Molecular Interaction of Agouti Protein and Agouti-Related Protein with Human Melanocortin Receptors

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ABSTRACT: Agouti protein and the Agouti-related protein (AGRP) are antagonists of the melanocortin-3 receptor and melanocortin-4 receptor. Both proteins contain 10 cysteines in the C-terminal domain arranged in five disulfide bonds. One possible arrangement of the disulfide bonds predicts an octapeptide loop, and the chemical properties of four residues within this loop (residues 111−114 in human AGRP) bear striking resemblance to those of several melanocortin peptides, including α-MSH, MT-II, and SHU-9119. We showed that cyclic synthetic octapeptides based on the sequence of this loop from Agouti protein or human AGRP are functional antagonists of the human melanocortin-4 receptor. All peptides had a lower affinity for the melanocortin-3 receptor than for the melanocortin-4 receptor. Substitution of serines for cysteines resulted in linear peptides which had reduced binding affinities for both receptors. Mutational analysis of human AGRP indicated that its C-terminal domain is functionally equivalent to the intact human AGRP. The RFF111−113 triplet appears to be the most critical portion of AGRP in determining the binding affinity for both melanocortin-3 and melanocortin-4 receptors. These data strongly suggest that the loop defined by Cys-110 and Cys-117 is critical in determining the antagonist activity of human AGRP. Our data provide indirect evidence for the suggestion that the Cys-110 to Cys-117 octapeptide loop of human AGRP mimics the conformation of α-MSH, MT-II, and SHU-9119.

In wild-type mice, the Agouti protein regulates coat coloration by binding to and antagonizing the melanocortin-1 receptor (MC-1R). In the obese yellow A^y mice, mutations in the regulatory region of the Agouti locus result in ectopic expression of the Agouti protein (*1*−*4*). The Agouti protein also binds to the melanocortin-3 receptor (MC-3R)[1] and melanocortin-4 receptor (MC-4R), although it appears to have a lower affinity for MC-3R than for MC-4R (*5*, *6*). Deletion of the MC-4R gene in mice results in an obese phenotype similar to that observed in the A^y mouse (*7*), implicating MC-4R in obesity development. This hypothesis is supported by the observation that intracerebroventricular administration of a MC-3(4)R agonist can significantly suppress feeding (*8*).

Nucleotide sequence homology searches identified an Agouti-related transcript (ART) expressed in the hypothalamus of the rodent brain (*9*). The level of ART expression is elevated in the hypothalamus of *ob/ob* and *db*/*db* obese mice, while overexpression of ART in transgenic mice resulted in obesity (*10*, *11*). The protein product of the ART gene (human Agouti-related protein or hAGRP) can bind to and antagonize the hMC-3R and hMC-4R with high affinity, although its affinity is much lower for MC-1R and MC-5R (*11*, *12*). One hypothesis is that AGRP functions as a natural antagonist at the MC-3R and MC-4R to regulate feeding behavior.

The sequence homology between Agouti and the AGRP includes conserved Cys residues. The cysteines are located in the C-terminal domain of the protein where most of the sequence homology exists (Figure 1). The C-terminal region of Agouti has a potency similar to that of the full-length protein (*13*). The C-terminal region of AGRP is also a potent inhibitor of pigment dispersion in melanophore cells (*14*). Similarities in the Cys spacing pattern may also be seen with several toxins, including several spider toxins, suggesting a conserved three-dimensional motif. These Cys-constrained loops may function as "fingers" or contact points with the receptor. Mutagenesis data and a comparison of known structure of some spider toxins have helped in predicting the disulfide structure of Agouti and the AGRP (*15*). One of the predicted loops of hAGRP contains the amino acid sequence CRFFNAFC. We have observed that within this region, the Arg side chain and the two aromatic side chains of RFF have properties resembling the general chemical properties of some small peptides (*8*), including HFRW in α-MSH, H(D-Phe)RW in MT-II, or H(D-Nal)RW in SHU-9119. This resemblance suggests that this loop in AGRP may mimic the binding interaction of melanocortin peptides. In this study, synthetic cyclic peptides based on this AGRP loop or the equivalent region of human or mouse Agouti protein were prepared and shown to selectively bind the hMC-4R, and to a lesser extent the hMC-3R. Linear peptides of the same sequence (except for the substitution of the terminal cysteines for serine) were essentially inactive. In addition, mutational analysis of hAGRP indicated that when residues within the region of RFFN111−114 were substituted with Ala, the binding affinity for the hMC-3R or hMC-4R was substantially reduced, while mutations in other regions had only a

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[1] Abbreviations: α-MSH, α-melanocyte-stimulating hormone; NDP-α-MSH, [Nle-4,D-Phe-7]-α-melanocyte-stimulating hormone; AGRP, Agouti-related protein; MC-3R, melanocortin-3 receptor; MC-4R, melanocortin-4 receptor; GPCR, G protein-coupled receptor.

