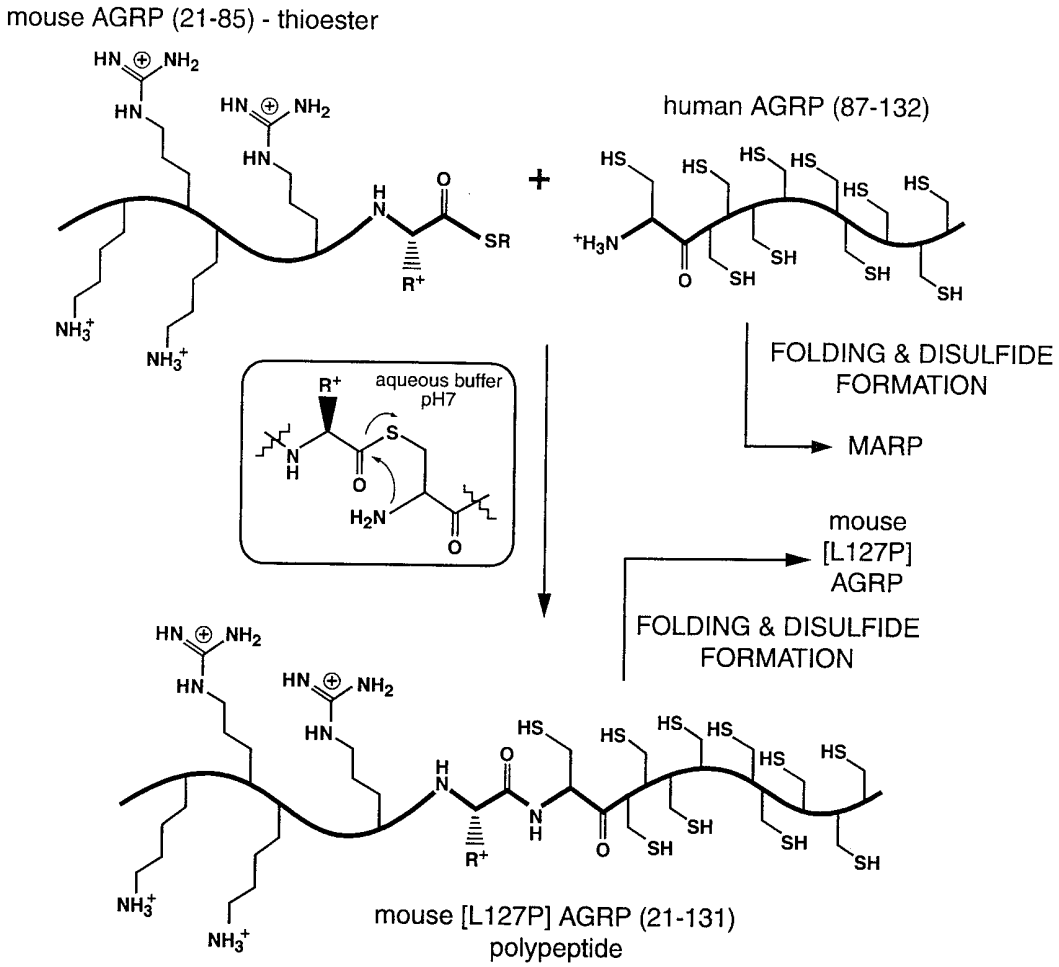

mouse AGRP (21-85) - thioester
human AGRP (87-132)
aqueous buffer
pH7
FOLDING & DISULFIDE
FORMATION
MARP
mouse
[L127P]
AGRP
FOLDING & DISULFIDE
FORMATION
mouse [L127P] AGRP (21-131)
polypeptide

