Melanocortin Receptor Binding Determinants in the Agouti Protein†

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ABSTRACT: The agouti protein plays an important role in the development of diabetes and obesity in rodents and has been shown to be a potent antagonist of melanocortin receptors. For this reason alanine-scanning mutagenesis was performed on the agouti protein carboxyl terminus to locate residues important for melanocortin receptor binding inhibition. When agouti residues Arg116 and Phe118 are changed to alanine, very large decreases in agouti affinity for melanocortin receptor 1, 3, and 4 result. Mutation of Phe117 to alanine causes a similar increase in agouti $K_{I\ app}$ at melanocortin receptor 4. Substitution of agouti residue Asp108 with alanine results in large increases in $K_{I\ app}$ for all three melanocortin receptors examined. All of these residues are conserved in the agouti-related transcript, ART, whose expression is up-regulated in animal models of obesity. The three-dimensional structure of the agouti carboxyl terminus was modeled, and residues which decrease receptor binding by a factor of ≥15 when mutated to alanine localize to one side of the structure. These agouti variants with altered receptor selectivity may be useful in determining the role of melanocortin receptors in diabetes and obesity.

Dominant mutations at the agouti locus result in yellow-haired mice that become obese and hyperinsulinemic (*1*−*3*). These mutations are all in the regulatory region and cause ectopic expression of the wild-type agouti protein. The murine agouti gene encodes for a 131-amino acid secreted protein expressed in the skin. During hair growth, agouti functions to regulate coat coloration (*4*), and the abnormal expression of the agouti protein causes the yellow phenotype. Ubiquitous expression of the agouti protein also causes diabetes (*4, 5*), suggesting that ectopic agouti protein has a role in altering metabolic regulation. Research examining the mechanism of action of agouti may lead to a better understanding of the causative factors of diabetes and obesity.

The murine agouti protein is a paracrine signaling molecule that regulates coat coloration through competitive antagonism ($K_{I\ app}$[1] = 1 nM) of α-melanocyte stimulating hormone (α-MSH) binding to its seven-transmembrane receptor (MC1-R) (*4, 6*). Agouti protein antagonism prevents the α-MSH-mediated increase in intracellular cAMP that results in the cell switching from production of black pigment, eumelanin, to yellow pigment, phaemelanin (*4*). We hypothesize that the obese/diabetic phenotype induced by ectopic agouti expression may occur through altered signaling of other melanocortin receptor family members, as these receptors are expressed in tissues involved in energy regulation such as brain, adipose, and muscle (*7*−*11*). Furthermore, mice which lack the MC4-R gene show an obese/diabetic phenotype similar to that of mice which display ubiquitous up-regulation of agouti protein expression (*12*). In addition, agonists of MC3-R and MC4-R inhibit feeding, whereas antagonists of these receptors stimulate feeding (*13*).

It has been demonstrated that the carboxyl terminus (Val83−Cys131) of the mouse agouti protein is as equally potent an antagonist of cAMP production induced by MCR-1 as the full-length protein (*14*). Furthermore, the carboxyl-terminal cysteine residues are necessary for full biological activity, while part of the lysine-rich basic domain is dispensable for normal function (*15*). We performed alanine-scanning mutagenesis on the agouti carboxyl terminus to identify the specific amino acids important for inhibition of melanocortin receptors 1 and 3−5. Residues involved in discrimination between the melanocortin receptor subtypes were also identified, and these agouti variants may be useful in deciphering the role of individual melanocortin receptors in agouti-induced diabetes and obesity. A model of the agouti carboxyl terminus based on the *ω*-conotoxin and *ω*-agatoxin structures indicates that all residues which decrease melanocortin receptor binding by a factor of ≥15 when mutated to alanine map to one side of the protein.

EXPERIMENTAL PROCEDURES

Construction and Baculovirus Expression of Mouse Agouti Proteins. Cloning and baculovirus expression of wild-type agouti protein were performed as described (*16*). Site-specific agouti variants were produced by cassette mutagenesis of the agouti gene containing six "silent" restriction sites in the carboxyl terminus as described (*17*). The accuracy

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[1] Abbreviations: α-MSH, α-melanocyte stimulating hormone; ART, agouti-related transcript; HEK 293 cells, human embryonic kidney 293 cells; $K_{I\ app}$, apparent inhibition dissociation constant; $K_{D\ app}$, apparent equilibrium dissociation constant; MC1-R, melanocortin receptor 1; MC3-R, melanocortin receptor 3; MC4-R, melanocortin receptor 4; MC5-R, melanocortin receptor 5; NDP-α-MSH, [Nle^4, D-Phe^7]-α-melanocyte stimulating hormone.

