Mutations in the Carboxyl Terminus of the Agouti Protein Decrease Agouti Inhibition of Ligand Binding to the Melanocortin Receptors

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ABSTRACT: Several mutations that cause ectopic expression of the agouti gene result in obesity, hyperinsulinemia, and yellow coat color. A candidate pathway for agouti induced obesity and hyperinsulinemia is through altered signaling by melanocortin receptors, as agouti normally regulates coat coloration through antagonism of melanocortin receptor 1. Furthermore, melanocortin peptides mediate functions including steroidogenesis, lipolysis, and thermoregulation. We report apparent inhibition dissociation constants for mouse and human agouti protein inhibition of ligand binding to the melanocortin receptors, to determine which of these receptors might be involved in agouti induced diabetes. The similarity in the apparent K_I values for agouti inhibition of ligand binding to the brain melanocortin receptors 3 and 4 (mouse: $K_{I\ app} = 190 \pm 74$ and 54 ± 18 nM; human: $K_{I\ app} = 140 \pm 56$ and 70 ± 18 nM, respectively) suggests that the MC3-R is a potential candidate for a receptor mediating the effects of agouti protein overexpression. Agouti residues important for melanocortin receptor inhibition were identified through the analysis of deletion constructs and site-specific variants. Val83 is important for inhibition of binding to MC1-R ($K_{I\ app}$ for Val83Ala agouti increased 13-fold relative to wild-type protein). Arg85, Pro86, and Pro89 are important for selective inhibition of binding between MC1-R and MC3-R and MC4-R as their apparent K_I values are essentially unchanged at MC1-R, while they have increased 6−10-fold relative to wild-type protein at MC3-R and MC4-R.

The murine agouti gene encodes for a 131 amino acid secreted protein normally expressed in the skin during hair growth (Bultman *et al*., 1992). Dominant mutations such as lethal yellow (A^Y) and viable yellow (A^{VY}) which cause the agouti gene to be expressed in most, if not all, tissues result in yellow coat color (Yen *et al*., 1994; Michaud *et al*., 1993, 1994). Mice with these mutations also become obese and hyperinsulinemic, suggesting that ectopic agouti protein expression has a role in altering metabolic regulation. Indeed, the diabetic as well as the yellow phenotype is a result of the abnormal expression of the agouti protein, as it is reproduced when the murine agouti cDNA is placed under the transcriptional control of a ubiquitous promoter in a transgenic mouse (Bultman *et al*., 1992; Klebig *et al*., 1995).

The murine agouti protein is a paracrine signaling molecule that regulates coat coloration through competitive antagonism [inhibition dissociation constant ($K_{I\ app}$)[1] = 1 nM] of α-melanocyte stimulating hormone (α-MSH) binding to its seven transmembrane receptor (MC1-R) (Blanchard *et al*., 1995; Bultman *et al*., 1992). Agouti protein antagonism prevents the increase in intracellular cAMP produced upon α-MSH receptor binding which results in the cell switching from production of black pigment to yellow pigment (Bultman *et al*., 1992). The obese/diabetic phenotype induced by ectopic agouti expression probably does not result from altered signaling of this receptor, as the *e* recessive mouse lacks a functional MC1-R (Robbins *et al*., 1993) and the crossing of these and A^{VY} animals results in obese mice (Lamoreux, 1973). This phenotype may instead occur through altered signaling of other melanocortin receptor family members, as they are expressed in energy-relevant tissues such as brain, adipose, and muscle (Roselli-Rehfuss *et al*., 1993; Mountjoy *et al*., 1994; Gantz *et al*., 1993a,b; Labbe *et al*., 1994). Furthermore, the pre-peptide, pro-opiomelanocortin, from which α-MSH is derived also gives rise to a variety of other biologically active substances. These peptides mediate a broad array of physiological functions including steroidogenesis, lipolysis, and thermoregulation (Schwyzer & Eberle, 1977; Di Wied & Jolles, 1982; O'Donohue & Dorsa, 1982; Ramachandran, 1987; Chretien, 1974; Murphy *et al*., 1983; Martin *et al*., 1990). Mouse agouti protein has been shown to antagonize the melanocortin receptor 4 (MC4-R), but no effect was observed on MC3-R or MC5-R (Lu *et al*., 1994). Here we report $K_{I\ app}$ values for mouse and human agouti protein inhibition of [^{125}I]-NDP-α-MSH binding to melanocortin receptors 1, 3, 4, and 5.

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[1] Abbreviations: α-MSH, α-melanocyte stimulating hormone; NDP-α-MSH, [Nle^4, D-Phe^7]-α-melanocyte stimulating hormone; MC1-R, melanocortin receptor 1; MC3-R, melanocortin receptor 3; MC4-R, melanocortin receptor 4; MC5-R, melanocortin receptor 5; $K_{D\ app}$, apparent equilibrium dissociation constant; $K_{I\ app}$, apparent inhibition dissociation constant; HEK 293 cells, human embryonic kidney 293 cells; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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The similarity in agouti affinity for MC3-R and MC4-R suggests that MC3-R is also a potential candidate for the receptor mediating the effects of agouti protein overexpression.

In addition, we have localized residues in the agouti protein that are important for selective melanocortin receptor inhibition. As there is no significant amino acid sequence homology between agouti and the melanocortin peptides, these residues were localized with deletion constructs, a proteolytic fragment consisting of the carboxyl terminus of agouti, and point mutations. The mouse agouti protein consists of a 22 amino acid secretion signal sequence, an internal basic region (Lys 57−Arg85), and a carboxyl-terminal cysteine-rich region (Cys92−Cys131). The data indicate that Val83, Arg85, Pro86, and Pro89 are important for inhibition of binding to melanocortin receptors 3, 4, and 5 while Val83 is also important for inhibition of MC1-R.