FIG. 3A hMC1R

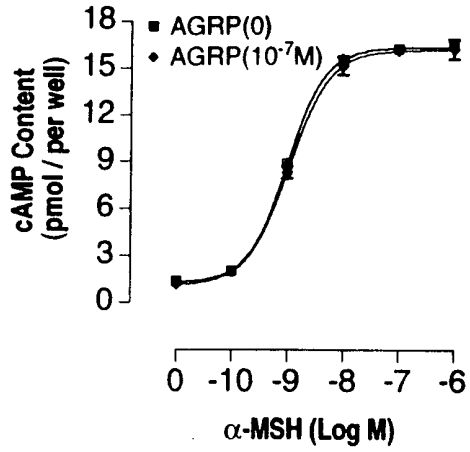


FIG. 3B hMC2R

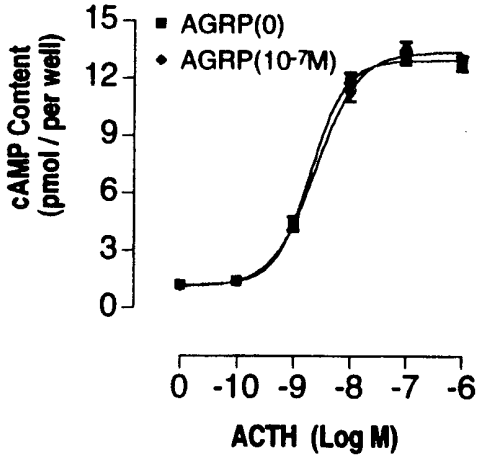


FIG. 3C hMC5R

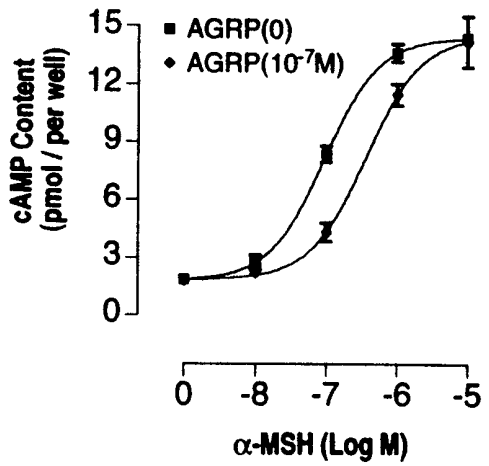


FIG. 3D

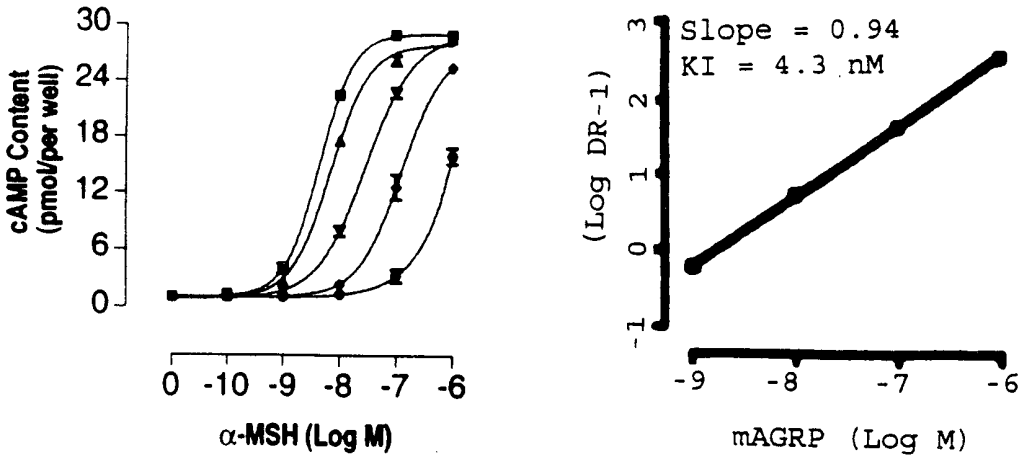


FIG. 3E

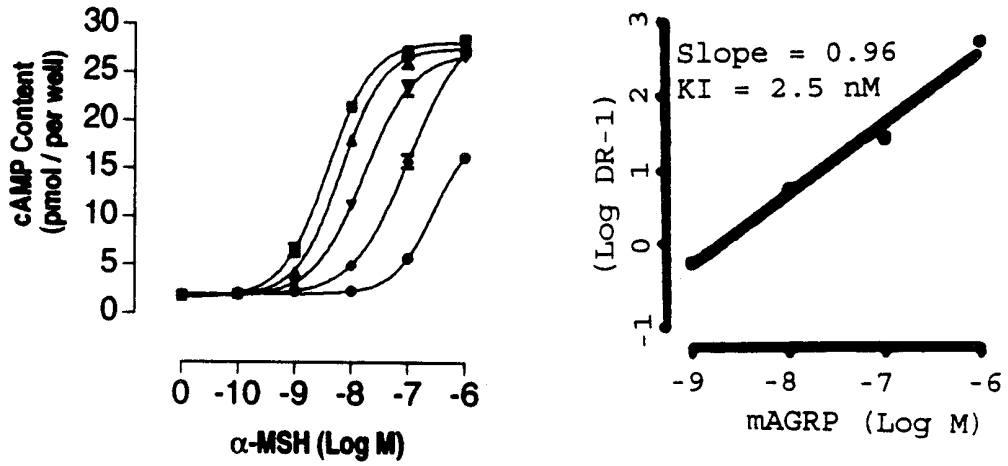


FIG. 3F

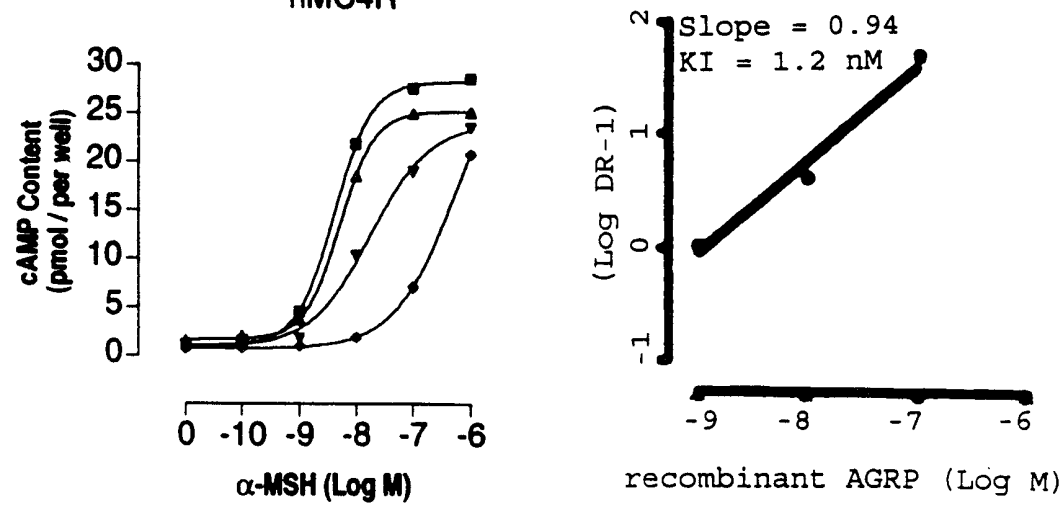


FIG. 4A

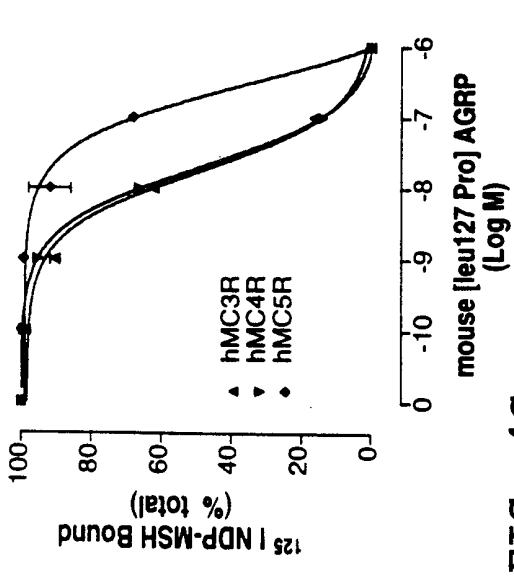


FIG. 4B

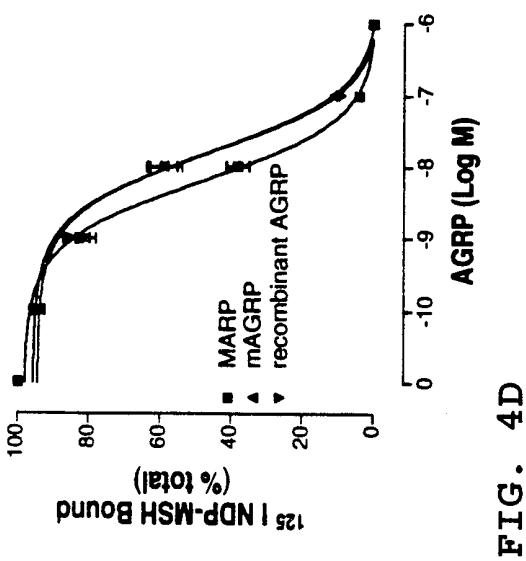


FIG. 4C

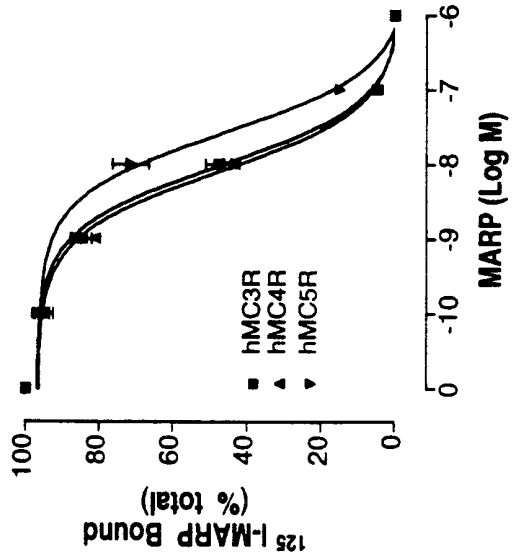
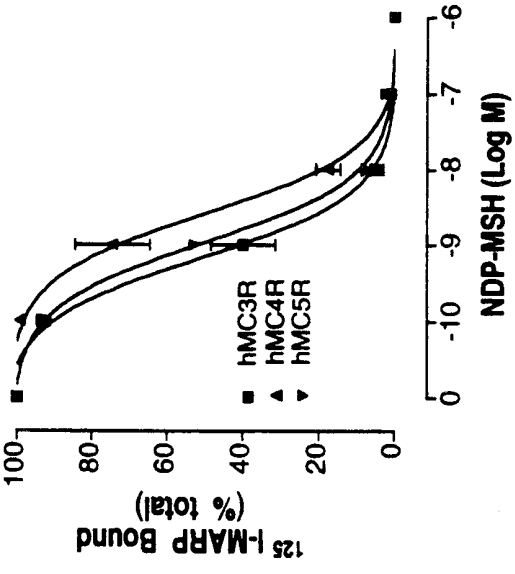