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of the agouti coding region was confirmed for all the constructs by Taq Dyedeoxy cycle sequencing using Amplitaq polymerase to synthesize DNA chains terminated by fluorescent dye-labeled ddNTPs (GlaxoWellcome Sequencing Facility). The variant agouti proteins were expressed using the baculovirus Bac-To-Bac system (*18*) (Gibco BRL). To express the agouti proteins, *Trichiplusia ni* cells (10^6 cells/mL) were infected (MOI = 2) with the agouti recombinant baculovirus and cultured for an additional 24- 48 h [120 rpm, 27 °C in Ex-Cell 405 media (JRH Biosciences) plus 50 μg/mL gentamycin].

Purification of Agouti Proteins. Wild-type agouti protein was partially purified (~80%) from the *T. ni* media on a Poros-20 HS cation-exchange column and subsequently dialyzed into phosphate-buffered saline (PBS) as described (*16*). The site-specific agouti variants were purified (~50−90%) by S Sepharose cation-exchange chromatography. Two milliliters of the S Sepharose resin equilibrated in PBS was gently shaken with 150 mL of the agouti-containing *T. ni* media for 45 min. The chromatography matrix was transferred to a plastic disposable column (0.5 × 5 cm). Typically, the column was washed with 0.5 M NaCl and 50 mM HEPES at pH 7.5, and the agouti proteins were eluted with 1 M NaCl and 50 mM HEPES at pH 7.5. The agouti charge variants, Arg85Ala, Arg96Ala, and Arg125Ala, were eluted from the S Sepharose column with the high-salt wash. For this reason, the wash was skipped, resulting in less pure samples. NaCl was removed from protein samples by PD-10 (Pharmacia) gel filtration chromatography in PBS. The agouti protein concentration was determined by first estimating the percentage of agouti protein present in a sample (50−90%) by visual examination of an SDS−PAGE gel stained with ProBlue (Integrated Separation Systems). This percentage was then multiplied by the total protein content measured in triplicate by BCA assay (Pierce) using bovine serum albumin as the standard and normalized to a sample containing a known concentration of wild-type agouti protein (*14*).

Melanocortin Receptor Clones and Cell Culture. $B_{16}F_{10}$ monolayers were cultured as previously described (*6*). Human embryonic kidney (HEK) 293 cell lines (ATCC) stably expressing mouse melanocortin receptor 3, 4, or 5 were prepared and cultured as previously described (*17*).

Binding Assays. $B_{16}F_{10}$ monolayers were plated at 10^5 (1 day) or 2.5×10^4 (2 days) cells/well before use. HEK 293 cells stably expressing one of the mouse melanocortin receptors were plated at 10^5 cells/well 2 days before use. $K_{I\ app}$ values for agouti protein inhibition of receptor binding were determined by incubating cells with ~0.1 nM [^{125}I]-NDP-α-MSH [prepared as described (*14*)] and agouti protein (0−2.8 μM) in a medium containing 25 mM Hepes (pH 7.5) and 0.1% BSA for 2 h at room temperature (total volume = 100 μL). After incubation, samples were cooled on ice and the medium was removed by rapid aspiration. Scintillation cocktail (125 μL) was added, and bound radioactive ligand was measured using a Wallac 1450 Microbeta Plate counter. Data were fit to eq 1 in which b_{max} is the amount of specific [^{125}I]NDP-α-MSH bound in the absence of agouti protein and I is agouti protein. IC_{50} values were converted to $K_{I\ app}$ values using eq 2.

$$\text{fraction bound} = b/b_{max} = 1 - [[I]/(IC_{50} + [I])] \quad (1)$$

$$K_{I\ app} = IC_{50}/(1 + [[^{125}I]NDP\text{-}\alpha\text{-}MSH]/K_{D\ app}) \quad (2)$$

cAMP Assays. $B_{16}F_{10}$ cells were plated as described above and then incubated with 0.1 nM NDP-α-MSH and agouti protein in the same medium for 1 h at 37 °C (total volume = 100 μL). The reaction was terminated by addition of 50 μL of 50 mM sodium acetate (pH 4) containing 0.1% Triton X-100. The cAMP content of the samples was measured with scintillation proximity assay using a commercially available kit (Amersham, Arlington Heights, IL). Data were fit to eq 3 in which b_{max} is the amount of cAMP in the absence of agouti protein, I is agouti protein, and Y2 is a constant.

