A synthetic human Agouti-related protein-(83–132)-NH_2 fragment is a potent inhibitor of melanocortin receptor function

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Abstract Chemical synthesis of Agouti proteins – Agouti and Agouti-related proteins – is complicated by their large size and by multiple cysteine residues located in the carboxyl terminal regions. Three human Agouti-related protein (AGRP) fragments, two of which correspond to a proposed endoprotease cleavage site between amino acids 82 and 83, were synthesized and tested for anti-melanotropic activity using *Xenopus laevis* dermal melanophores. Amino-terminal fragments AGRP(25–51) and (54–82) were devoid of significant antagonist activity, whereas the amidated carboxyl-terminal AGRP fragment (83–132)-NH_2 was potently active with an inhibitory equilibrium dissociation constant (K_i) of 0.7 nM. The ability to synthesize functionally active AGRP should help unravel its role in the central nervous system and its unusual properties with respect to interaction with the melanocortin family of G-protein coupled receptors.

Key words: Agouti-related protein; Protein synthesis; Melanocortin receptor antagonist; Melanocyte-stimulating hormone; Frog melanophore; (*Xenopus laevis*)

1. Introduction

The Agouti protein is perhaps best known for its modulation of coat color in mice. Normally expressed in skin cells, the protein effects this function by regulating eumelanin pigment synthesis via its antagonism of melanocortin subtype-1 (MC1) receptors. Ectopically expressed, Agouti can lead to a condition characterized by yellow coat color, where the normal pigmentation response to α-melanocyte-stimulating hormone (α-MSH) in dermal papilla cells of the hair follicle has been disrupted [1–6]. Paradoxically, mutant alleles of the *Agouti* gene affect not just processes of the skin but also broadly based characteristics such as obesity and hyperinsulinemia. A clue to the puzzle of such widespread effects may lie in a recently identified relative of Agouti, called Agouti-related protein (AGRP), which appears to be expressed primarily in the adrenal gland and the hypothalamus [1,2]. AGRP seems to play its role in the nervous system by inhibiting MC4 receptors, which are key players in energy homeostasis, food intake and obesity, and the leptin hormonal pathway [1,3,4]. Unraveling the molecular specifics of AGRP's activity may suggest a means by which to treat malfunction in some of these key physiological processes. Our strategy to achieve such molecular level inquiry is to chemically synthesize a bioactive fragment of the 132 amino acid parent human AGRP molecule. This synthesis gives us access to a host of structural and mechanistic studies of AGRP, while bypassing the potentially laborious synthetic and purification procedures that accompany the recombinant expression systems currently used to obtain AGRP [1,5].

While the structural features that determine potency and receptor specificity of human Agouti and AGRP remain elusive, the cysteine-rich carboxy terminus of each appears important for activity. Indeed, it is in this region that the proteins are most strikingly similar, exhibiting better than 50% identity for portions of their carboxyl halves. (Global sequence identity across their entire 132 amino acids is 30%.) Site-directed modification of four residues in this domain of mouse Agouti – Val(83), Arg(85), Pro(86) and Pro(89) – reduced binding of iodinated [Nle^4,D-Phe^7]-α-MSH to MC receptors [7], suggesting a key role for the carboxy terminus in Agouti's biological activity. Ollman et al. [1] have demonstrated analogous results for AGRP; partially purified fractions of AGRP containing C-terminal fragments (residues 69–132 or 71–132) antagonized α-MSH induced pigment dispersion in *Xenopus* melanophores with a potency similar to or slightly greater than fractions containing longer fragments (residues 24–132, 46–132, 48–132, or 50–132). It may well be that proteolytic processing of these proteins occurs in vivo. Based on analogy to the processing pattern of atrial-natriuretic factor [8,9], we propose that Pro(81)-Arg(82) of human AGRP, and Pro(85)-Arg(86) of human Agouti, may act as cleavage sites for the generation of biologically active protein fragments. We therefore synthesized a fragment corresponding to residues 83–132 of the AGRP protein, mimicking the purported proteolytic processing; peptides corresponding to more amino terminal parts of AGRP (residues 25–51 and 54–82) were generated to serve as controls.

Production of AGRP by any means, chemical or biological, is complicated by the protein's numerous cysteine residues. AGRP bears 11 cysteines in total, 10 of which are confined to the carboxyl half of the molecule; the potential for disulfide mismatches is large. Assignments of disulfide connectivities in the native protein are yet to be made. The task is complicated by the sheer number of cysteines and by the laboriousness of procedures currently employed to obtain purified protein. Some hints may be derived from the observation that cysteine residues found in Agouti, and therefore AGRP, lie in homologous positions to those found in ω-conotoxin GVIA, the N-type calcium channel antagonist isolated from *Conus geographus* [10]. We have employed a biological assay using *Xenopus* melanophores to test the activity of synthetic fragments, positing that the successful chemical synthesis of an active fragment implies that its folding pattern mimics the normal conformation. We suggest that chemical synthesis offers reliable means to manipulate protein structure and to obtain the ma-

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terial so crucial to the determination of endogenous disulfide arrangements and biological mechanism.