FIG. 4D



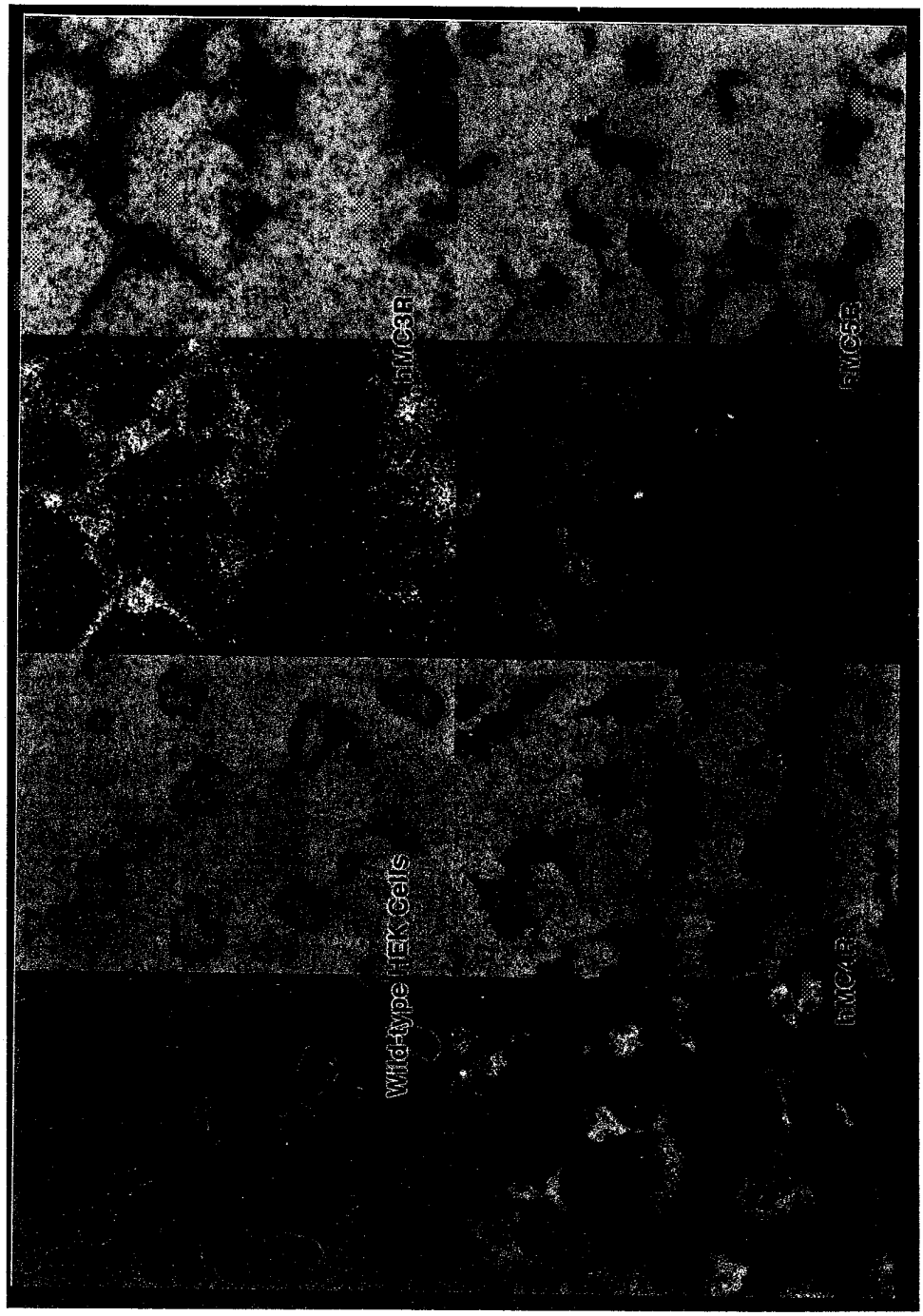


FIG. 5

FIG. 6

Table 1

		hMC3R	hMC4R
Ki (nM)	mouse [Leu127Pro] AGRP	4.3±0.6	2.5±0.25
	MARP	3.3±0.28	2.6±0.21
	Recombinant Form A+B	ND	1.2±0.17
IC_{50} (nM)	^{125}I NDP-MSH*	17.4±3.7	15.7±4.1
	^{125}I MARP#	11.2±3.1	9.0±1.7