MATERIALS AND METHODS

Construction and Baculovirus Expression of Mouse Agouti Proteins. Cloning and baculovirus expression of wild-type agouti protein were performed as previously described (Lu *et al*., 1994). Two agouti deletion constructs, Δbasic and ΔC-term (Figure 1), were constructed using an mp18/agouti single-strand DNA template and a mutagenesis kit from Amersham (Amersham Life Science). Antisense oligonucleotides composed of nucleotide sequences flanking the agouti basic domain were used to loop-out this domain generating Δbasic (Δ57−85). Truncated agouti without carboxyl-terminal sequences (ΔC-term) was generated by introducing a stop codon at Ser90.

Site-specific agouti variants were produced by cassette mutagenesis of the agouti gene containing six "silent" restriction sites in the carboxyl terminus. These restriction sites were incorporated by annealing 6 synthetic oligonucleotides containing 48−62 nucleotides each which spanned the carboxyl-terminal coding region of the agouti gene. The 5′ ends of all of the oligonucleotides (except the two most 5′ on the sense and antisense strands) were phosphorylated using T4 polynucleotide kinase and then annealed by combining the oligonucleotides in an equal molar ratio (6 μg in 10 μL of 10 mM Tris-HCl, pH 7.5, 50 mM NaCl) and heating at 95 °C for 5 min followed by slow cooling to RT. The annealed oligonucleotides were ligated using T4 DNA ligase (Promega), and the full-length construct was purified by electrophoresis on a 5% acrylamide gel. This carboxyl-terminal construct was placed behind the amino-terminal region of the mouse agouti cDNA which had been cloned into pCR-Script Direct SK(+) (Stratagene) after PCR amplification.

Mutagenesis was performed by first cutting out the desired cassette from the agouti cDNA with the appropriate restriction enzymes, selectively precipitating the plasmid DNA away from the liberated cassette (use of ammonium acetate), and ligation of the synthetic cassette DNA (20−66 nucleotides in length) back into the cDNA. The accuracy of the agouti coding region was confirmed for all of 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 (Luckow *et al*., 1993) (Gibco BRL). In this system, recombinant baculoviruses are generated efficiently by site-specific transposition of a DNA cassette containing the gene of interest into a baculovirus shuttle vector (bacmid) propagated in *Escherichia coli.* To express the agouti proteins, *T. 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]. *T. ni* cells infected with wild-type baculovirus were used as a control. Media containing secreted agouti protein were separated from the cells by centrifugation.

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 previously (Willard *et al*., 1995). The ΔC-term construct was similarly purified (∼60%) using S Sepharose cation-exchange chromatography followed by desalting on a G-10 column (Pharmacia) equilibrated in PBS. As the Δbasic construct lacks the basic region, it could not be purified by cation-exchange chromatography. Instead, the Δbasic-conditioned media was concentrated first using an Amicon YM2 filter and then by ammonium sulfate precipitation. The precipitate was resuspended (3.7 mL) and chromatographed on a 2.6 × 66 cm Superose 75 gel filtration column (Pharmacia) equilibrated in 25 mM HEPES, pH 7.1, 150 mM NaCl which resulted in a sample consisting of ∼20% Δbasic protein and ∼80% of one other high molecular weight protein. The control media from wild-type baculovirus-infected cells were concentrated by ammonium sulfate precipitation, and without further purification, desalted by G-10 chromatography as described for ΔC-term. The site-specific agouti variants were partially purified from the *T. ni* media by S Sepharose cation-exchange chromatography. The column was washed with 0.5 M NaCl, 50 mM HEPES, pH 7.5, and the agouti protein eluted with 1 M NaCl, 50 mM HEPES, pH 7.5. The salt wash was skipped in the case of Arg85Ala agouti. The NaCl was removed by G-25 (Pharmacia) gel filtration chromatography in PBS. Agouti protein concentration was determined by first estimating the percentage of agouti protein present in a sample 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 by BCA assay (Pierce) using bovine serum albumin as the standard.

Isolation of Melanocortin Receptor Clones and Cell Culture. $B_{16}F_{10}$ monolayers were cultured as described previously (Blanchard *et al*., 1995). Human embryonic kidney (HEK) 293 cells (ATCC) were cotransfected with one of the human melanocortin receptor cDNA's in pMT4 and pRSV-Neo using Transfectam Reagent (Promega). The cDNA's were obtained from genomic DNA using PCR with primers designed specifically for each of the four melanocortin receptors (1, 3, 4, and 5) according to the published sequences (Chhajlani & Wikberg, 1992; Mountjoy *et al*., 1992; Gantz *et al*., 1993a,b; Labbe *et al*., 1994). The coding sequences of the mouse melanocortin receptors (3, 4, and 5) were subcloned into pcDNAIneo vector (InVitrogen), and these were stably transfected into HEK 293 cells using a modified $CaPO_4$ procedure (Chen & Okayama, 1987). The coding sequences of mouse MC3-R and MC4-R were obtained by probing a genomic DNA library, 129 strain (Stratagene), with the corresponding rat receptor DNA

sequences. The mouse MC5-R cDNA was obtained from brown fat cDNA using PCR with primers specific for this gene (Labbe *et al*., 1994). Stable clones were selected using 600 μg/mL Geneticin (Gibco BRL) and subsequently cultured in Minimum Essential Media with Earle's Salts, 2 mM L-glutamine, 50 μg/μL penicillin/streptomycin, 250 μg/mL Geneticin, and 10% fetal bovine serum (Hyclone) in a humidified 5% CO_2 air atmosphere.