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A

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ω-agaTX IVA   1          KKKCIAKDYGRCKWGGTPCCRGRG CICSIMGTNCECKPRLIMEGLGL
ω-agaTX IVB   1          EDNCIAEDYGKCTWGGTKCCRGRP CRCSMIGTNCECTPRLIMEGLSF
  AGRP HUM   76 LQDREPRSSRRCVRLHES CLGQQVPCCDPCATCYCRFFNAFCYCRKLGTAMNPCS
AGOUTI HUM   82 VVRPRTPLSAPCVATRNS CKPPAPACCDPCASCQCRFFRSACSCRVL   SLNC
AGOUTI MOU   83 VARP  PPPSPCVATRDS CKPPAPACCDPCASCQCRFFGSACTCRVL   NPNC
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B

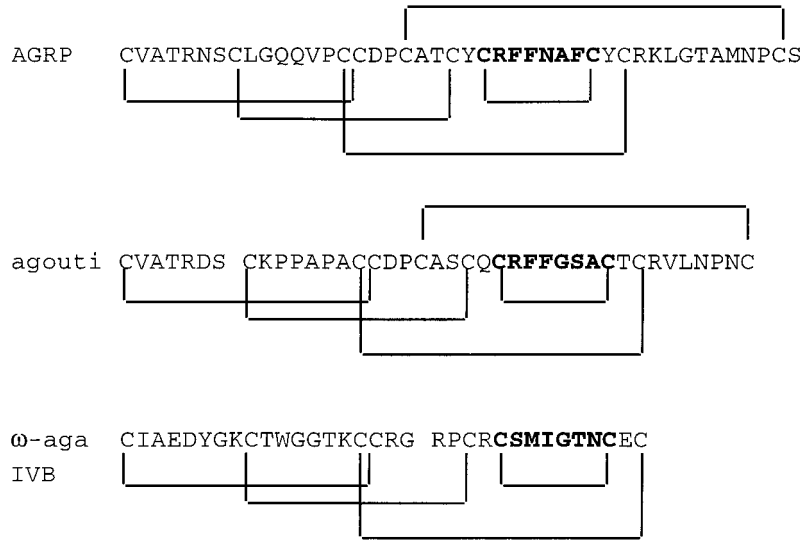

FIGURE 1: (A) Sequence alignment of the C-terminal regions of mouse Agouti, human Agouti, human AGRP, and ω-agatoxin IVA and IVB. Synthetic peptides were based on the bold sequences. (B) The disulfide patterns of human AGRP (*23*) and mouse Agouti (*15*) are shown along with the disulfide structure of ω-agatoxin IVB (*24*).

small effect on hAGRP binding. The general implications of ligand mimicry and ligand−receptor interaction are discussed.

MATERIALS AND METHODS

Materials. Cyclic and linear peptides derived from Agouti and AGRP were custom synthesized by Research Genetics. NDP-α-MSH, α-MSH, and γ2-MSH were purchased from Peninsula Labs. [^{125}I]NDP-α-MSH was from Amersham. Synthetic hAGRP(83−132)-NH_2 or human Agouti(87−132)-NH_2 was purchased from Phoenix Pharmaceuticals.

[^{125}I]NDP-α-MSH Binding Assays. Competition binding assays in which [^{125}I]NDP-α-MSH was used were performed essentially as described previously (*12*). Briefly, membranes were prepared from CHO cells expressing hMC-3R or hMC-4R (obtained from I. Gantz, University of Michigan, Ann Arbor, MI) and incubated in 50 mM Tris (pH 7.2), 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, 0.1% BSA, 4 μg/mL leupeptin, 40 μg/mL bacitracin, 5 μg/mL aprotinin, and 10 mM Pefabloc, along with 100 000 cpm of [^{125}I]NDP-α-MSH, for 2 h at room temperature in the presence of the indicated concentration of peptides. The reaction was terminated by filtration over GF/C filter plates. Competition curves were analyzed in GraphPad Prism, and the inhibition was described by IC_{50} values. Similar results were obtained using intact cells in suspension followed by filtration or using cell monolayers followed by washing.

cAMP Assays. CHO cells expressing an MC receptor were used for the functional assay. Cells were dissociated from tissue culture flasks by rinsing with Ca^{2+} and Mg^{2+} free phosphate-buffered saline, and these were detached following 5′ incubation with enzyme free dissociation buffer (Specialty Media, Lavellette, NJ). Cells were collected by centrifugation and resuspended in Earle's Balanced Salt with additions of 10 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 1 mM glutamine, and 1 mg/mL bovine serum albumin. Cells were counted and diluted to $1-5 \times 10^6$ per milliliter. The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine was added to cells to a concentration of 0.6 mM.