*^{125}I NDP-MSH was displaced by mouse [Leu 127Pro] AGRP

#^{125}I MARP were displaced by unlabeled MARP

FIG. 7

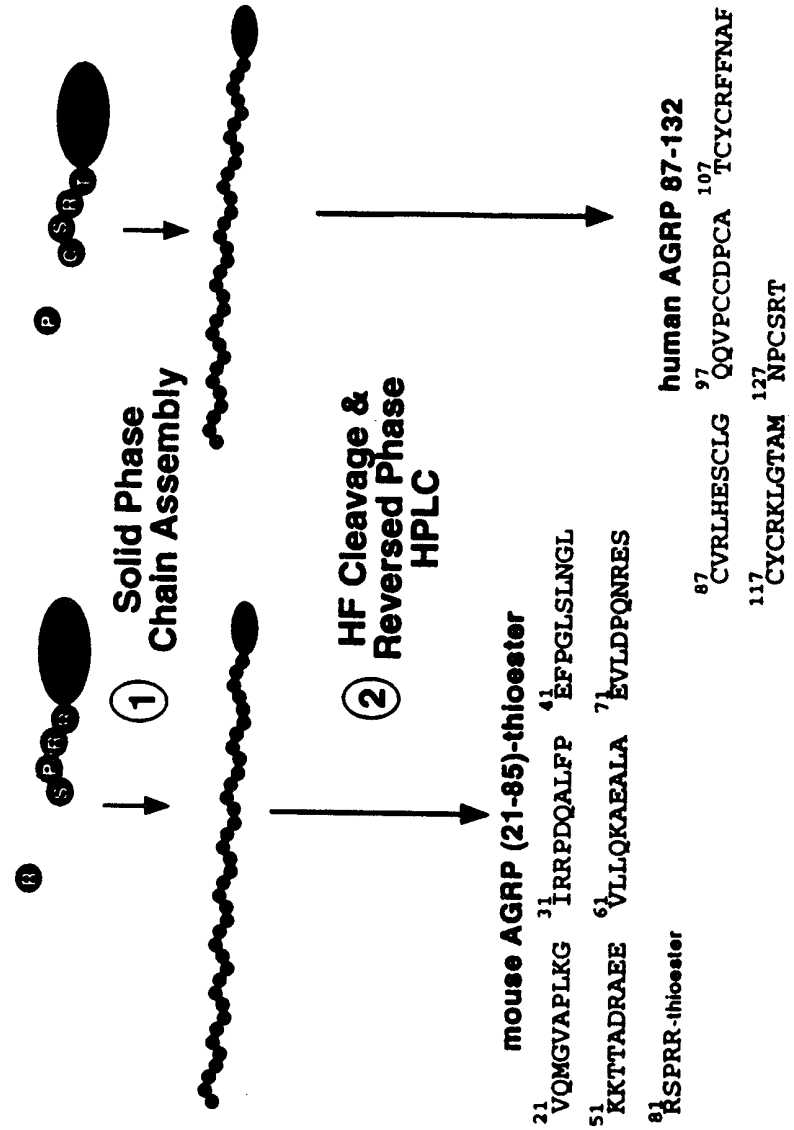
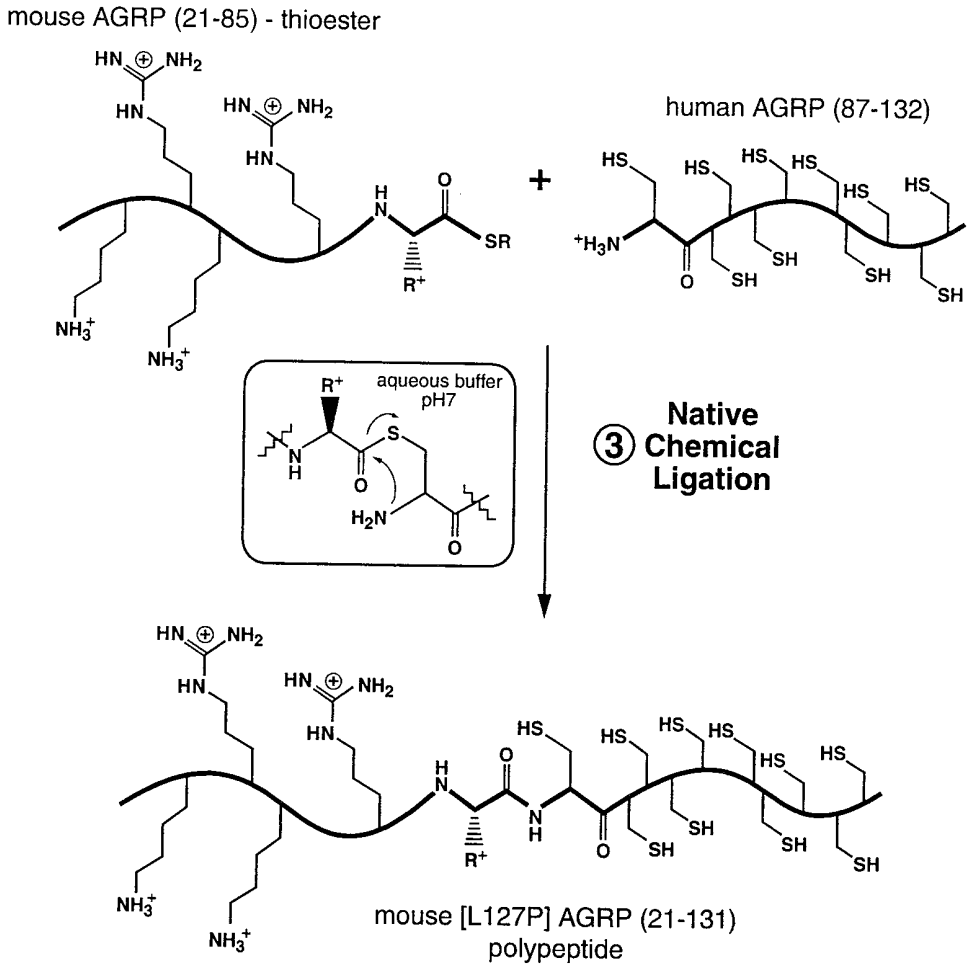

Solid Phase
Chain Assembly
HF Cleavage &
Reversed Phase
HPLC
mouse AGRP (21-85)-thioester
21 VQMGVAPLKG 31 IRRPDQALFP 41 EFPGLSLNGL
51 KKTTADRAEE 61 VLLQKAEALA 71 EVLDPQNRES
81 RSPRR-thioester
human AGRP 87-132
87 CVRLHESCLG 97 QQVPCCDPCA 107 TCYCRFFNAF
117 CYCRKLGTAM 127 NPCSRT

FIG. 8



(SEQ ID NO: 3)

^{21}VQNGVAPLKG	^{31}IRRPDQALFP	^{41}EFPGLSLNGL	^{51}KKTTADRAEE
^{61}VLLQKAEALA	^{71}EVLDPQNRES	^{81}RSPRRCVRLH	^{91}ESCLGQQVPC
^{101}CDPCATCYCR	^{111}FFNAFCYCRK	^{121}LGTAMNPCSR	^{131}T

FIG. 9A

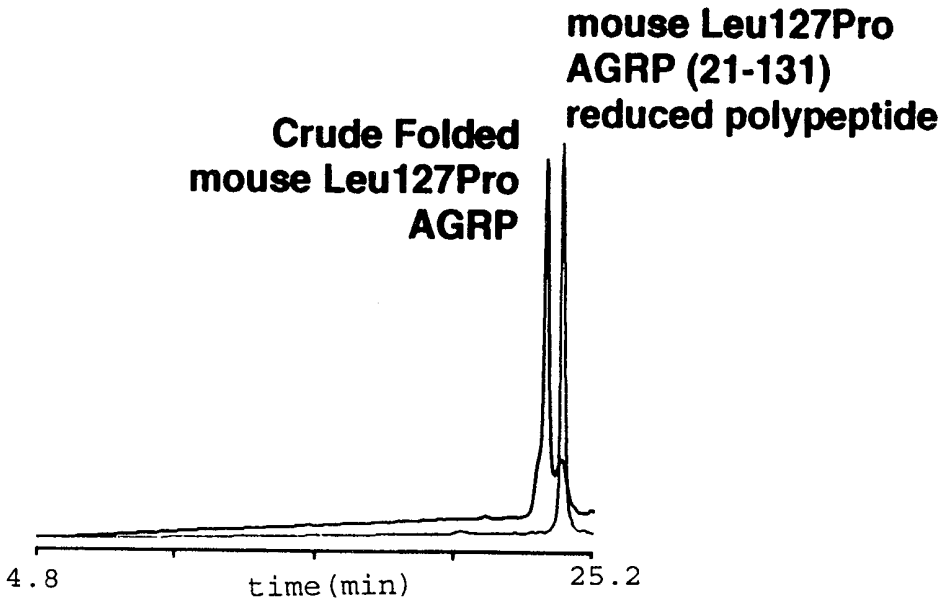

mouse Leu127Pro
AGRP (21-131)
reduced polypeptide
Crude Folded
mouse Leu127Pro
AGRP
4.8
time(min)
25.2