Binding Assays. $B_{16}F_{10}$ cells were plated at 10^5 (1 day) or 2.5×10^4 (2 days) cells/well prior to use, and HEK 293 cells stably expressing one of the human or mouse melanocortin receptors were plated at 10^5 cells/well 2 days prior to use. $K_{D\ app}$ values were determined by saturation binding of [^{125}I]-NDP-α-MSH to whole cell monolayers under equilibrium conditions (25 °C, 2 h) in RPMI 1640 containing 50 mM HEPES, pH 7.5, and 1 mg/mL BSA (total volume = 100 μL). $K_{I\ app}$ values were determined similarly by incubating cells with ~0.1 nM [^{125}I]-NDP-α-MSH [prepared as described in Willard *et al*. (1995)] and agouti protein. After incubation, samples were cooled on ice, and the medium was removed by rapid aspiration; 125 μL of scintillation cocktail was added, and bound radioactive ligand was measured using a Wallac 1450 Microbeta Plate counter. Nonspecific binding (determined by addition of 1 μM NDP-α-MSH) ranged from 5−15% and was subtracted from all data. $K_{D\ app}$ values for [^{125}I]-NDP-α-MSH binding were calculated by nonlinear least-squares curve fitting to a simple one-site model. IC_{50} values for agouti inhibition of binding were calculated similarly by fitting 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 concentration. IC_{50} values were converted to $K_{I\ app}$ values using eq 2 (Cheng & Prusoff, 1973):

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

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

Measurement of k_{off} for [^{125}I]-NDP-α-MSH. HEK 293 cells plated as described above were incubated with ~0.1 nM [^{125}I]-NDP-α-MSH for 30 min at 25 °C in binding medium (total volume = 100 μL). After removal by rapid aspiration, this medium was replaced with 100 μL of the same medium containing 1 μM unlabeled NDP-α-MSH. Bound radioactive ligand was measured as a function of time as described above. The amount of nonspecific binding was assessed by adding 1 μM unlabeled NDP-α-MSH at the same time as the [^{125}I]-NDP-α-MSH. Dissociation rate constants (k_{off}) were calculated by nonlinear least-squares curve fitting to a single exponential decay.

cAMP Assays. $B_{16}F_{10}$ cells were plated as described above and then incubated with 1 nM α-MSH and agouti protein in the culture medium without serum 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 by 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 concentration, and Y2 is a constant:

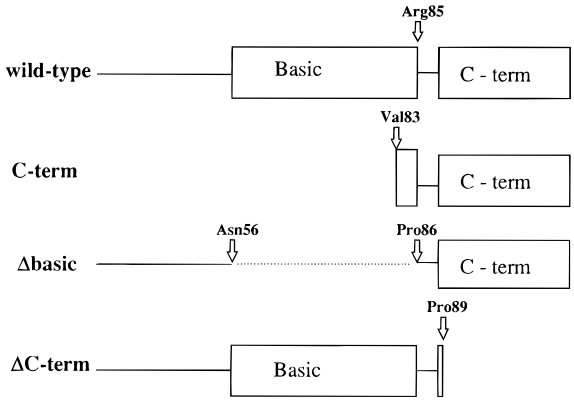

FIGURE 1: Mouse agouti protein deletion constructs. The wild-type agouti protein contains an internal highly basic region and a carboxyl-terminal cysteine-rich domain. The C-term construct is a proteolytic fragment of the wild-type protein consisting of residues Val83−Cys131 purified to homogeneity as described in Willard *et al*. (1995). Δbasic is a deletion construct lacking the internal highly basic region (Δ Lys57−Arg85) which has been partially purified as described under Materials and Methods. Another deletion construct, ΔC-term, lacks the carboxyl-terminus (Δ Ser90−Cys131).

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

RESULTS

We have analyzed the ability of mouse and human agouti proteins to inhibit ligand binding to their respective mouse and human MC1, MC3, MC4, and MC5 receptors. To localize the region of the mouse agouti protein necessary for receptor inhibition, two deletion constructs were analyzed after partial purification (Materials and Methods). The first construct, Δbasic, lacks the internal highly basic region (Δ Lys57−Arg85), while ΔC-term lacks the carboxyl-terminus (Δ Ser90−Cys131) (Figure 1). These proteins differ from the carboxyl terminal, proteolytic fragment of the agouti protein (C-term, Val83−Cys131; Willard *et al*., 1995) in that Δbasic lacks only the first three amino acids, Val83−Arg85, while ΔC-term contains only the first seven residues, Val83−Pro89. Inhibition of ligand binding to the melanocortin receptors by the C-term fragment was also measured.