Peptides were diluted in 10% dimethyl sulfoxide (DMSO) (10^{-5} to 10^{-10} M); 0.1 volume of compound was added to 0.9 volume of cells ($1-5 \times 10^5$ cells; final DMSO concentration of 1%). Following incubation for 15 min, agonist (α-MSH or γ2-MSH) was added to the cells at the indicated concentrations. The assays were allowed to proceed at room temperature for 45 min, at which point the cells were lysed by incubation at 100 °C for 5 min to release accumulated cAMP. The amount of cAMP was measured in an aliquot of the cell lysate with the Amersham cAMP detection assay (*16*).

Construction and Expression of hAGRP Mutants. The expression plasmid for all hAGRP mutants was constructed by modifying the wild-type hAGRP expression plasmid via PCR (*12*). As described previously, the hAGRP expression construct contains the Myc epitope which allows ELISA quantitation and analysis of the protein by Western blotting. The hAGRP-c sequence differs from that of hAGRP in that residues 27−75 were deleted. The QQ97−98AA mutant was constructed by substituting Gln-97 and Gln-98 with Ala. The RFFN111−114AAAA mutant was constructed by substituting residues 111−114 with Ala. The RKLGTAMN120−127AALGAAAA mutant was constructed by substituting residues 120, 121, 124, 126, and 127 with Ala. The entire coding region of all hAGRP mutants was confirmed by nucleotide sequencing. All mutants were expressed in COS-7 cells, prepared, and quantitated as previously described (*12*).

Computational Studies. The initial structure of peptide 5 (Ac-cyclo[CRFFGSAC]-NH_2) was generated using the backbone conformation of the ω-agatoxin IVA disulfide-linked octapeptide fragment CSIMGTNC as determined from NMR studies (*17*). This backbone conformation was then held rigid while an additional 200 conformations were generated using a proprietary distance geometry algorithm. One thousand conformations of MT-II were similarly generated although without incorporating any structural information. Both sets of conformers were then energy minimized using MMFF94s (*18*) with a distance-dependent dielectric of 2*r*.

In the development of the superpositions depicted in Figure 6, centroids for the aromatics and the Cξ of the arginines were used as match centers to create a series of alignments between the two peptides. Viable matches that were considered included aromatic to aromatic, guanidino to guanidino, and aromatic to guanidino. These were then examined to determine how well they meet the available structural information (the aforementioned backbone conformation of the agatoxin for peptide 5) while falling within 10 kcal/mol of the lowest-energy conformer for each compound. The two alignment families which appear to best meet these criteria (residues are numbered according to MT-II sequence) are one in which His-6 of MT-II aligns with the first Phe of peptide 5, D-Phe-7 with the second Phe, and Arg-8 with Arg (Figure 6A) and the other in which D-Phe-7 aligns with the first Phe, Trp-9 with the second Phe, and Arg-8 with Arg (Figure 6B). The conformer of the MT-II peptide model depicted in Figure 6 was identified in both alignment families as the lowest-energy conformer of MT-II which, additionally, has ϕ and φ angles similar to those reported for models of a series of potent melanotropin peptides (*19*).

RESULTS

Inhibition of [^{125}I]NDP-α-MSH Binding by AGRP-Derived Peptides. Cyclic peptides were generated corresponding to the proposed loop formed between cysteines 110 and 117 of hAGRP, cysteines 115 and 122 of mouse Agouti, or cysteines 116 and 123 of human Agouti (Figure 1 and Table 1). Peptide 1 corresponds to residues 109−118 in hAGRP and is a cyclic peptide as a consequence of the disulfide bond. Peptide 2 is also a cyclic peptide corresponding to hAGRP residues 110−117 and is acylated and amidated. Peptide 3 is similar to peptide 2 except it contains a free amino and carboxylic acid group. Peptide 4 has serines substituted for the cysteines and is linear. A similar set of peptides was generated corresponding to mouse Agouti (peptides 5−7) and human Agouti (peptides 8 and 9). All peptides had a higher affinity for MC-4R than for MC-3R. The linear peptides (peptide 4 derived from AGRP and peptide 7 derived from Agouti) show a significantly reduced affinity compared to their cyclic counterparts (peptides 1−3, and 5 and 6, respectively). Acylation and amidation provided a modest increase in binding affinity for MC-4R. For peptide 3, addition of naturally occurring adjacent tyrosines (as seen in peptide 1) resulted in a significantly increased binding affinity for the hMC-3R and -4R, suggesting these tyrosines may be involved in making a contact with the receptor. Variants of peptide 1 in which three residues were substituted for alanine individually in position 3, 4, or 5 (RFF) resulted in peptides with an affinity of less than 20 μM for MC-3R and MC-4R (data not shown). Thus, the RFF residues are critical for binding.