$$\text{picomoles of cAMP} = (b_{max}[I])/(IC_{50} + [I]) + Y2 \quad (3)$$

Construction of an Agouti Homology Model. A homology model was built for the carboxyl terminus of the agouti protein (residues 92−125) based on the structures of *ω*-conotoxin GVIA (*19*) and *ω*-agatoxin IVB (*20*) as determined by NMR. The alignments used for construction of the model are as follows:

ω-CgTx	C-KSZ G SSC SZT SYN CC - - - RSCNZYT - - - -KRCY
ω-AgaIVB	EDNCIAEDYGKCTWGGTKCCRG- RPCRCSMIGTNCECTPRLIMEGLSFA
Agouti	C-VATRDSC KPP APA CCDPC ASCQCRFFGSACTCRVLNPNC

While the NMR structures show the same general fold, *ω*-CgTx was specifically used as the basis for the agouti model for residues 92−106 and *ω*-AgalVB was used for residues 106−125. Once the initial model was constructed, multiple side chain rotamers were examined followed by 200 steps of conjugate gradient energy minimization.

RESULTS

Alanine-Scanning Mutagenesis of the Agouti Carboxyl Terminus. The carboxyl terminus (Val83−Cys131) of the mouse agouti protein is as equally potent an antagonist of MCR-1 as the full-length protein (*14*). To determine which amino acids in the carboxyl terminus of the mouse agouti protein are important for inhibition of binding to melanocortin receptors 1 and 3−5, site-directed mutagenesis was performed on full-length agouti to replace individual residues with alanine or glycine. Alanine was used in most cases because it can be expected to cause minimal perturbation in the folding of the protein, due to its small size and its presence in both buried and exposed positions in proteins (*21*, *22*).

Agouti protein competes with the high-affinity α-MSH analogue, NDP-α-MSH, for binding to melanocortin receptors. $B_{16}F_{10}$ cells expressing high concentrations of MC1-R (*23*, *24*) and HEK 293 cells stably expressing mouse MC3-R, MC4-R, or MC5-R were used for these assays. These melanocortin receptor-expressing cell lines have been characterized for [^{125}I]NDP-α-MSH binding and agouti inhibition (*6*, *17*). Wild-type agouti inhibition of ligand binding to these melanocortin receptors is shown in Figure 1. The relative ability of mouse agouti protein to inhibit ligand binding to the mouse melanocortin receptors is MC1-R > MC4-R > MC3-R > MC5-R. IC_{50} values were calculated by curve fitting using a nonlinear regression algorithm and eq 1. $K_{I\ app}$ values (Table 1) were calculated with eq 2 and

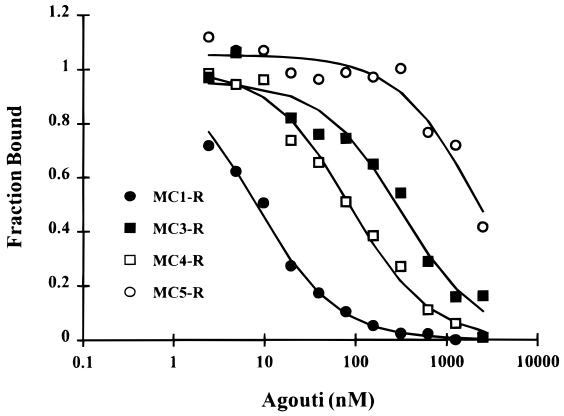

FIGURE 1: Mouse agouti inhibition of [^{125}I]NDP-α-MSH binding to cells stably expressing the mouse MC1-R, MC3-R, and MC4-R. Curves are representative of the two to four experiments performed, and each point is the mean of duplicate experimental values. The ordinate is expressed as a fraction of the total specific binding. IC_{50} values were calculated by curve fitting using a nonlinear regression algorithm and eq 1. $K_{I\ app}$ values were calculated with eq 2 and are listed in Table 1.