FIG. 9C

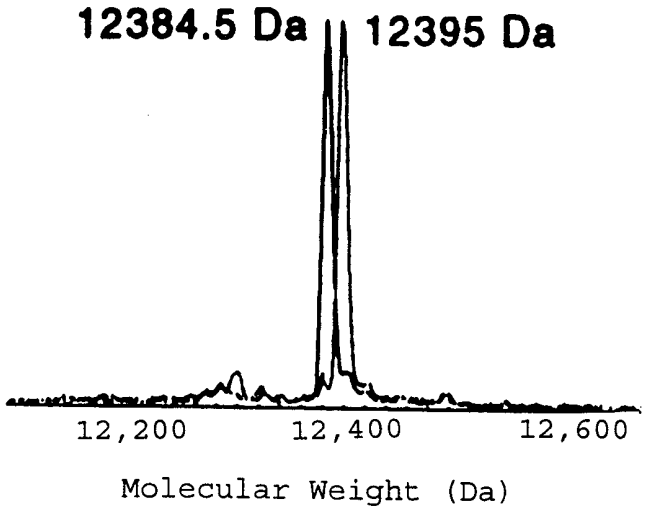

12384.5 Da
12395 Da
12,200
12,400
12,600
Molecular Weight (Da)

FIG. 9B

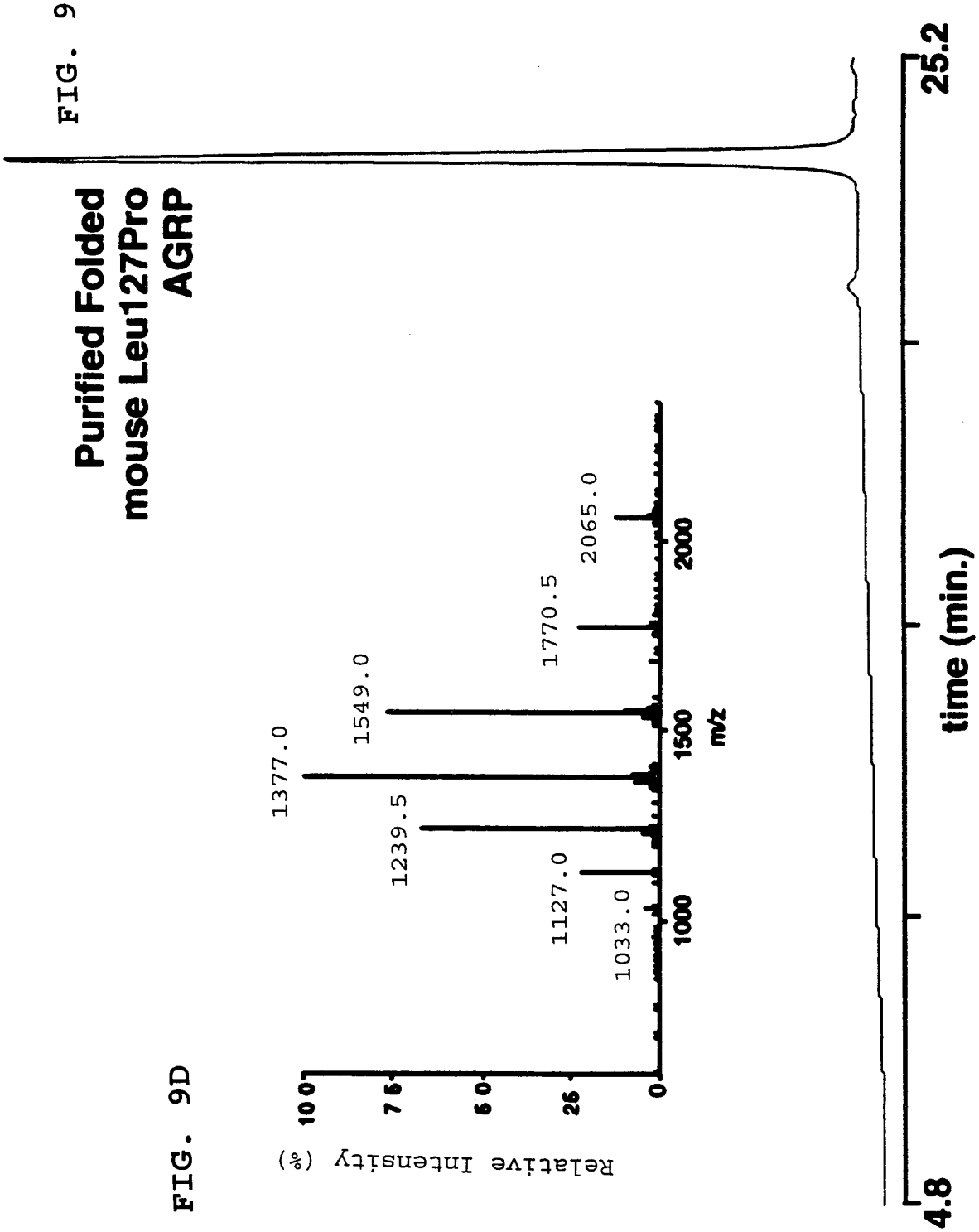

Purified Folded
mouse Leu127Pro
AGRP
25.2
4.8
time (min.)

FIG. 9D

Relative Intensity (%)
100
75
50
25
0
1000
1500
2000
m/z
1377.0
1549.0
1239.5
1127.0
1033.0
1770.5
2065.0

FIG. 10A

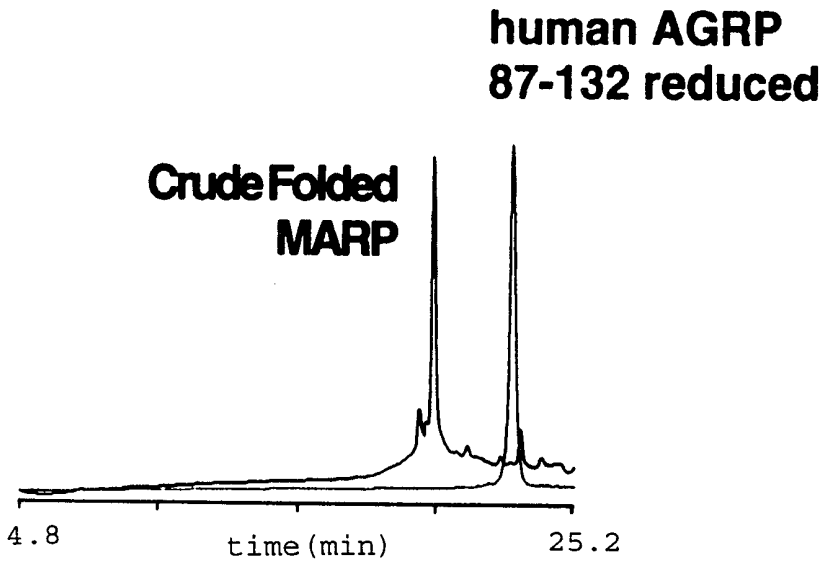
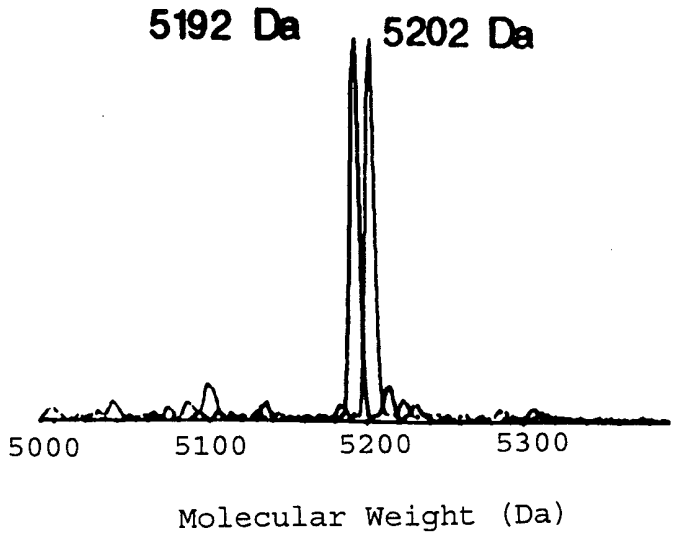