We (Blanchard *et al*., 1995) have demonstrated that the agouti protein is a competitive inhibitor ($K_{I\ app}$ = 1.9 nM) of the high-affinity α-MSH analogue, [^{125}I]-NDP-α-MSH, for binding to mouse melanoma $B_{16}F_{10}$ cells. Studies indicate that the $B_{16}F_{10}$ cell line expresses only a single melanocortin receptor, MC1-R (Solca *et al*., 1991; Lunec *et al*., 1993). We similarly analyzed the ability of full-length and partially deleted mouse agouti proteins to inhibit ligand binding to the mouse MC1-R. The relative ability of the agouti proteins to compete with 0.1 nM [^{125}I]-NDP-α-MSH for binding to $B_{16}F_{10}$ cells is depicted in Figure 2. The data were analyzed by nonlinear least-squares curve fitting to a competitive model (eq 1) and $K_{I\ app}$ values (Table 1) for this inhibition were calculated using eq 2 and the $K_{D\ app}$ value for [^{125}I]-NDP-α-MSH determined previously (Blanchard *et al.*, 1995). Control medium (medium from wild-type baculovirus-infected cells that was concentrated and desalted as described for Δbasic and ΔC-term) was assayed similarly and showed no inhibition of binding (data not shown). We (Blanchard *et al*., 1995) have also measured a $K_{I\ app}$ = 0.9 nM for agouti antagonism of cAMP production induced by α-MSH in $B_{16}F_{10}$ cells (evidence that cAMP production is proportional

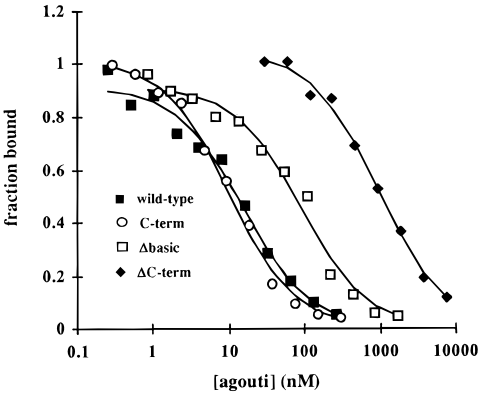

FIGURE 2: Mouse agouti deletion construct inhibition of [^{125}I]-NDP-α-MSH binding to $B_{16}F_{10}$ cells expressing the mouse MC1-R. Curves are representative of the 2−4 experiments performed, and each point is the mean of duplicate experimental values. The ordinate is expressed as a fraction of the total specific binding. IC_{50} values were calculated by curve fitting using a nonlinear regression algorithm and eq 1. Apparent K_I values were calculated with eq 2 and are listed in Table 1.

Table 1: Antagonism of cAMP Production and Inhibition of [^{125}I]-NDP-α-MSH Binding to the Mouse MC1 Receptor by Mouse Agouti Proteins

	$K_{I\ app}$ (nM)[a]			
	wild-type	C-term	Δbasic	ΔC-term
cAMP	0.8 (0.3)[b]	0.8[c]	13 (6.0)	360 (220)
binding	2.6 (0.8)	2.9 (0.6)	16 (6.6)	230 (11)

[a] All values are the average of 2−4 experiments. [b] Standard error values are reported within the parentheses. [c] Taken from Willard *et al*. (1995).

to receptor occupancy is provided). Again the data are best fit to a competitive model of inhibition. The agouti antagonism is reversible, and agouti has no effect on basal cAMP accumulation (Blanchard *et al*., 1995). We have similarly measured the ability of the agouti deletion mutants to antagonize cAMP production induced by 1 nM α-MSH. The $K_{I\ app}$ values for this inhibition, calculated from eq 2 and the EC_{50} value of 0.9 nM for α-MSH determined previously (Blanchard *et al*., 1995), are listed in Table 1. The $K_{I\ app}$ values for both binding and cAMP production are increased 5−15-fold and 100−500-fold relative to wild-type agouti protein for Δbasic and ΔC-term, respectively. As demonstrated previously, C-term has activity equivalent to wild-type agouti protein at this receptor (Willard *et al*., 1995).

To investigate the ability of the mouse agouti deletion mutants and the human agouti protein to inhibit ligand binding to other melanocortin receptor family members, HEK 293 cells stably expressing the human MC1-R, MC3-R, MC4-R, or MC5-R were generated. Clones were selected based on their ability to bind [^{125}I]-NDP-α-MSH. The binding experiments were performed under equilibrium conditions (a time and temperature after which the amount of [^{125}I]-NDP-α-MSH bound to the cells remained constant; 25 °C, 2 h. Additionally, the rate at which [^{125}I]-NDP-α-MSH comes off MC1-R (k_{off} = 0.85 h^{-1}; $t_{1/2}$ = 49 min) was measured to demonstrate that the binding is reversible. It was assumed that binding of [^{125}I]-NDP-α-MSH to the remaining human melanocortin receptors is also reversible because this has been demonstrated for the corresponding mouse receptors, MC3-R (Desarnard *et al*., 1994), MC4-R

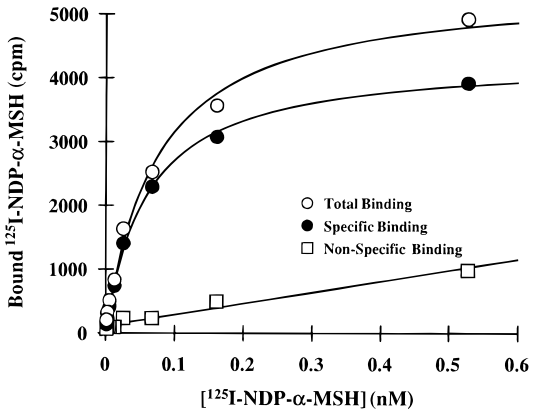

FIGURE 3: Saturation binding of [^{125}I]-NDP-α-MSH to HEK 293 cells stably expressing the human MC1-R. Curves are representative of the 2−4 experiments performed, and each point is the mean of duplicate experimental values. Curve fitting using a nonlinear regression algorithm and a single-site model yielded a $K_{D\ app}$ of 0.06 nM (Table 2).