Cyclic Octapeptides Are Effective MC-4R Antagonists. Agonist activation of the MC-4 receptor by either α-MSH or γ2-MSH leads to stimulation of adenylyl cyclase and accumulation of intracellular cAMP. All of the cyclic peptides in Table 1 were able to block α-MSH or γ2-MSH activation of the MC-4R, and none of the cyclic peptides possessed any agonist activity of their own (data not shown). Schild analyses were performed with the most potent peptides (peptides 1, 2, 5, and 8; Figure 2 and Table 2). The calculated K_B values were within a factor of 4−14 of the IC_{50} values for binding. Peptides 1 and 5 had the lowest K_B values, consistent with results from inhibition of [^{125}I]NDP-α-MSH binding. All of the peptides tested had a slope close to unity, consistent with a competitive inhibitor.

Mutational Analysis of hAGRP. To further investigate the binding interaction of hAGRP with human MC receptors, a series of mutant hAGRPs were tested. The functional role of the C-terminal half of hAGRP was demonstrated by testing the hAGRP-c mutant which was expressed from the cDNA encoding only residues 1−26 and 76−132 of hAGRP. Since the signal peptide sequence is predicted to consist of residues 1−20, the mature hAGRP-c mutant is expected to contain residues 21−26 and 76−132 of hAGRP. As shown in Table 3, the hAGRP-c mutant has essentially the same binding affinity as hAGRP for the hMC-3R or hMC-4R, suggesting that the N-terminal half of hAGRP is not required for high-affinity binding to these receptors. Furthermore, the recombinant hAGRP-c was functionally equivalent to the synthetic hAGRP(83−132)-NH_2.

To determine the contribution of various residues within the C-terminal domain of hAGRP to receptor binding, a homology model of hAGRP-c based on the ω-agatoxin structure (*20*) was used to guide the identification of potential receptor-interacting regions. In addition to RFFN111−114, two loops were identified that protrude on the protein surface (QQ97−98 and RKLGTAMN120−127) (Figure 3). Three Ala substitution mutants in which amino acids encoded in each of these proposed loops of intact hAGRP were replaced were constructed. The QQ97−98AA mutant contains two substitutions at positions 97 and 98; the RFFN111−114AAAA mutant contains four substitutions at positions 111−114, and the RKLGTAMN120−127AALGAAAA mutant contains five substitutions at positions 120−127. When

Table 1: Inhibition of [^{125}I]NDP-α-MSH Binding to Melanocortin Receptors by Synthetic Peptides

Peptide	Sequence	IC_{50} (nM) ± SEM (*n*) MC-3R	MC-4R
	Peptides Derived from hAGRP		
1	Ycyclo[CRFFNAFC]Y	1890 ± 127 (3)	57 ± 15 (5)
2	Ac- cyclo[CRFFNAFC]-NH_2	>10000 (3)	127 ± 22 (3)
3	cyclo[CRFFNAFC]	> 20000 (3)	621 ± 156 (4)
4	SRFFNAFS	> 20000 (3)	>20000 (3)
	Peptides Derived from mAgouti		
5	Ac- cyclo[CRFFGSAC]- NH_2	> 20000 (3)	130 ± 25 (3)
6	cyclo[CRFFGSAC]	> 20000 (3)	351 ± 40 (4)
7	SRFFGSAS	> 20000 (3)	>20000 (3)
	Peptides Derived from hAgouti		
8	Ac- cyclo[CRFFRSAC]- NH_2	10200 ± 4500 (3)	141 ± 21 (3)
9	cyclo[CRFFRSAC]	8633 ± 619(3)	299 ± 45 (3)
10	hAGRP(83-132)- NH_2	4.5 ± 1.3 (3)	3.5 ± 1.2 (3)
11	Ac-SYSMEHFRWGKPV- NH_2 (αMSH)	12 ± 1.4 (3)	21 ± 0.5 (3)

expressed in COS-7 cells, each of the mutants can be detected by the anti-Myc epitope antibody, and all three mutants had a similar apparent MW as the wild-type hAGRP (Figure 4).

The QQ97−98AA mutant bound to both hMC-3R and hMC-4R with essentially the same affinity as the wild-type hAGRP (Table 3). The RKLGTAMN120−127AALGAAAA mutant had a slightly reduced affinity compared to the wild-type hAGRP (Figure 5). Because the affinities of hAGRP and its mutants for hMC-3R parallel those for hMC-4R, these data do not suggest that hAGRP interacts differently with either hMC-3R or hMC-4R in either of these loop regions. In contrast, the RFFN111−114AAAA mutant had a substantially reduced affinity for both hMC-3R and hMC-4R, indicating that these four amino acid residues are critical in determining the high-affinity binding of hAGRP. In addition, none of these modifications converted the hAGRP mutants into an agonist of MC-3R or MC-4R.