FIG. 10C



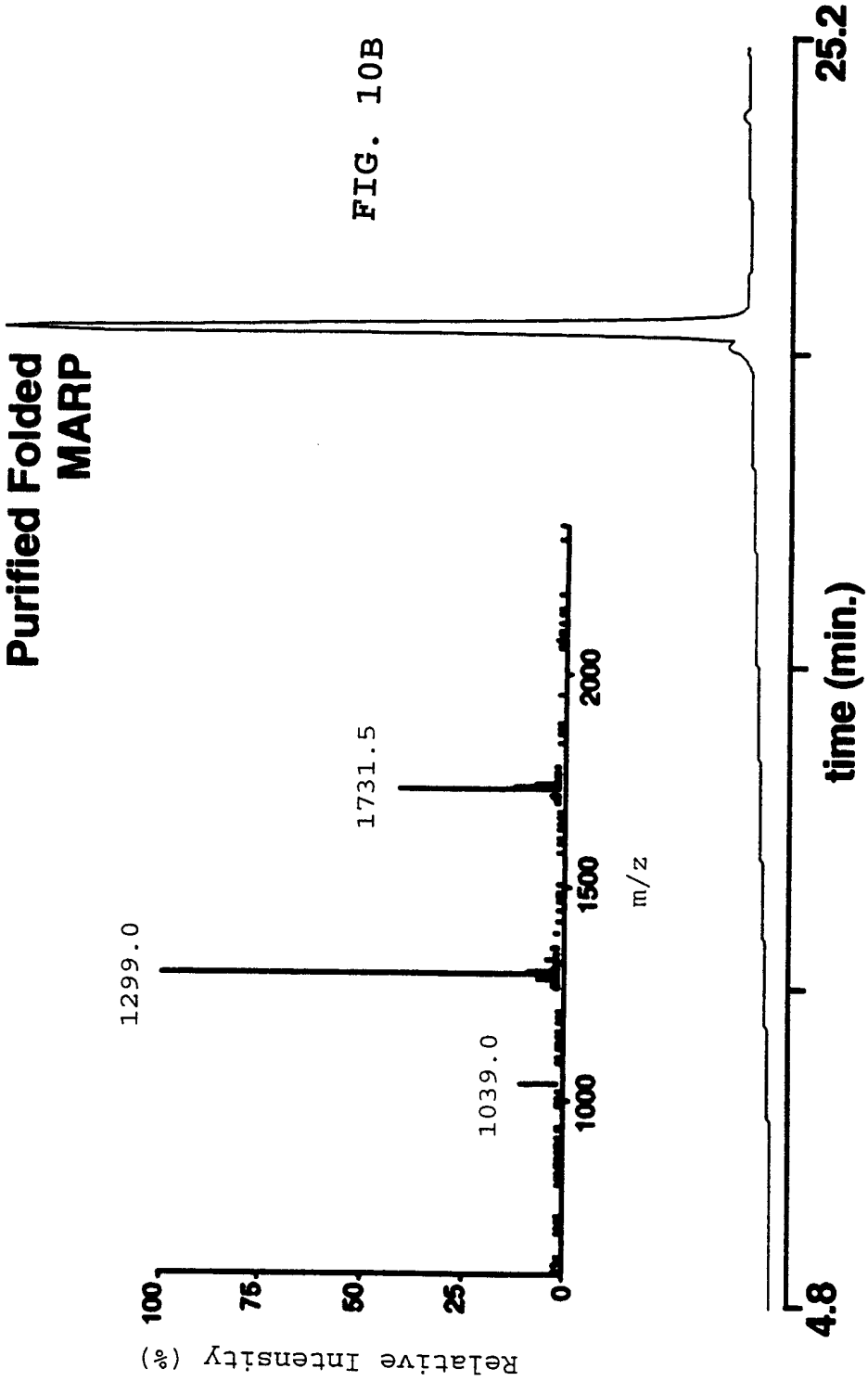


FIG. 10B

FIG. 10D

FIG. 11

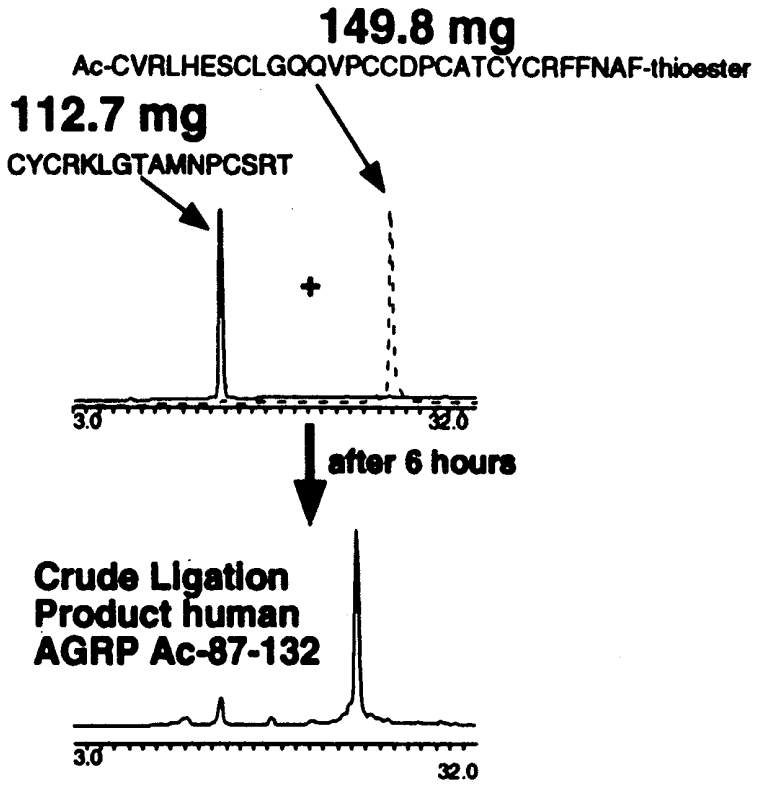

149.8 mg
Ac-CVRLHESCLGQQVPCCDPCATCYCRFFNAF-thioester
112.7 mg
CYCRKLGTAMNPCSRT
3.0
32.0
after 6 hours
Crude Ligation
Product human
AGRP Ac-87-132
3.0
32.0