Table 2: [^{125}I]-NDP-α-MSH Binding to the Human and Mouse Melanocortin Receptors

	$K_{D\ app}$ (nM)[a]			
	MC1	MC3	MC4	MC5
human	0.1 (0.05)[b]	0.2 (0.1)	0.5 (0.2)	0.9 (0.1)
mouse	0.2[c]	0.6 (0.2)	0.2 (0.1)	0.2 (0.1)

[a] Performed under equilibrium conditions (25 °C, 2 h). [b] Standard error values from 2−4 experiments are reported within the parentheses. [c] Taken from Blanchard *et al*. (1995).

Table 3: Inhibition of [^{125}I]-NDP-α-MSH Binding to the Human Melanocortin Receptors by Mouse and Human Agouti Proteins

	$K_{I\ app}$ (nM)[a]			
	MC1	MC3	MC4	MC5
human agouti	23 (3.3)[b]	140 (56)	70 (18)	>1000
mouse agouti	2.1 (0.9)	140 (22)	59 (39)	>1000
C-term	2.9 (0.1)	230 (40)	71 (4.5)	
Δbasic	39 (22)	2000 (590)	290 (81)	
ΔC-term	770	>21000	6600	

[a] All values are the average of 2−4 experiments except in the case of ΔC-term. [b] Standard error values are reported within the parentheses.

(k_{off} = 1.7 h^{-1}; $t_{1/2}$ = 24 min), and MC5-R (Labbe *et al*., 1994). The number of receptors per cell for our HEK 293 cell lines was estimated by saturation binding analysis at approximately 14 000, 25 000, 15 000, and 20 000 for MC1-R (Figure 3), MC3-R, MC4-R, and MC5-R, respectively. The $K_{D\ app}$ values for [^{125}I]-NDP-α-MSH binding to human MC1-R, MC3-R, MC4-R, and MC5-R ($K_{D\ app}$ = 0.1, 0.2, 0.5, and 0.9 nM, respectively; Table 2) are in reasonable agreement with those in the literature (Schioth *et al*., 1995, 1996).

The ability of the mouse and human agouti proteins to compete with 0.1 nM [^{125}I]-NDP-α-MSH for binding to HEK 293 cells stably expressing the human melanocortin receptors was analyzed. IC_{50} values were calculated by curve fitting to a competitive model (eq 1). Again, the control medium showed no inhibition of binding. $K_{I\ app}$ values (Table 3) were generated using eq 2 and the $K_{D\ app}$ values for [^{125}I]-NDP-α-MSH listed in Table 2. The human agouti protein antagonizes the human MC1-R approximately 10-fold more weakly than the mouse protein (human: $K_{I\ app}$ = 23; mouse: $K_{I\ app}$ = 2.1), although the relative affinity of human

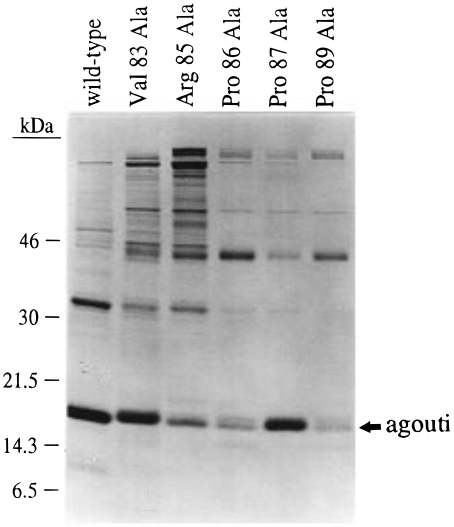

FIGURE 4: SDS−PAGE of wild-type and site-specific mouse agouti protein variants. To prevent loss of the agouti charge variant Arg85Ala from the S Sepharose column, the high-salt wash was skipped, resulting in a less pure sample.

and mouse agouti proteins for the human melanocortin receptors is the same: MC1-R > MC4-R > MC3-R > MC5-R. The agouti proteins inhibit binding to MC4-R better than MC3-R although the difference in $K_{I\ app}$ is only 2-fold (human: $K_{I\ app} = 140 \pm 56$ and 70 ± 18 nM; mouse: $K_{I\ app} = 140 \pm 22$ and 59 ± 39 nM, respectively). A $K_{I\ app}$ value for agouti protein antagonism of the MC5-R was not determined due to weak binding ($K_{I\ app} > 1\ \mu$M).

The $K_{I\ app}$ values for inhibition of ligand binding to the human melanocortin receptors by the various mouse agouti deletion mutants are also shown in Table 3. The C-term fragment inhibits binding to these receptors as well as wild-type agouti with the same order of potency: MC1-R > MC4-R > MC3-R. Indeed, all of the agouti proteins analyzed show this selectivity, even though ΔC-term contains only the first seven amino acids of C-term. The $K_{I\ app}$ values for Δbasic inhibition at each of the three receptors are increased 5−20-fold relative to wild-type agouti protein, which is very similar to the effect of Δbasic on the mouse MC1-R. Likewise, the $K_{I\ app}$ values for ΔC-term at these receptors are at least 100-fold less potent than those for the wild-type protein. When compared to the fully active C-term fragment, the combination of the reduced ability of Δbasic and ΔC-term to inhibit binding to the melanocortin receptors, with their retained selectivity between receptors, suggests that some of residues Val83−Pro89 are involved in receptor affinity. To identify which of these seven amino acids is involved, they were each changed to alanine except for Ala84 and Pro88. The five variant proteins were expressed using a baculovirus system and partially purified by S Sepharose chromatography (Figure 4). Agouti protein concentration was estimated by a combination of SDS−PAGE and BCA assay.