Table 1: Mutant Agouti Protein Inhibition of [^{125}I]NDP-α-MSH Binding to Melanocortin Receptors

	$K_{I\ app}$ (nM)			
	MC1-R	MC3-R	MC4-R	MC5-R
wild type	2.6 (0.8)[a]	190 (74)	54 (18)	12000 (340)
Val83Ala	33 (5)	1200 (430)	240 (90)	>wt[b]
Arg85Ala	3.4 (1.5)	1100 (350)	310 (65)	>wt
Pro86Ala	3.7 (0.8)	2900 (1800)	470 (160)	>wt
Pro87Ala	3.3	170	90	+[c]
Pro89Ala	5.8 (0.5)	1100 (310)	310 (57)	>wt
Pro91Ala	4.8 (0.8)	1100 (400)	250 (56)	>wt
Val93Ala	2.5 (0.2)	400 (200)	65 (16)	+
Arg96Ala	9.9 (0.2)	>3000	450 (130)	>wt
Ser98Ala	2.8 (0.1)	920 (250)	150 (36)	>wt
Lys100Ala	3.8	220	110 (28)	+
Pro101Ala	5.8	210	180 (3)	+
Pro102Ala	2.9	240	58 (10)	+
Pro104Ala	4.3	120	81 (2.5)	+
Asp108Ala	34 (4)	>3000	960 (430)	>wt
Pro109Ala	6.8	120	100 (8)	+
Ser112Gly	13 (1)	3200 (52)	250 (55)	>wt
Gln114Ala	9.3 (0.6)	250 (49)	88 (39)	>wt
Arg116Ala	>1900 (140)	>8000	>7100	>wt
Phe117Ala	19 (2)	820 (150)	3400 (1300)	>wt
Phe118Ala	640 (34)	>8000	2200 (850)	>wt
Ser120Ala	12 (0.5)	390 (180)	92 (48)	+
Thr123Gly	20 (2.5)	2900 (470)	82 (19)	>wt
Arg125Ala	13 (1)	1300 (170)	380 (94)	>wt
Val126Gly	18 (4)	1700 (940)	230 (14)	>wt
Leu127Phe	15 (4)	1500 (500)	400 (200)	>wt
Asn128Ala				
Asn128Ala				
Pro129Ala	4.9 (0.2)	350 (130)	100 (2)	+
Asn130Ala	5.5 (0.7)	320 (40)	160 (15)	+

[a] Standard error values are reported within the parentheses and were generated from two to six experiments. [b] The $K_{I\ app}$ value is greater than that of wild-type agouti, but could not be determined due to the high concentration. [c] The $K_{I\ app}$ value could not be distinguished from that of wild-type agouti.

the $K_{D\ app}$ values for [^{125}I]NDP-α-MSH binding published previously (*17*).

Variant agouti proteins containing a single alanine or glycine mutation were expressed using the baculovirus system and partially purified from media by S Sepharose chromatography. The ability of these mutant agouti proteins to inhibit melanocortin receptor binding was analyzed as described above for the wild-type protein.[2] The $K_{I\ app}$ values for all the agouti variants are summarized in Table 1. Due to the high $K_{I\ app}$ value (1200 nM) for wild-type agouti binding inhibition at MC5-R, the exact increase in the $K_{I\ app}$ value for the agouti variants at this receptor was not determined. The increases in $K_{I\ app}$ values for the agouti variants at MC1-R, MC3-R, and MC4-R relative to that of the wild-type protein are depicted in Figure 2. Very large decreases in agouti affinity for melanocortin receptor 1, 3, and 4 ($K_{I\ app\ mut}/K_{I\ app\ wt} > 40$) result when residues Arg116 and Phe118 are changed to alanine. Replacing Phe117 with alanine causes a very large increase in $K_{I\ app}$ value at MC4-R ($K_{I\ app\ mut}/K_{I\ app\ wt} = 63$), but has only small effects at MC1-R and MC3-R ($K_{I\ app\ mut}/K_{I\ app\ wt} \sim 5$). Substitution of agouti residue Asp108 with Ala results in large increases in $K_{I\ app}$ relative to that of wild-type agouti (~15-fold) at all three melanocortin receptors examined. In contrast, Pro86 is more important for inhibition at MC3-R and MC4-R than at MC1-R as $K_{I\ app\ mut}/K_{I\ app\ wt}$ for Pro86Ala agouti is 9−15 at MC3-R and MC4-R but only 1.4 at MC1-R. Similarly, changing Ser112 to glycine has a larger effect at MC3-R ($K_{I\ app\ mut}/K_{I\ app\ wt} = 17$) than at MC1-R and MC4-R ($K_{I\ app\ mut}/K_{I\ app\ wt} = 5$). The substitution of Thr123 to glycine increases $K_{I\ app\ mut}/K_{I\ app\ wt}$ by a factor of 8−15 at MC1-R and MC3-R but only by a factor of 2 at MC4-R.