FIG. 12A

142.4 mg

Ac-CVRLHESCLGQQVPCCDPCATCYCRFFNAFCYCRKLGTAMNPCSRT

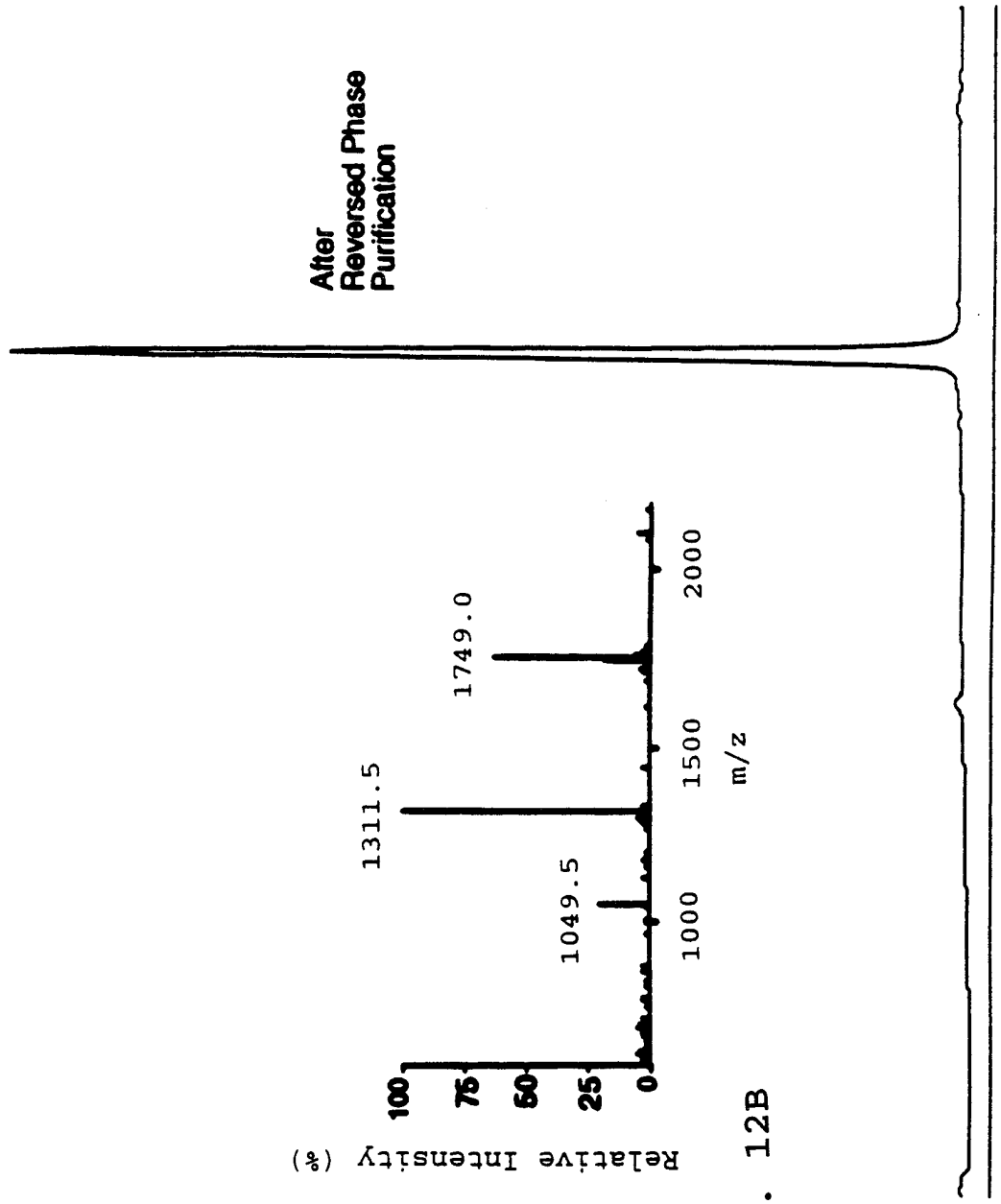


FIG. 12B

FIG. 13A

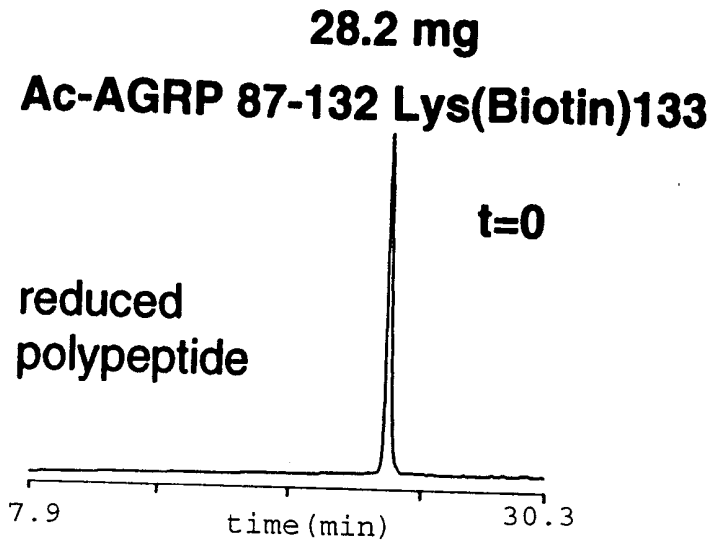


FIG. 13B

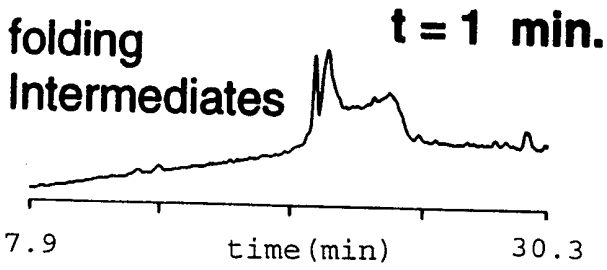
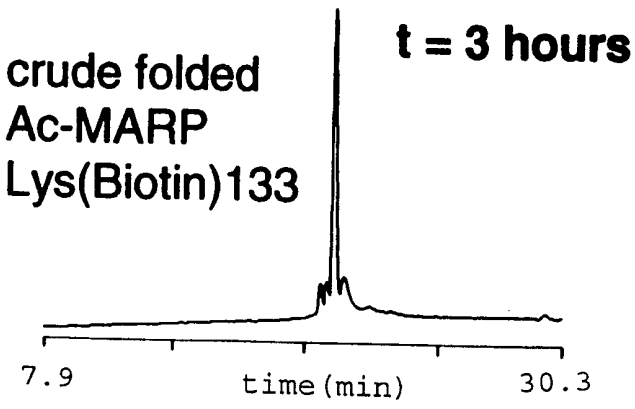