As the site-specific mutations were made in mouse agouti, we wanted to analyze their ability to inhibit ligand binding to the mouse melanocortin receptors. HEK 293 cells stably expressing mouse MC3-R, MC4-R, or MC5-R were generated, while $B_{16}F_{10}$ cells were used for mouse MC1-R. As described for the human melanocortin receptors, clones were selected based on their ability to bind [^{125}I]-NDP-α-MSH, and the number of receptors per cell was estimated by saturation binding under equilibrium conditions. All of these cell lines contain approximately 10 000 receptors per cell. The $K_{D\ app}$ values for [^{125}I]-NDP-α-MSH binding to mouse MC1-R, MC3-R, MC4-R, and MC5-R are 0.2, 0.6, 0.2, and 0.2 nM, respectively (Table 2). These $K_{D\ app}$ values are in reasonable agreement with those previously reported for mouse MC1-R, MC3-R, and MC5-R (Blanchard *et al*., 1995; Desarnard *et al*., 1994; Labbe *et al*., 1994).

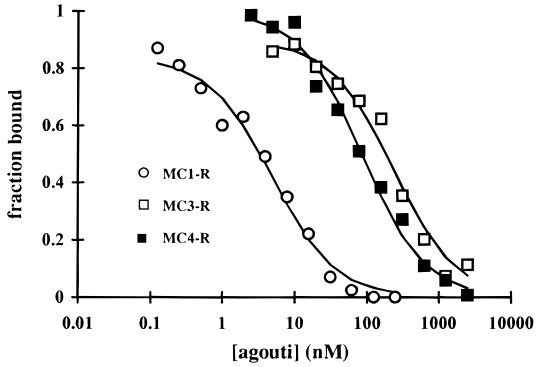

FIGURE 5: Mouse agouti inhibition of [^{125}I]-NDP-α-MSH binding to cells stably expressing the mouse MC1-R, MC3-R, and MC4-R. Curves are representative of the 2−4 experiments performed, and each point is the mean of duplicate experimental values. The ordinate is expressed as a fraction of the total 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 4.

Table 4: Inhibition of [^{125}I]-NDP-α-MSH Binding to the Mouse Melanocortin Receptors by Mouse Agouti Proteins

	$K_{I\ app}$ (nM)[a]			
	MC1	MC3	MC4	MC5
mouse agouti	2.6 (0.8)[b]	190 (74)	54 (18)	1200 (340)
Val83Ala agouti	33 (5)	1200 (430)	240 (90)	>wt[c]
Arg85Ala agouti	3.4 (1.5)	1100 (350)	310 (65)	>wt
Pro86Ala agouti	3.7 (0.8)	>2000	470 (160)	>wt
Pro87Ala agouti	3.3	170	90	+[d]
Pro89Ala agouti	5.8 (0.5)	1100 (310)	310 (57)	>wt

[a] All values are the average of 2−4 experiments except in the case of Pro87Ala agouti. [b] Standard error values are reported within the parentheses. [c] The $K_{I\ app}$ value is greater than that of wild-type agouti. [d] The $K_{I\ app}$ value could not be distinguished from that of wild-type agouti.

Titration curves for wild-type mouse agouti inhibition of [^{125}I]-NDP-α-MSH binding to the mouse melanocortin receptors are shown in Figure 5. The corresponding $K_{I\ app}$ values, calculated as described for the human melanocortin receptors, are listed in Table 4, and indicate that the mouse agouti protein has equal affinity for the mouse and human melanocortin receptors. Also listed in Table 4 are the $K_{I\ app}$ values for the site-specific agouti variants. The only mutation that significantly affects activity at MC1-R is Val83Ala; the $K_{I\ app}$ for this variant is increased 13-fold relative to wild-type agouti. Conversely, activity was decreased 5−10-fold relative to wild-type agouti at MC3-R and MC4-R for all of the agouti variants except Pro87Ala agouti.

DISCUSSION

Lu *et al*. (1994) observed that the agouti protein, whose overexpression in mice leads to obesity and hyperinsulinemia, is a potent antagonist of the MC4-R. This, coupled with

the fact that melanocortin peptides mediate steroidogenesis, lipolysis, and thermoregulation (Schwyzer & Eberle, 1977; Di Wied & Jolles, 1982; O'Donohue & Dorsa, 1982; Ramachandran, 1987; Chretien, 1974; Murphy *et al*., 1983; Martin *et al*., 1990), indicates that the disease state may be mediated through a melanocortin receptor(s). The human homologue of the mouse agouti gene (85% homology with conservative changes) has been cloned and is expressed in fat and testes (Kwon *et al*., 1994). This leaves open the possibility that altered agouti expression or altered regulation of the agouti pathway is also involved in human obesity and diabetes, making the melanocortin receptors prime physiological targets for disease therapy. Here we report $K_{I\ app}$ values for human and murine agouti protein inhibition of ligand binding to melanocortin receptors to elucidate which receptor(s) might be involved in agouti protein-induced diabetes. We have also identified some of the amino acids in agouti that are responsible for melanocortin receptor affinity and selectivity.