To further define the role of individual residues of RFFN111−114 in receptor binding, four single residue substitutions of the full-length human AGRP were created and analyzed. As shown in Table 3, the R111A and F112A mutants led to an approximately 50-fold reduction in the affinity of hAGRP, and the F113A mutant led to an approximately 10- or 25-fold reduction in the affinity for hMC-3R and hMC-4R, respectively. In contrast, the N114A mutant only slightly affected the receptor binding affinity. These data demonstrated that the RFF triplet within the loop defined by Cys-110 and Cys-117 appears to be most critical for binding to both MC-3R and MC-4R.

Molecular Models of Cyclic Peptides. Two molecular models of peptide 5 and the potent cyclic peptide melanocortin agonist MT-II are shown in Figure 6. In the first model (Figure 6A), MT-II Arg-8 aligns with the Arg of the RFF triplet in Agouti or AGRP (Figure 1), MT-II His-6 aligns with the first Phe of the RFF triplet, and MT-II D-Phe-7 aligns with the second Phe. In the second model (Figure 6B), MT-II Arg-8 aligns with the Arg of the RFF triplet, MT-II D-Phe-7 aligns with the first Phe, and MT-II Trp-9 aligns with second Phe. Each residue of the RFF triplet is important for both full-length AGRP (Table 3) and peptide 1 (data not shown). The resolution of the alignment model and the limited structure−activity relatioship available for both peptides do not allow us to conclusively select one alignment over the other as the most reasonable, although each provides the foundation for further studies. However, it should be pointed out that the ability to mutate Phe-118 to Trp in the full-length Agouti peptide with no impact on the ability to inhibit [^{125}I]NDP-α-MSH binding to MC-4R (*21*) is consistent with the second alignment, in which Trp-9 of MT-II aligns with the second Phe. As shown in Figure 6A, superposition of the Arg side chains occurs from opposite sides of the turn conformation found in each peptide.