FIG. 13C



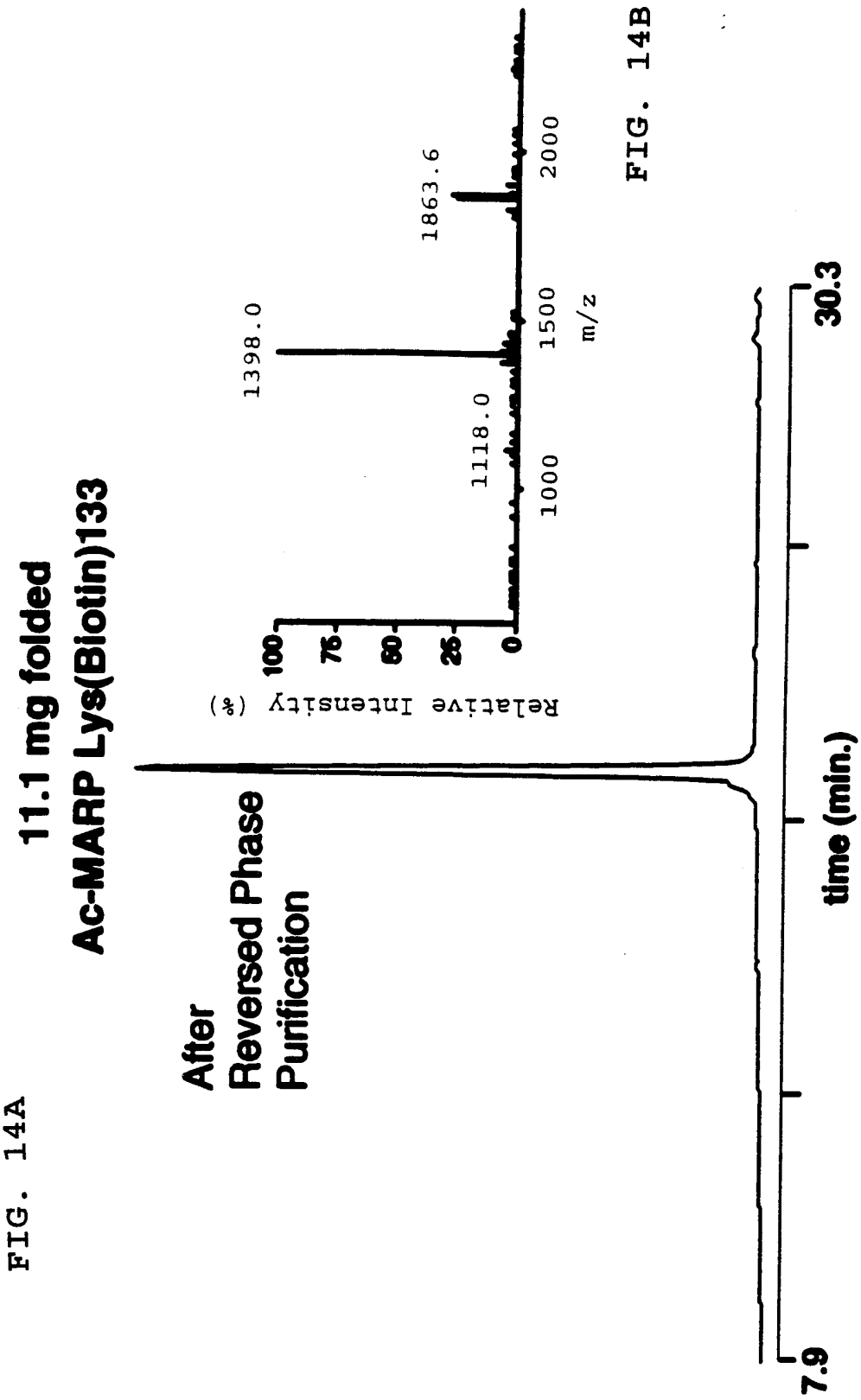


FIG. 14A

FIG. 14B

FIG. 15

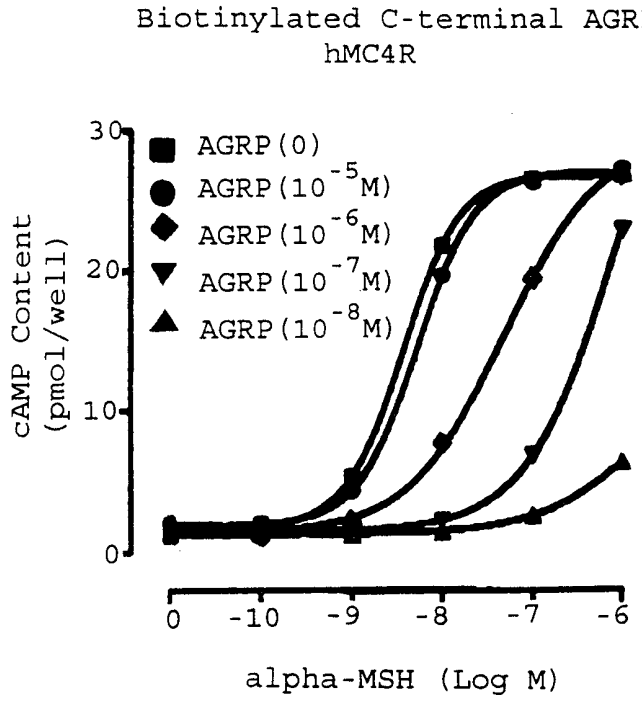
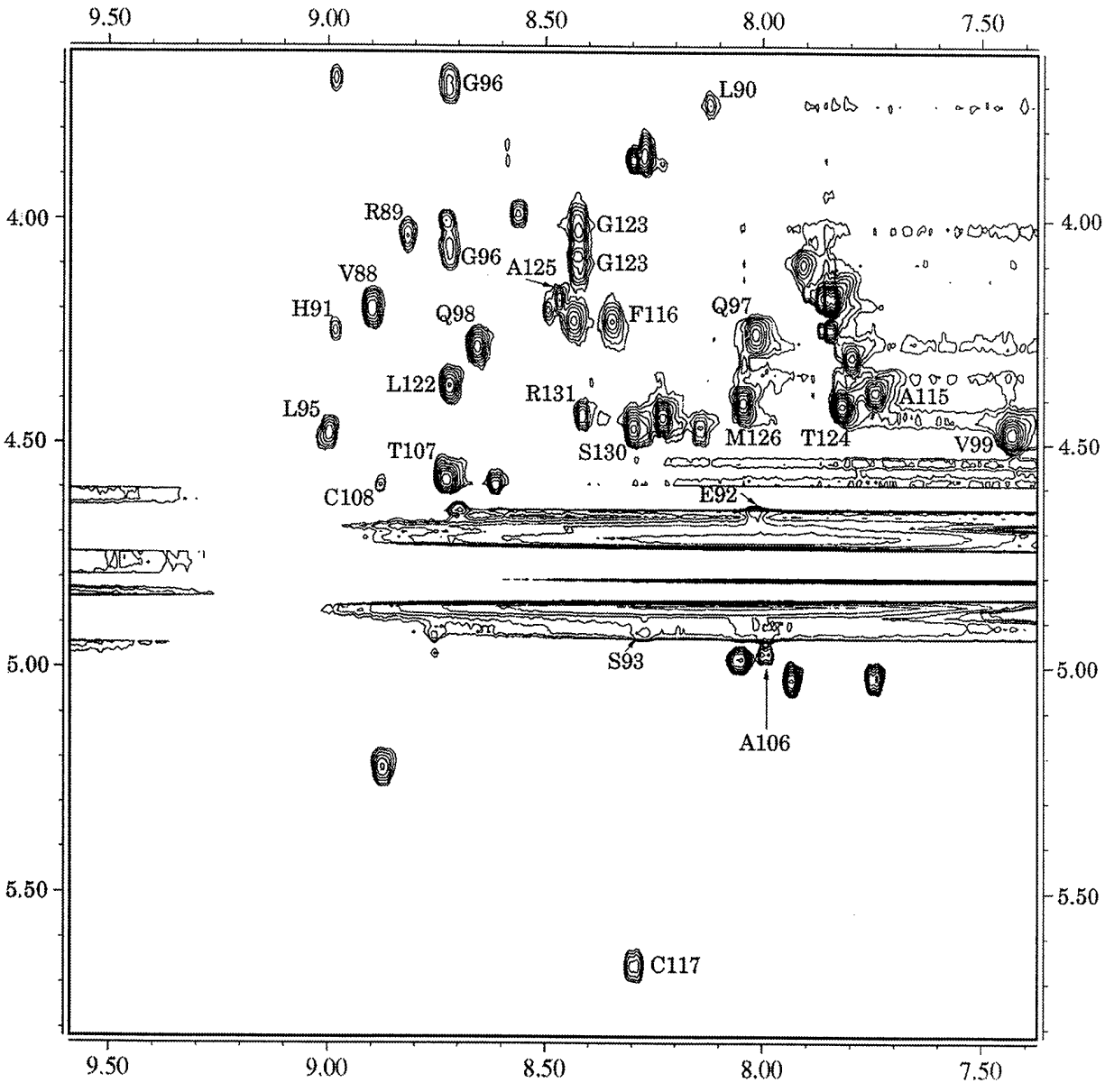

Biotinylated C-terminal AGRP
hMC4R
cAMP Content
(pmol/well)
30
20
10
0
AGRP(0)
AGRP(10-5M)
AGRP(10-6M)
AGRP(10-7M)
AGRP(10-8M)
0
-10
-9
-8
-7
-6
alpha-MSH (Log M)

FIG. 16



Ac-CVRLHESCLG QQVPCCDPCA TCYCRFFNAF CYCRKLGTAM NPCSRT
87 97 107 117 127