Both mouse and human agouti proteins inhibit ligand binding to the melanocortin receptors with the same order of selectivity (MC1-R > MC4-R > MC3-R > MC5-R) and affinity except that the $K_{I\ app}$ for the human protein at the human MC1-R is approximately 10-fold higher than that for the mouse protein at the same receptor. 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; Blanchard *et al*., 1995; $K_{I\ app}$ for adenylyl cyclase activation = 0.3 nM; Lu *et al.*, 1994). This is not the case at MC4-R. We report a $K_{I\ app}$ of 59 nM for agouti inhibition of binding to human MC4-R, while Lu *et al.* (1994) reported an increase in the EC_{50} value for α-MSH activation of adenylyl cyclase through MC4-R with the addition of only 0.7 nM agouti protein. Perhaps binding of the agouti protein changes the equilibrium between active and inactive MC4 receptors, allowing it to be a better functional antagonist. Lu *et al.* (1994) did not observe a similar increase in EC_{50} for α-MSH at MC3-R with the addition of 0.7 nM agouti. We have shown that agouti inhibits ligand binding at MC4-R only 2−4-fold better than at MC3-R. The small difference in agouti affinity for MC3-R and MC4-R indicates that agouti may also antagonize cAMP production through MC3-R at only slightly higher concentrations than for MC4-R. For this reason, the MC3-R should be included as a potential candidate for a receptor mediating the effects of agouti protein overexpression. Indeed, the expression of both MC3-R and MC4-R in the hypothalamus, which regulates thermogenesis and feeding, makes these receptors good prospects for being biological mediators of agouti-induced obesity and diabetes (Roselli-Rehfuss *et al.*, 1993; Mountjoy *et al*., 1994; Low *et al.*, 1994). Although the MC5-R is present in energy-relevant tissues such as muscle, liver, and adipose (Labbe *et al*., 1994), we observed only weak inhibition of binding to this receptor by agouti protein ($K_{I\ app}$ > 1 μM), consistent with previously published results (Lu *et al.*, 1994; Boston & Cone, 1996). In addition, it is not likely that agouti is an extremely abundant protein, as we were unable to detect it by Western Blot analysis of A^Y and transgenic BAPa[2] (Klebig *et al*., 1995) mouse tissues including muscle, adipose, skin, and liver (unpublished results). This makes it unlikely that MC5-R mediates agouti effects.

mouse	Val83	Ala84	Arg85	Pro86	Pro87	Pro88	Pro89
human		Val			Arg	Thr	

FIGURE 6: Comparison of amino acids Val83−Pro89 in mouse and human agouti proteins.

The melanocortin receptor(s) important for agouti-induced obesity/diabetes cannot be identified based on agouti protein affinity alone. This will have to await the generation of small molecule antagonists or mutant agouti proteins which are highly specific for one of these receptors and can be introduced into the appropriate animal model. With the generation of such a variant agouti protein as the goal, the region of the agouti protein necessary for melanocortin binding inhibition was localized through the use of deletion constructs. The carboxyl-terminal region of the agouti protein (Val83−Cys131) retains full inhibitory activity at the human MC1-R, MC3-R, and MC4-R's (MC1-R > MC4-R > MC3-R). This is consistent with previous data showing that this region is fully active against the mouse MC1-R (Willard *et al*., 1995). The slight decrease in activity for Δbasic and the large decrease for ΔC-term are consistent with the C-term fragment having full activity, as Δbasic is missing only a few C-term residues while ΔC-term is lacking almost the entire region. The fact that removing a few amino acids in C-term results in a loss of activity while having only a few residues of this region results in significant residual activity suggests that some or all of the residues, Val83−Pro89, interact with the receptor. On the other hand, it is possible that the decreased activity of Δbasic is simply due to a protein structural change induced by the juxtaposition of Asn56 and Pro86 rather than the absence of amino acids Val83−Val85. However, the significant remaining affinity of ΔC-term for MC1-R ($K_{I\ app}$ = 230 nM from Table 1) favors the former hypothesis. Furthermore, the fact that ΔC-term inhibits ligand binding to the melanocortin receptors with the same order of potency as the wild-type protein indicates that the observed receptor selectivity is also due in part to residues Val83−Pro89. Indeed, the retained receptor specificity is further evidence that this protein interacts in a manner similar to the wild-type protein, and does not have a grossly altered protein structure.

To confirm that the region of agouti, Val83−Pro89, is important for inhibition of receptor binding, each of these residues was changed to alanine except for Ala84 and Pro88. Pro88 was not altered because it is not conserved between the mouse and human agouti proteins (Figure 6) which bind the melanocortin receptors with similar affinities. Three of the variant agouti proteins, Arg85Ala, Pro86Ala, and Pro89Ala, were expressed at low levels which can correlate with improper folding (Figure 4). We believe that the proteins are folded correctly, however, and that the low expression is due instead to a problem with the baculovirus expression system (these three proteins were expressed separately from Val83Ala and Pro87Ala agouti proteins). The data that support correct folding for these agouti variants are (i) they inhibit ligand binding at MC1-R significantly better than at MC3-R and MC4-R ($K_{I\ app\ mut}/K_{I\ app\ wt}$ = 1.3−2.2 at MC1-R, while 5.7−>10 at MC3-R and MC4-R), and (ii) they have essentially wild-type activity at MC1-R (Table 4). The

[2] The mouse agouti cDNA was placed under the transcriptional control of the ubiquitous β-actin promoter in a transgenic mouse. Northern Blot analysis indicated that large amounts of agouti message were produced in tissues including muscle, liver, adipose, and skin.

decreased ability of four of the five site-specific agouti variants to inhibit ligand binding to some or all the melanocortin receptors indicates that this region does contain determinants for receptor affinity and selectivity. For example, the increase in $K_{I\ app}$ for binding inhibition at MC1-R for Val83Ala agouti ($K_{I\ app\ mut}/K_{I\ app\ wt} = 13$) indicates that Val83 is an important binding determinant for this receptor. Arg85, Pro86, and Pro89 are important for selective inhibition of binding between MC1-R and MC3-R and MC4-R as their apparent K_I values are essentially unchanged at MC1-R, while they have increased 6−10-fold relative to wild-type protein at MC3-R and MC4-R.