2. Materials and methods

2.1. Protein synthesis and purification

Protein fragments of human AGRP consisting of residues (25–51) with the sequence LAPMEGIRRPDQALLPELPGLGLRAPL, (54–82) with the sequence TTAEQAEEDLLQEAQALAEVLDLQ-DREPR, and the amidated form of (83–132) with the sequence SSRRCVRLHESCLGQQVPCCDPCATCYCRFFNAFCYCRKLG-TAMNPCSRT-NH_2 were synthesized on an Applied Biosystems 433A automated peptide synthesizer (Foster City, CA), using hydroxymethyl or *para*-methyl benzylhydrylamine resin as the solid support and standard solid-phase Fmoc methods. N-terminal Fmoc amino acids were deprotected with piperidine in dimethylformamide (20%, v/v) and coupled using *o*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate in 1-hydroxybenzotriazole (1:1 molar equivalents, in 5 molar excess). Protein fragments were cleaved from resin with hydrogen fluoride containing anisole (10%, v/v) and dimethyl sulfide (1%, v/v) for 45 min at 0°C, washed with diethylether, and extracted with water/acetonitrile (50%, v/v). AGRP-(83–132)-NH_2 fragments were cyclized in an acetonitrile/H_2O solution, adjusted to pH 8.5 with ammonium hydroxide, for 72 h, and extracted with Bio-Rex 70 cation exchange resin for 24 h. The Bio-Rex resin was then rinsed with H_2O, cyclized protein eluted from the resin with 50% glacial acetic acid in H_2O (v/v), and major fractions analyzed by Pauly spray. Major fractions were purified by HPLC using a Vydac C-18 column (5 micron pore size) over a standard linear gradient of 100% H_2O (0.1% trifluoroacetic acid) to 60% acetonitrile (0.1% trifluoroacetic acid). Samples were then roto-evaporated, lyophilized, and analyzed using matrix assisted laser desorption ionization-time of flight (MALDI-TOF) and electrospray mass spectrometry.

2.2. Testing synthetic AGRP fragments for functional antimelanotropic activity

Synthetic human AGRP fragments were assayed for antagonist activity using *Xenopus laevis* dermal melanophore cell preparations as described previously [11,12]. Cultured melanophores can translocate cytoplasmic pigment vesicles in response to hormonal stimulation by α-MSH, norepinephrine, vasotocin, pituitary adenylylcyclase activating peptide (PACAP), and serotonin. These compounds cause pigment dispersion and cell darkening. Pigment aggregation and cell lightening can be stimulated by melatonin. Melanophore responses were determined by measuring absorbance (OD) ratios taken through a monolayer of cells using a FisherBiotec BT2000 Microplate Reader (Pittsburgh, PA). Absorbance was measured before and after a 90-min exposure to test peptides and then expressed as percent of initial absorbance. Measurements were taken after 60 min pretreatment with 10 nM melatonin to initiate readings from an aggregated state.

3. Results

3.1. Protein synthesis and mass spectra analysis

Three preparatory HPLC separations of synthetic extracts yielded protein fragments which were 99% pure by analytical HPLC (Fig. 1). MALDI-TOF mass spectrometric analysis of the linear AGRP fragments gave a molecular mass of 2894.5 Da for (25–51) and 3282.5 Da for (54–82), both of which correspond to expected theoretical values. The molecular mass of cyclized AGRP(83–132)-NH_2 as determined by MALDI-TOF and electrospray mass spectra was 5678.04 (S.E. 0.1%) and 5677.0 (S.E. 0.01%) Da, respectively (Fig. 1). These values are consistent with the calculated theoretical