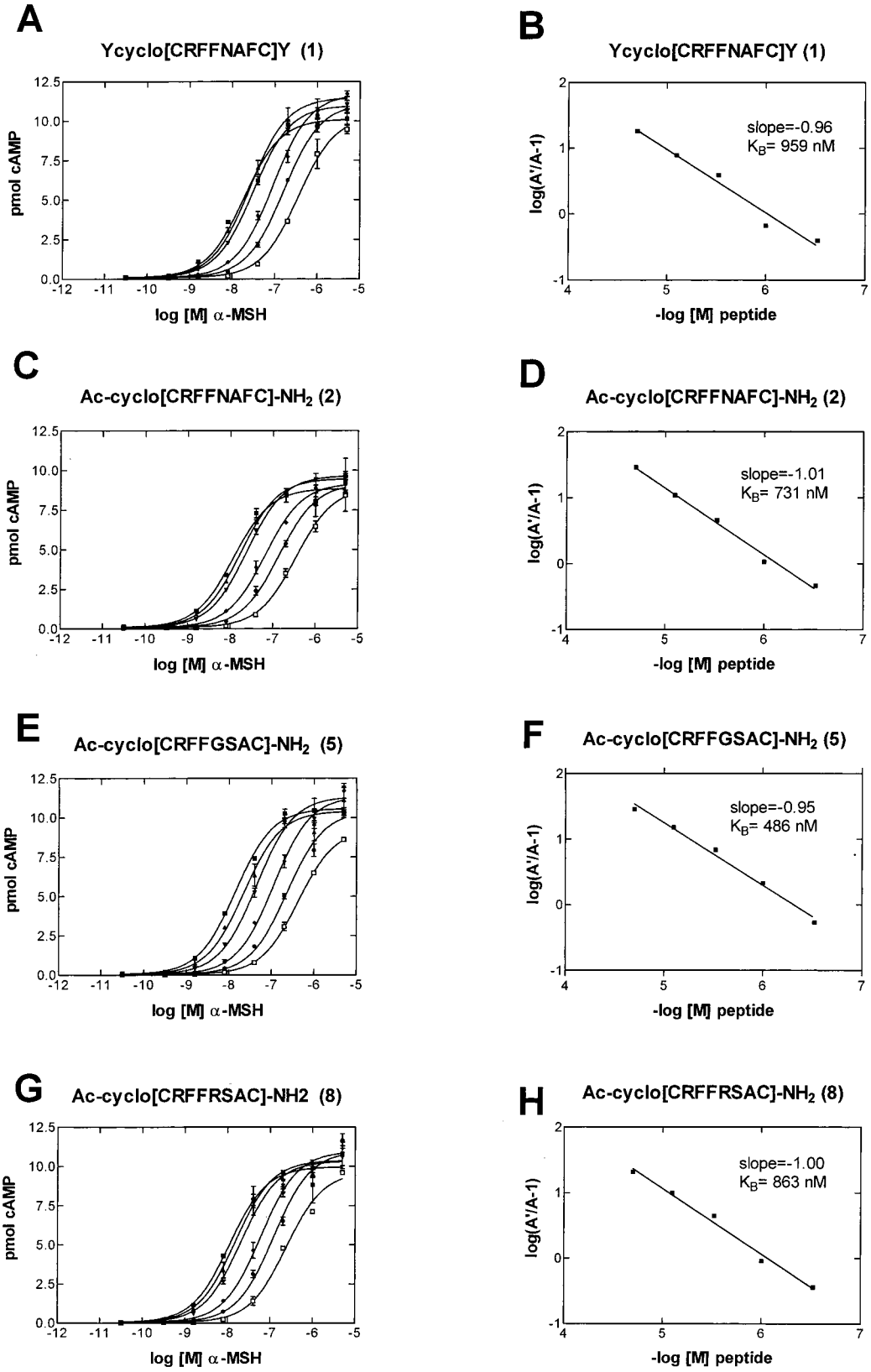

FIGURE 2: Peptide inhibition of agonist activation of MC-4R. Cells expressing the MC-4R were stimulated with α-MSH to produce cAMP in the presence of the indicated peptides as described in Materials and Methods. Panels A, C, E, and G depict data for activation of cells with α-MSH in the presence of peptides 1, 2, 5, and 8, respectively. The concentrations of inhibiting peptide used were 0, 0.3, 1, 3, 8, and 20 μM. Schild analyses for peptides 1, 2, 5, and 8 are shown in panels B, D, F, and H, respectively. On the *Y* axis, *A* is the EC_{50} of α-MSH in the absence of competing peptide and *A*′ is the EC_{50} of α-MSH in the presence of peptide.

DISCUSSION

We have identified cyclic peptides derived from Agouti and AGRP which act as inhibitors of [^{125}I]NDP-α-MSH binding and which are antagonists of α-MSH at MC-4R, effectively reducing a large protein ligand to an eight-residue peptide ligand. The functional significance of these peptide

Table 2: Functional Antagonism of MC-4R by Synthetic Peptides[a]

	Sequence	Schild Analysis			
		K_B, nM	SEM (n)	Slope	SEM (n)
	Peptides Derived from hAGRP				
1	Ycyclo[CRFFNAFC]Y	785	± 135(6)	-1.05	± 0.05(6)
2	Ac- cyclo[CRFFNAFC]-NH_2	1018	± 120(7)	-1.06	± 0.02(7)
	Peptide Derived from mAgouti				
5	Ac- cyclo[CRFFGSAC]- NH_2	564	± 80(7)	-1.07	± 0.08(7)
	Peptide Derived from hAgouti				
8	Ac- cyclo[CRFFRSAC]- NH_2	1847	± 451(7)	-1.17	± 0.08(7)

[a] Schild analyses were the average of independent experiments such as those shown in Figure 2.

Table 3: Apparent Binding Affinity (IC_{50}) of Recombinant hAGRP and Its Mutants for the hMC-3R or hMC-4R[a]

ligand	IC_{50} (nM) ± SEM (*n*)	
	hMC-3R	hMC-4R
hAGRP	0.9 ± 0.3 (5)	0.5 ± 0.1 (4)
hAGRP-c	1.9 ± 0.5 (3)	1.6 ± 0.2 (3)
QQ97−98AA	0.9 ± 0.4 (3)	1.2 ± 0.3 (3)
RFFN111−114AAAA	>20 (3)	>20 (3)
RKLGTAMN120−127AALGAAAA	3.3 ± 1.5 (3)	2.6 ± 0.6 (3)
R111A	45 ± 17 (3)	67 ± 46 (3)
F112A	62 ± 16 (3)	61 ± 35 (3)
F113A	11 ± 3 (3)	25 ± 13 (3)
N114A	2.0 ± 0.6 (3)	3.3 ± 0.3 (3)

[a] Data were obtained from at least three independent experiments such as those shown in Figure 5.

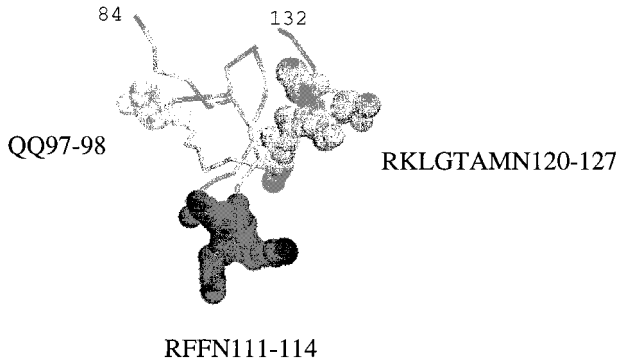

FIGURE 3: Homology model of hAGRP-c encompassing residues 84−132. Selected side chains that are targeted for mutational analysis are depicted in a space-filling representation. The α carbon backbone conformation is based on the *ω*-agatoxin NMR structure (*20*), while the side chain conformation is hypothetical.