We have reported $K_{I\ app}$ values for agouti inhibition of ligand binding to the melanocortin 1, 3, 4, and 5 receptors, and have localized four amino acids necessary for receptor affinity and selectivity. Future experiments will include generating agouti mutants with even greater selectivity between the melanocotin receptors that can be used as tools for elucidating the mechanism of agouti-induced diabetes and obesity.

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REFERENCES

Blanchard, S. G., Harris, C. O., Ittoop, O., Nichols, J. S., Parks, D. J., Truesdale, A. T., & Wilkison, W. O. (1995) *Biochemistry 34*, 10406−10411.

Boston, B. A., & Cone, R. D. (1996) *Endocrinology 137*, 2043−2050.

Bultman, S. J., Michaud, E. J., & Woychik, R. P. (1992) *Cell 71*, 1195−1204.

Chen, C., & Okayama, H. (1987) *Mol. Cell. Biol. 7*, 2745−2752.

Cheng, Y., & Prusoff, W. H. (1973) *Biochem. Pharmacol. 22*, 3099.

Chhajlani, V., & Wikberg, J. E. S. (1992) *FEBS Lett. 309*, 417−420.

Chretien, M. (1974) *Triangle 13*, 63−71.

Desarnaud, F., Labbe, O., Eggerickx, D., Vassart, G., & Parmentier, M. (1994) *Biochem. J. 299*, 367−373.

Di Wied, D., & Jolles, J. (1982) *Physiol. Rev. 62*, 976−1059.

Gantz, I., Konda, Y., Tashiro, T., Shimoto, Y., Miwa, H., Munzert, G., Watson, S. J., DelValle, J., & Yamada, T. (1993a) *J. Biol. Chem. 268*, 8246−8250.

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

Klebig, M. L., Wilkinson, J. E., Geisler, J. G., & Woychik, R. P. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 4728−4732.

Kwon, H. Y., Bultman, S. J., Loffler, C., Chen, W.-J., Furdon, P. J., Powell, J. G., Usala, A.-L., Wilkison, W. O., Hansmann, I., & Woychik, R. P. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 9760−9764.

Labbe, O., Desarnaud, F., Eggerickx, D., Vassart, G., & Parmentier, M. (1994) *Biochemistry 33*, 4543−4549.

Lamoreux, M. L. (1973) Doctoral dissertation, The Jackson Laboratory, Bar Harbor, ME.

Low, M. J., Simerly, R. B., & Cone, R. D. (1994) *Curr. Opin. Endocrinol. Diabetes* 79−88.

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.

Luckow, V. A., Lee, S. C., Barry, G. F., & Olins, P. O. (1993) *J. Virol. 67*, 4566−4579.

Lunec, J., Pieron, C., Bal, W., MacNeil, S., & Thody, A. J. (1993) *Melanoma Res. **3***, 99−106.

Martin, S. M., Malkinson, T. J., Veale, W. L., & Pittman, Q. J. (1990) *Brain Res. 526*, 351−354.

Michaud, E. J., Bultman, S. J., Stubbs, L. J., & Woychik, R. P. (1993) *Genes Dev. 7*, 1203−1213.

Michaud, E. J., Bultman, S. J., Klebig, M. L., van Vugt, M. J., Stubbs, L. J., Rusell, L. B., & Woychik, R. P. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 2562−2566.

Mountjoy, K. G., Robbins, L. S., Mortrud, M. T., & Cone, R. D. (1992) *Science 257*, 1248−1251.

Mountjoy, K. G., Mortund, M. T., Low, M. J., Simerly, R. B., & Cone, R. D. (1994) *Mol. Endocrinol. 8*, 1298−1308.

Murphy, M. T., Richards, D. B., & Lipton, J. M. (1983) *Science 221*, 192−193.

O'Donohue, T. L., & Dorsa, D. M. (1982) *Peptides 3*, 353−395.

Ramachandran, J. (1987) in *Hormonal Proteins and Peptides* (Li, C. H., Ed.) Vol. XIII, pp 31−57, Academic Press, Orlando, FL.

Robbins, L. S., Nadeau, J. H., Johnson, K. R., Kelly, M. A., Roselli-Rehfuss, L., Baack, E., Mountjoy, K. G., & Cone, R. D. (1993) *Cell 72*, 827−834.

Roselli-Rehfuss, L., Mountjoy, K. G., Robbins, L. S., Mortrud, M. T., Low, M. J., Tatro, J. B., Entwistle, M. L., Simerly, R. B., & Cone, R. D. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 8856−8860.

Schioth, H. B., Muceniece, R., Wikberg, J. E., & Chhajlani, V. (1995) *Eur. J. Pharmacol. 288*, 311−317.

Schioth, H. B., Muceniece, R., & Wikberg, J. E. S. (1996) *Pharmacol. Toxicol. 79*, 161−165.

Schwyzer, R., & Eberle, A. (1977) *Front. Horm. Res. 4*, 18−25.

Solca, F. F. A., Salomon, Y., & Eberle, A. N. (1991) *J. Recept. Res. 11*, 379−390.

Willard, D. H., Bodnar, W., Harris, C., Nichols, J., Kiefer, L. L., Hoffman, C., Moyer, M., Burkhart, W., Weiel, J., Wilkison, W. O., & Rocque, W. (1995) *Biochemistry 34*, 12341−12346.

Yen, T. T., Gill, A. M., Frigeri, L. G., Barsh, G. S., & Wolff, G. L. (1994) *FASEB J. 8*, 479−488.

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