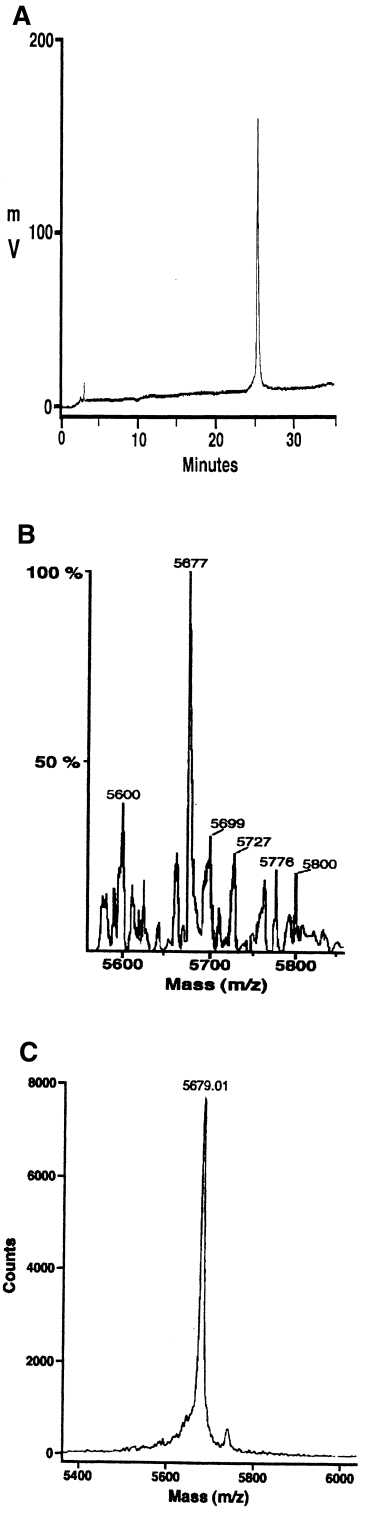

Fig. 1. A: Analytical HPLC of the purified fraction of AGRP-(83–132)-NH_2 used in mass spectrometric and in vitro testing. Tracing is for a linear gradient from 0% Buffer B to 100% Buffer B/40 min, where Buffer A = 100% water/0.1% trifluoroacidic acid (v/v) and Buffer B = 60% acetonitrile/0.1% trifluoroacidic acid (v/v). Flow rate was set at 1 ml/min and the detector set to 220 nm. Sample injection volume was 0.02 ml onto a 27.5×0.5 cm Vydac C-18 column with a 5-μ pore size. B: Electrospray mass spectrum of AGRP-(83–132)-NH_2 reveals one major peak at 5677.0 Da, which corresponds to the expected theoretical mass of a fully cyclized protein. C: MALDI-TOF mass spectrometric analysis was used to confirm that the minor peaks in the electrospray analysis were due to multiply charged ions or fragment ions. The final mass was calculated by subtracting one proton mass unit from the indicated value of 5679.04 Da.

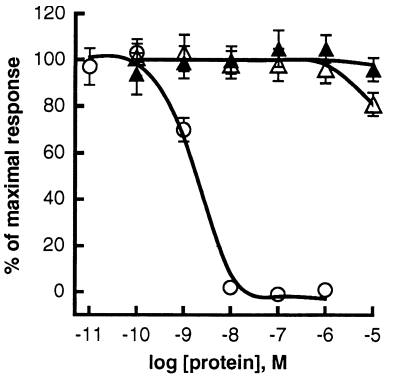

Fig. 2. AGRP-(83–132)-NH_2 (○) potently inhibits activation of pigment dispersion in melanophore cells by 300 pM α-MSH with an IC_{50} of 1.7 (S.E. 1.5) nM. N-terminal fragments AGRP-(25–51) (△) and AGRP (54–82) (▲) were inactive or had IC_{50} values in excess of 10 μM. Each symbol represents the mean and standard deviation of four independent measurements.

mass of 5676.7 Da for protein containing a full complement of five disulfide bonds.

3.2. Antagonist activity of AGRP fragments

Synthetic AGRP-(83–132)-NH_2 produced a dose-dependent suppression of α-MSH-induced dispersion of pigment in melanophore cells (Fig. 2). Under the same experimental conditions, AGRP(25–51) and AGRP(54–82) were inactive. The inhibitory equilibrium dissociation constant (K_i) of human AGRP(83–132)-NH_2, as determined by Schild regression analysis, was 0.7 (S.E. 0.4) nM (Fig. 3), which is comparable to K_d values reported for AGRP and Agouti protein purified by recombinant means [1,6]. Each of the three protein fragments had no 'agonist like' activity in melanophore cells. When applied to melanophores in the absence of α-MSH, the peptides did not cause pigment dispersion in the presence of 10 nM melatonin (data not shown). The specificity of AGRP for MC receptor-mediated response was tested by examining AGRP(83–132)-NH_2 for interaction with other ligands known to induce pigment dispersion. AGRP(83–132)-NH_2 (30 nM) had no effect on concentration-response curves evoked with norepinephrine, serotonin, PACAP-27, and [Arg^8]-vasotocin, indicating that antagonism is selectively directed towards responses initiated by α-MSH stimulation of MC receptors.