Mouse agouti inhibits binding of [^{125}I]NDP-α-MSH and cAMP accumulation induced by α-MSH at mouse MC1-R with similar potencies [$K_{I\ app} = 1.9$ and 0.9 nM, respectively (*6*)]. The ability of the agouti point mutants to antagonize cAMP production induced by 0.1 nM NDP-α-MSH in $B_{16}F_{10}$ cells was similarly analyzed. Indeed, the $K_{I\ app}$ values for inhibition, calculated from eq 2 and the EC_{50} value of 0.1 nM determined for NDP-α-MSH, are within a factor of 3 of the corresponding $K_{I\ app}$ values for inhibition of ligand binding listed in Table 1 (data not shown). The wild-type agouti protein does not alter basal concentrations of cAMP in $B_{16}F_{10}$ cells (*6*). Similarly, the amount of cAMP accumulation in $B_{16}F_{10}$ cells observed in the presence (1 μM) or absence of the agouti point mutants remained constant.

Additional Mutagenesis at Positions 116−118. As replacement of Arg116, Phe117, and Phe118 with alanine results in >40-fold decreases in agouti affinity for the melanocortin receptors examined, additional amino acid substitutions were made at these positions to confirm their role in melanocortin receptor binding inhibition. Arg116 was replaced with glutamine, histidine, and lysine to determine whether the positive charge is important for receptor inhibition. The $K_{I\ app}$ values for these agouti variants are summarized in Table 2. It appears that the positive charge is important, as the ability of the agouti variants to inhibit binding to all three receptors improves as the positive charge on the side chain increases (Arg116Lys > Arg116His > Arg116Gln > Arg116Ala). Substitution of Phe117 with tryptophan results in wild-type activity at MC1-R and MC3-R and, surprisingly, 6-fold increased activity at MC5-R relative to that of the wild-type protein. Activity is increased for this protein at MC4-R relative to Phe117Ala agouti, but not restored to wild-type levels ($K_{I\ app\ mut}/K_{I\ app\ wt} = 14$). Substitution of Phe118 with tryptophan restores wild-type activity

[2] It has been demonstrated previously that contaminating proteins in the agouti preparations do not interfere with the assay (*17*).