domains was corroborated following mutagenesis of the relevant amino acid residues in the proposed Cys-110−Cys-117 octapeptide loop of hAGRP. Cyclic peptides 1−3 derived from hAGRP bind to hMC-4R with an affinity of 57−621 nM. The most potent cyclic peptides (1, 2, 5, and 8) were subjected to Schild analysis, which demonstrated that the peptides are antagonists for MC-4R, with Schild slopes close to 1 (Table 2 and Figure 2). Compared to the C-terminal domain of AGRP, which has an IC_{50} of 1.6−3.5 nM for MC-4R, the activities of the cyclic peptides are reduced by more than 1 order of magnitude (Tables 1 and 3). To establish the functional significance of these cyclic octapeptides, hAGRP was mutated at four amino acid residues predicted to be of significance, on the basis of the data obtained with the cyclic octapeptides. The affinity of

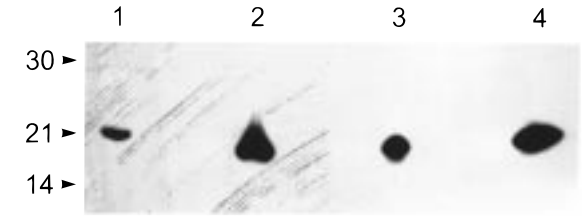

FIGURE 4: Western blot of recombinant hAGRP and its mutants. The protein was visualized with anti-Myc antibody and horseradish peroxidase. The MW markers are indicated on the left. Lane 1, human AGRP; lane 2, QQ97−98AA mutant; lane 3, RFFN111−114AAAA mutant; and lane 4, RKLGTAMN120−127AALGAAAA mutant. Each lane contains approximately 1 pmol of recombinant AGRP or its mutant.

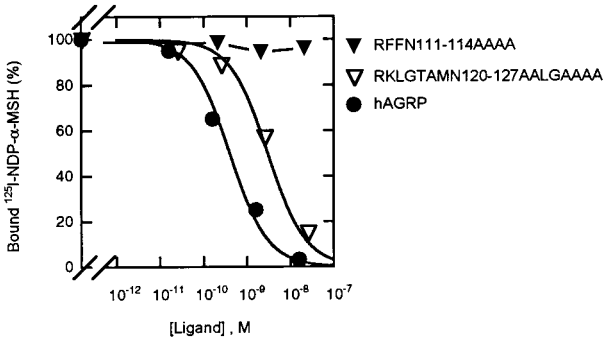

FIGURE 5: Inhibition of [^{125}I]NDP-α-MSH binding to the human MC-4R by the human AGRP, the RKLGTAMN120−127AALGAAAA mutant, or the RFFN111−114AAAA mutant. The average from duplicate measurements in each experiment is shown. Specific binding was typically 5500 cpm, representing 10% of the total radioligand added. The nonspecific binding (typically 500 cpm) was determined in the presence of 1 μM NDP-α-MSH. Data from multiple independent experiments are summarized in Table 3.

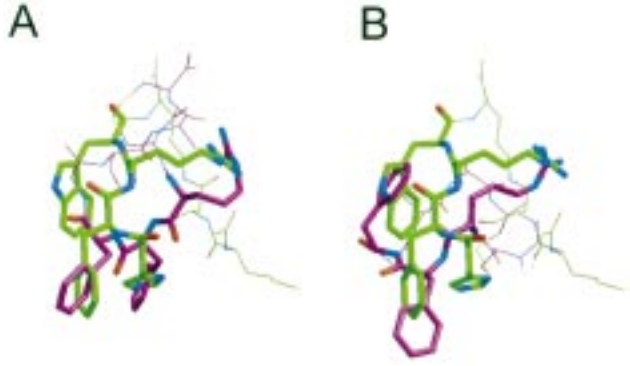

FIGURE 6: View of two putative superpositions of the MT-II model (green carbons) with peptide 5 (purple carbons). Residues which form the basis of the alignment are shown as stick bonds.

hAGRP mutant RFFN111−114AAAA for the hMC-4R was substantially reduced, while mutations at other regions did not significantly affect hAGRP binding. Subsequent single-residue substitutions confirmed that residues RFF111−113 are the most critical ones within this region (Table 3), which is also consistent with alanine substitutions at analogous positions in peptide 1 (each >20 μM, data not shown).