4. Discussion

Our results demonstrate that a synthetic fragment of human AGRP based on the amino acid sequence (83–132) can potently antagonize MC receptor function in *Xenopus* melanophores; the amino-terminal fragments (25–51) and (54–83) are inactive as antagonists. Although it is not known what enzymatic processes shape AGRP in vivo, we have surmised by analogy to atrial-natriuretic peptide, that AGRP may contain an endoprotease cleavage site between amino acids 82 and 83. This hypothesis is strengthened by our finding that the amidated carboxyl protein fragment that corresponds to this proposed cleavage site, i.e. AGRP(83–132)-NH_2, is fully active and has a K_i similar to, or perhaps even less than, values reported for longer fragments containing greater portions of the amino-terminus [1]. Thus, it appears that the cysteine-rich carboxyl-terminal region of AGRP, the region with highest homology to Agouti protein, accounts for all of its anti-melanotropic activity in melanophores. We are currently in the process of producing antibodies to AGRP(83–132), and this may help address whether AGRP fragments which correspond to this cleavage site can actually be found in vivo.

Electrospray mass spectrometry is an accurate method of determining protein molecular mass and purity, but proteins can produce multiply charged ions and fragment ions. This problem is accentuated with AGRP(83–132)-NH_2 because of its numerous disulfide sites. Although the major peak in the electrospray mass spectrum was 5677.0 Da, it also contained a number of minor peaks which were difficult to interpret. The major peak was consistent with protein containing five disulfide bonds; which was expected since cyclization reactions usually proceed to completion. To better resolve the origin of the minor peaks in the electrospray analysis, we retested AGRP(83–132)-NH_2 using MALDI-TOF mass spectrometry, a procedure less prone to fragmentation. The MALDI-TOF mass spectrogram contained only one major peak and confirmed our suspicion that minor peaks in the electrospray procedure were likely due to fragmentation. Thus, an

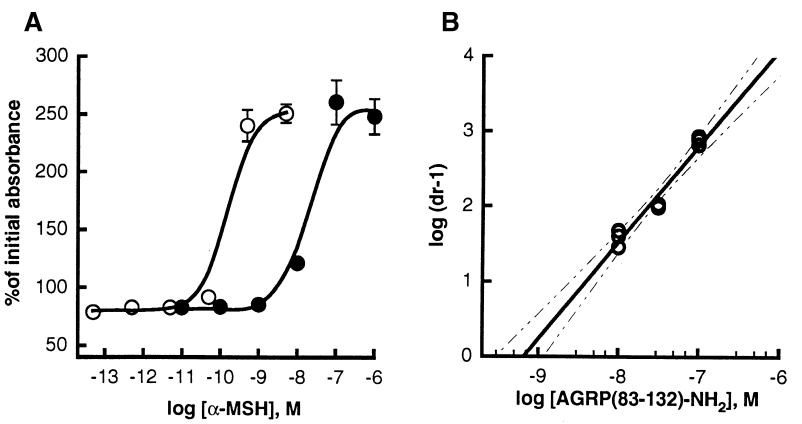

Fig. 3. A: Synthetic APR-(83–132)-NH_2 caused a 100-fold shift in the concentration-response curve to α-MSH. The EC_{50} of α-MSH was 0.16 (S.E. 0.03) nM in the absence (○), and 19 (S.E. 5) nM in the presence (●), of 100 nM AGRP-(83–132)-NH_2. Each symbol represents the mean and standard deviation of four independent measurements. B: Schild regression analysis indicates the pK_i of synthetic AGRP-(83–132)-NH_2 is 9.18 (S.E. 0.25). The slope of the regression is 1.3 (S.E. 0.1) and the correlation coefficient 0.96. Broken lines indicate the 95% confidence interval.

AGRP(83–132)-NH_2 structure containing a full complement of disulfide bonds appears to account for the biological activity observed in *Xenopus* melanophores.

The arrangement of disulfide bonds in AGRP contributes to the protein's tertiary structure. Our ability to synthesize biologically active molecules of AGRP chemically will make the task of resolving these arrangements easier since disulfide bonds can now be assessed by systematically altering and rearranging protein structures without the need to rely on more limiting and time consuming recombinant expression systems. Despite current uncertainties about the pattern of disulfide bonding within AGRP, it is interesting to note that in vitro formation of biologically active structures appears to proceed spontaneously in the absence of the larger precursor protein. Thus, the carboxyl-terminal region of AGRP, and possibly Agouti protein as well, appear to contain all the necessary secondary structure needed for proper bond formation and folding into bioactive molecules.

As a selective and non-competitive inhibitor of MC4 receptors in humans, AGRP likely regulates some of the crucial physiological processes mediated by these receptors, including energy homeostasis and food intake. Structural studies of our chemically synthesized AGRP fragments may clarify the role this protein plays in the body. Further, structural insights gained from such research may facilitate the development of even more potent and selective MC receptor antagonists – both peptide and peptoid in nature. We hope that the ability to synthesize active fragments of AGRP and, we anticipate, Agouti as well will eventually elucidate their participation in obesity and diabetes [2,13], leading to better treatments for these conditions.

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