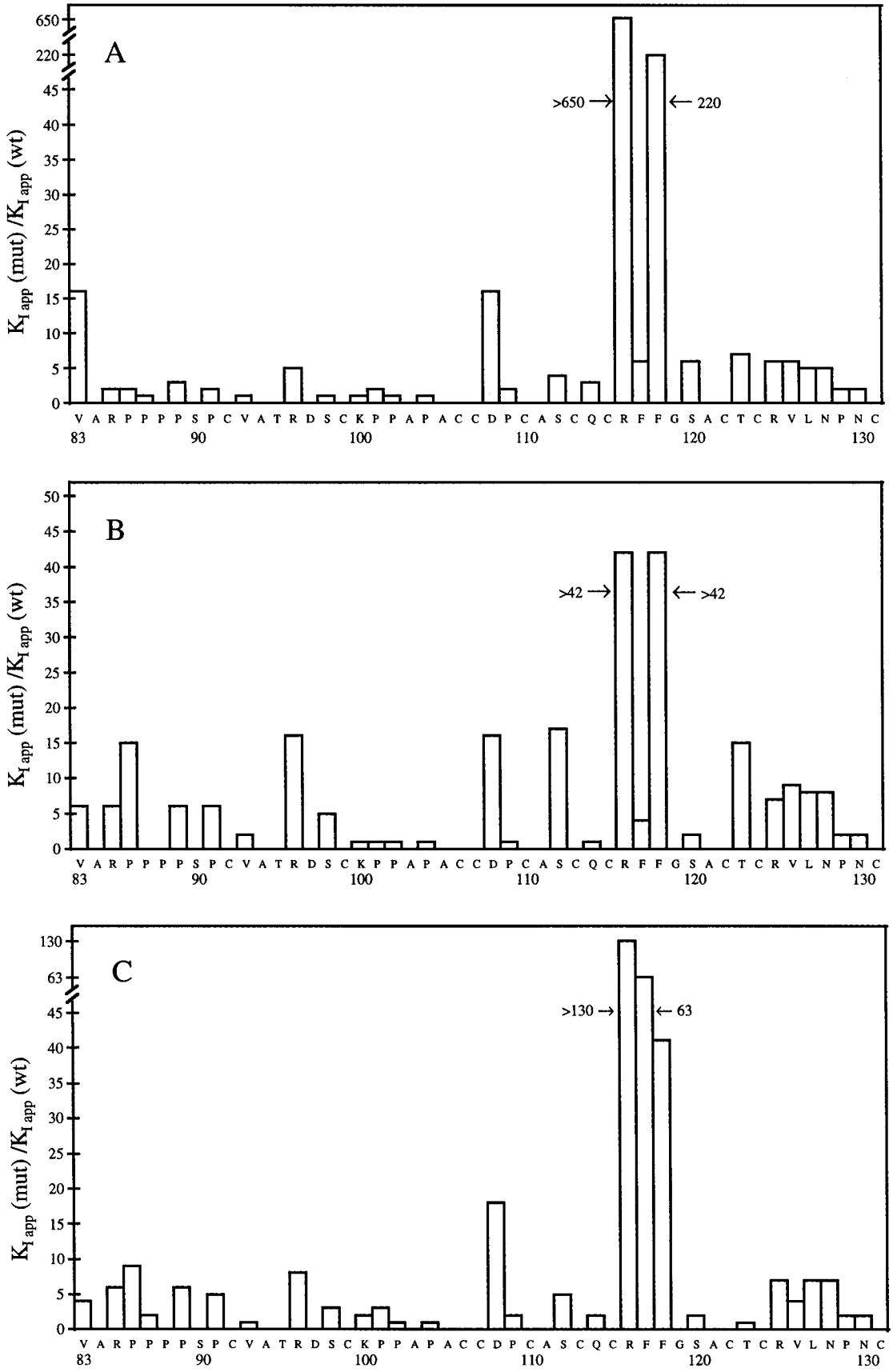

FIGURE 2: Histograms showing the effect on $K_{I\ app}$ at mouse melanocortin receptor 1 (A), 3 (B), and −4 (C) for alanine substitutions scanned over the carboxyl terminus of the mouse agouti protein. Values of $K_{I\ app\ mut}/K_{I\ app\ wt}$ were calculated from data in Table 1.

at MC1-R, MC3-R, and MC4-R. Similar to Phe117Trp agouti, activity for this protein has increased at MC5-R ($K_{I\ app\ mut}/K_{I\ app\ wt} = 0.11$).

Modeling of the Agouti Carboxyl Terminus. The agouti protein carboxyl terminus was modeled to determine the spatial arrangement of the residues implicated in binding

Table 2: Effects of Substitutions at Positions 116−118 on Agouti Protein Inhibition of [^{125}I]NDP-α-MSH Binding to Melanocortin Receptors

	$K_{I\ app}$ (nM)			
	MC1-R	MC3-R	MC4-R	MC5-R
wild type	1[a]	1	1	1
R116A	>650[b]	>42	>130	>1[c]
R116Q	>240	>21	>74	>1
R116H	50	21	>54	>1
R116K	30	16	20	>1
F117A	6	4	63	>1
F117W	1	0.5	14	0.17
F118A	220	>42	41	>1
F118W	0.5	1	1	0.11

[a] Values and standard error for wild-type agouti protein as reported in Table 1. Ratio = $K_{I\ app\ wt}/K_{I\ app\ wt}$. Standard error values for the agouti mutants are ≤40% except for F118W at MC1-R (80%). [b] $K_{I\ app\ mut}/K_{I\ app\ wt}$. [c] The mutant $K_{I\ app}$ value is greater than that of wild-type agouti but could not be determined due to the high concentration.