It is clear that the amino acid sequence represented by each of the synthetic hAGRP peptides is not completely sufficient to account for the binding affinity of the full-length hAGRP for the hMC-4R, and additional points of contact may exist between hAGRP and the MC-4R. Alternatively, the AGRP-derived octapeptides may not have adopted an

ideal conformation, thereby affecting their IC_{50} values. In addition to a reduction in affinity, the peptides appear to have different pharmacology for MC-4R and MC-3R when compared to the full-length proteins or C-terminal fragments. First, MC-4R is capable of distinguishing between Agouti and AGRP or between C-terminal Agouti and AGRP. On the basis of inhibition of radiolabeled NDP-α-MSH binding, K_i values of 70 nM for human Agouti protein and 59 nM for murine Agouti protein at the hMC-4R have been reported (*5*, *6*). The reported binding affinity of Agouti is clearly lower than that of hAGRP at the MC-3R and MC-4R (Table 3). The affinity of MC-4R for C-terminal Agouti (ref *5* and confirmed in our laboratory) was also lower than that for C-terminal AGRP (Tables 1 and 3). On the other hand, peptides 5, 6, 8, and 9 derived from mouse or human Agouti bind to hMC-4R with an affinity (130−350 nM) similar to that of the cyclic peptides derived from hAGRP (Table 1). Thus, the MC-4R cannot distinguish Agouti-derived peptides from AGRP-derived peptides. Second, all of the peptides have a lower affinity for MC-3R than for MC-4R. In contrast, the C-terminal domain of AGRP had an equal affinity for MC-3R and MC-4R (Tables 1 and 3), indicating that it encodes all additional amino acid residues essential for high-affinity MC-3R binding. The full-length myc-tagged AGRP also had approximately equal affinity for both receptors (*12*). Full-length and C-terminal domain Agouti protein also had similar affinities for the MC-3R and MC-4R (*5*, *21*). Therefore, the isolated peptide is more selective for MC-4R, presumably due to a unique conformation adopted only by the isolated peptide, or due to interactions involving adjacent amino acids. A similar phenomenon has also been observed for neurokinin A which is approximately 6-fold more potent on the NK2 receptor than on the NK1 receptor, whereas the peptide fragment neurokinin A(4−10) is 72-fold more potent on the NK2 receptor than on the NK1 receptor (*22*).

The importance of the C-terminal region of Agouti for binding and MC receptor antagonism was documented previously (*5*, *13*). The C-terminal domain of AGRP also appears to encode almost all information for high-affinity binding (Tables 1 and 3 and ref *11*). The conserved disulfide pattern in this cysteine-containing region is most likely important for its functional activity. Perry et al. (*15*) mutated the cysteines and determined that Cys-110 and -131 in Agouti were not as critical as other Cys residues; albeit, these were still required for full activity. Using the disulfide configuration of ω-agatoxin, a pattern can be proposed and experimentally verified for Agouti and AGRP in which Cys-110 and -117 of AGRP form a disulfide bond (*23*). The predicted octapeptide loop of AGRP residues 110−117 (Agouti residues 115−122) has basic and aromatic residues reminiscent of α-MSH and other melanocortin ligands. Alanine scanning mutations of the conserved RFF in this region of Agouti greatly reduce the extent of binding to the melanocortin receptors (*21*). Outside of this region, only one mutation (D108A) caused a greater than 10-fold reduction in the affinity for MC-4R. Interestingly, there were four additional point mutations which caused a 10−20-fold decrease in the affinity for MC-3R. The addition of the flanking tyrosines in peptide 1 resulted in the only peptide with a reasonable affinity for MC-3R. These residues outside of residues 110−117 might explain the high affinity of full-length Agouti or AGRP for MC-3R compared to the low affinity of isolated peptides for MC-3R.

We generated mutations in three different regions of hAGRP. Modification of QQ97−98 did not affect the hAGRP binding affinity. In this region, Agouti contains Pro-Ala, and the neighboring region lacks polar side chains. These data suggest that while this region may be exposed on the surface of hAGRP, it does not appear to interact directly with the MC-3R or MC-4R. Modification of several hydrophilic residues in the region of residues 120−127 in hAGRP led to a slight reduction of binding affinity (Table 3). This region is not highly conserved between Agouti and AGRP, although point mutations in this region of mouse Agouti also resulted in a slight reduction of the Agouti binding affinity (*21*). These data suggest that this region contributes only a small amount of binding energy for AGRP or Agouti binding. The most critical region within hAGRP for receptor binding appears to be residues RFF111−113 (Table 3). These data are consistent with the effect of similarly positioned point mutations in Agouti (*21*).

The cyclic peptides encompassing residues RFFN111−114 used in this study were able to bind to the MC-4R with, for the most part, sub-micromolar affinity and function as antagonists. The corresponding linear peptides were much less potent. The AGRP peptide data support the hypothesis that this region in AGRP forms a loop which binds at the same site as α-MSH and is responsible for much of the interaction between AGRP and melanocortin receptors. These results demonstrate reduction of a large GPCR protein ligand to a small eight-residue peptide ligand with sub-micromolar affinity, but with altered pharmacology compared to the parent protein.

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