inhibition. We used the fact that the spacing of the 10 cysteine residues in the carboxyl terminus of the agouti protein is characteristic of the "cystine knot" family of proteins (*25*) and is homologous to that of snail and spider neurotoxins (*26*). The ω-conotoxin GVIA contains 26 residues with 3 disulfide bonds, and the ω-agatoxin IVB has 48 residues and 4 disulfide bonds (*27*). All 10 cysteine residues in the agouti carboxyl terminus are disulfide-bonded (*14*). The NMR structures of ω-conotoxin GVIA and ω-agatoxin IVB have been solved (*19, 20*) and are remarkably similar despite low sequence homology. A homology model of residues 92−125 of the mouse agouti protein was built on the basis of the ω-conotoxin and ω-agatoxin structures (Figure 3A, B). This region contains 4 of the 5 disulfide bonds (9 of the 10 cysteine residues). In this structure, residues which result in large increases in $K_{I\ app}$ ($K_{I\ app\ mut}/K_{I\ app\ wt} \sim 15$) when mutated to alanine are shown in purple, and those resulting in very large increases ($K_{I\ app\ mut}/K_{I\ app\ wt} > 40$) are in yellow. These residues are localized to one side of the agouti protein that encompasses slightly more than half of the total surface area.

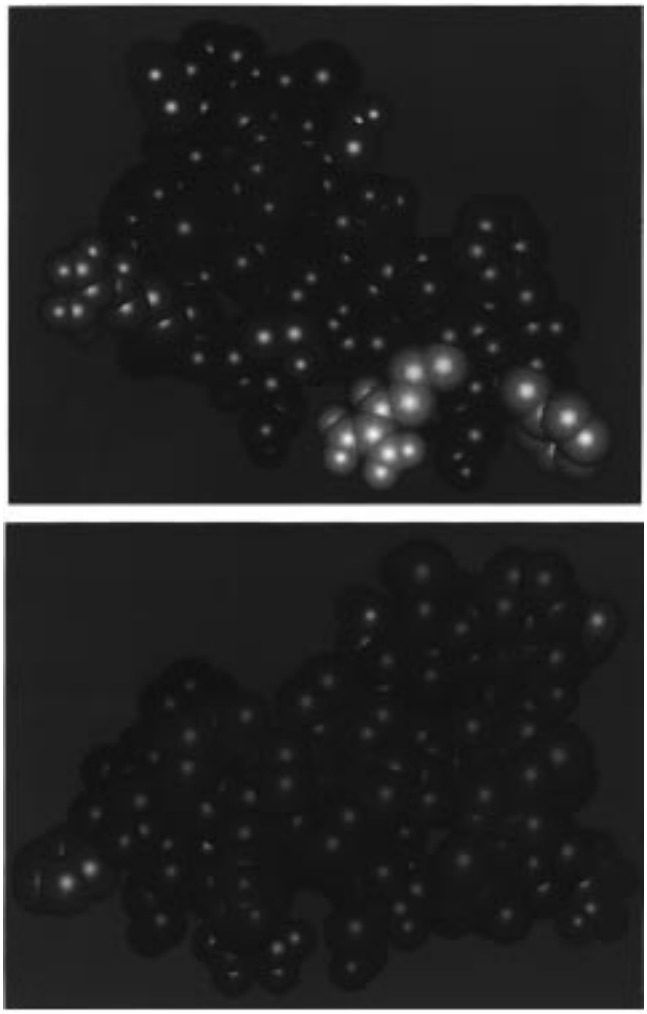

FIGURE 3: (A, top) Homology model of residues 92−125 of the mouse agouti protein built on the basis of the ω-conotoxin and ω-agatoxin structures (*19, 20*). Residues that result in large increases in $K_{I\ app}$ ($K_{I\ app\ mut}/K_{I\ app\ wt} \sim 15$) when mutated to alanine are shown in purple, and those resulting in very large increases ($K_{I\ app\ mut}/K_{I\ app\ wt} > 40$) are in yellow. The purple and yellow residues are localized to one side of the agouti protein that encompasses slightly more than half of the total surface area. (B, bottom) A 180° rotation of panel A.

DISCUSSION

Alanine-Scanning Mutagenesis of the Agouti Carboxyl Terminus. We have identified residues in the agouti protein that are important for melanocortin receptor binding inhibition by systematically replacing carboxyl-terminal residues with alanine. Of the 28 amino acids that were varied, 2 affected binding to MC1-R and MC3-R and 3 affected binding to MC4-R by factors of >40-fold. These mutations are a basic residue (Arg116Ala) and two large hydrophobic residues (Phe117Ala and Phe118Ala). There is evidence besides the large decreases in activity that these 3 amino acids are important binding determinants. For example, analysis of 3 additional agouti variants at position 116 demonstrates that the positive charge is important for receptor binding inhibition. The ability of the agouti variants, Arg116Lys, Arg116His, Arg116Gln, and Arg116Ala, to inhibit binding to all three receptors improves as the positive charge on the side chain increases. An alternative interpretation of these data is that a positive charge at position 116 is instead necessary for proper protein folding. However, this is probably not the case as the highest levels of protein expression were observed for Arg116Ala and the lowest for Arg116Lys agouti. It is also unlikely that Phe117Ala agouti is improperly folded, as it has near wild-type activity at MC1-R and MC3-R. Mutation of Phe118 to tryptophan results in a decrease rather than an increase in $K_{I\ app}$ relative to that of wild-type agouti protein at MC5-R (9−fold), indicating that Phe118 is an important binding determinant. Furthermore, all of the amino acids which decrease receptor binding inhibition by ≥15-fold when mutated to alanine localize to one side of the agouti homology modeled structure (Figure 3A,B). This suggests that they modulate binding by direct, or at least localized indirect, effects rather than through gross alteration of protein structure.

All residues except the 10 disulfide-bonded cysteines, the alanines, and some residues that are not conserved between mouse and human agouti proteins were altered. The cysteine residues were not mutated because disruption of any of the 5 disulfide bonds might alter protein structure. Residues not conserved between mouse and human agouti proteins were left unaltered, as both proteins inhibit binding to the

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magouti APACCDPCAS CQCRFFGSAC TCRVLNPNC

mART    QVPCCDPCAT CYCRFFNAFC YCRKLGTAT NLCSRT
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FIGURE 4: Amino acid sequence comparison of agouti (residues 103−131) and the agouti-related transcript ART (residues 103−137). Agouti residues that result in large increases in $K_{I\ app}$ for the melanocortin receptors analyzed when mutated to alanine are shown in large type.

melanocortin receptors with similar affinities (*17*). Furthermore, two variants with changes in amino acids not conserved between mouse and human agouti, Pro87Ala and Pro129Ala agouti, have essentially wild-type activity. Additionally, one of the residues altered (Asn128) in the double variant Leu127Phe/Asn128Ala agouti is not conserved between mouse and human agouti, and this protein has only slightly reduced activity relative to that of the wild-type protein. For these reasons, we believe we have identified most, if not all, of the important binding determinants possible, without a structure of the ligand−receptor complex.

A gene having sequence homology with agouti and a similar expression pattern in humans and mice has recently been cloned and characterized (*28*). The up-regulation of the expression of this gene, ART, in animal models of obesity gives further credence to a role for melanocortin receptors in the central control of feeding. The region of highest identity between ART and agouti is in the cysteine-rich carboxyl terminus. ART lacks the region of basic and polyproline residues found in the middle of the agouti protein. The agouti residues important for melanocortin binding inhibition discussed above are all conserved in ART (Figure 4). For this reason, we would predict that ART is able to antagonize the melanocortin receptors.

Mechanism of Agouti Antagonism of Melanocortin Receptors. The 3 agouti residues important for binding to all the melanocortin receptors analyzed, Asp108, Arg116, and Phe118, are identical to 3 of the 7 core amino acids of the melanocortin peptides, Met-Glu-His-Phe-Arg-Trp-Gly, with the substitution of aspartate for glutamate. Therefore, these agouti amino acids may interact with some of the same receptor residues involved in peptide agonist binding. However, it is unlikely that agouti and the melanocortin peptides interact with melanocortin receptors in exactly the same manner, as the distance between Asp108 and Arg116 or Phe118 in the agouti protein model is greater than in the melanocortin peptides. It is likely that the receptor residues that do interact with these three agouti residues will be conserved throughout the melanocortin receptor family, as the increases in mutant agouti $K_{I\ app}$ values are similar for all the receptors.

Mutagenesis experiments have identified human MC1-R residues Asp117, His 260, Ser6, Asp184, Glu269, and Thr272 as being important for agonist binding (*29*, *30*). Some of these residues might also be involved in agouti protein receptor antagonism. Modeling studies of a cyclic analogue of MSH docked into the human MC1-R indicate that Asp117 interacts with histidine in MSH, His260 with glutamate, and Asp121 in transmembrane 3 with arginine (*31*). The latter 3 MC1-R residues are candidates for interaction with agouti residues Asp108 and Arg116, as they are conserved in all 5 mouse and human melanocortin receptors.

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NOTE ADDED IN PROOF

ART recombinant protein has been shown to antagonize melanocortin receptor melanocortin-mediated cAMP accumulation (*32*).

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