Agouti Antagonism of Melanocortin Binding and Action in the $B_{16}F_{10}$ Murine Melanoma Cell Line

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ABSTRACT: Several dominant mutations at the murine agouti locus result in the expression of a number of phenotypic changes, including a predominantly yellow coat color, obesity, and hyperinsulinemia. The mutants exhibit ectopic overexpression of normal agouti protein, suggesting that agouti regulates coat coloration by direct antagonism of the α-melanocyte-stimulating hormone receptor. We have tested this hypothesis by examining agouti inhibition of both melanocortin-stimulated cyclic adenosine monophosphate production and the binding of a radioactive melanocortin analog in the murine $B_{16}F_{10}$ melanoma cell line. Inhibition of melanocortin-induced cyclic nucleotide accumulation did not require preincubation of the cells with agouti and was independent of the agonist used. Furthermore, inhibition of both agonist binding to and activation of melanocortin receptor could be described by a simple competitive model with similar inhibition constants of 1.9 and 0.9 nM, respectively. The mutually exclusive binding of agouti and melanocortin was verified by cross-linking experiments using a radiolabeled α-melanocyte-stimulating hormone analog. Competitive inhibition of α-melanocyte-stimulating hormone binding can account for the effects of agouti on coat coloration and suggests the possibility that the other phenotypic changes observed on agouti overexpression may be due to direct action of agouti at a novel melanocortin receptor-(s).

The mouse agouti gene is normally involved in regulating the production of pigment granules that give rise to the wild-type coat color (Silvers, 1979). Several dominant mutations at agouti, most notably lethal yellow (A^y) and viable yellow (A^{vy}), cause mice to develop a predominantly yellow coat color and become obese and hyperinsulinemic with age (Wolff *et al.*, 1986; Wolff, 1987; Klebig *et al.*, 1994; Yen *et al.*, 1994). The agouti gene has been cloned and encodes a 131-amino acid protein with a consensus signal sequence (Bultman *et al.*, 1992; Miller *et al.*, 1993). Agouti is normally expressed in the skin during hair growth (Bultman *et al.*, 1992); however, in the A^{vy} and A^y mutants, the agouti gene is expressed in most, if not all, tissues of the animal, including skin.

Agouti functions in a paracrine manner to regulate the differential production of melanin pigments by the melanocyte (Silvers, 1958a,b, 1961, 1979; Silvers & Russell, 1955). α-MSH[1] binds to its receptor (MSH-R) on the melanocyte and causes an increase in intracellular cAMP via activation of adenylate cyclase (Takeuchi *et al.*, 1989). When this occurs, the melanocyte synthesizes black pigment (eumelanin). When agouti is present within the hair follicle, it appears to block the ability of α-MSH to activate its receptor. Consequently, the adenylate cyclase within the melanocyte is not activated, and yellow pigment (phaemelanin) is synthesized (Takeuchi *et al.*, 1989), resulting in the yellow coat color of the A^{vy} and A^y mice. Therefore, in the melanocyte, agouti regulates the differential synthesis of eumelanin and phaeomelanin by antagonizing the action of α-MSH at its receptor (Bultman *et al.*, 1992). The obesity and hyperinsulinemia observed in yellow mice are also due to the ectopic overexpression of agouti protein (Bultman *et al.*, 1992).

Three distinct mechanisms of agouti antagonism of melanocortin action may be envisioned (Yen *et al.*, 1994; Bultman *et al.*, 1992): First, agouti may act directly on the melanocortin ligand, either via a stoichiometric binding event, resulting in a decrease in free ligand available for receptor activation, or by the catalytic degradation of agonist. A second possibility is direct antagonist interaction of agouti at the MSH receptor, while the third mechanism would be an interruption of the melanocortin signaling pathway via interaction of agouti with a distinct "agouti receptor".

Initial observations showed that agouti antagonized melanocortin action in vitro in an apparently competitive manner. That is, a single concentration of agouti protein induced a parallel rightward shift in the dose−response curve for melanocortin ligands with no apparent effect on the maximal stimulation (Lu *et al.*, 1994). The limited availability of

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[1] Abbreviations: α-MSH, α-melanocyte-stimulating hormone; MSH-R, melanocyte-stimulating hormone receptor; cAMP, adenosine 3′,5′-cyclic monophosphate; TSH, thyroid-stimulating hormone; β-MSH, β-melanocyte-stimulating hormone; ACTH (1−39), adrenocorticotropic hormone (residues 1−39); NDP-α-MSH, (norleucine)[4],(D-phenylalanine)[7]-α-melanocyte-stimulating hormone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; DNase I, deoxyribonuclease; EGS, ethylene glycol bis(succinimidyl succinate); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; IBMX, isobutylmethylxanthine; [^{125}I]-NDP-α-MSH, (norleucine)[4],(D-Phenylalanine)[7]-α-melanocyte-stimulating hormone iodinated at (tyrosine)[2]; PBS, phosphate-buffered saline; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; β-LPH, β-lipotropin.

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purified agouti, however, precluded verification of the competitive nature of this interaction over an extended concentration range. Furthermore, it is important to note that mechanisms in which agouti interacts directly with, or on, the agonist to reduce its effective concentration would result in apparently competitive behavior, i.e., a rightward shift in the dose−response curve for agonist with no decrease in maximal stimulation/binding.

In the present study, the competitive nature of the agouti inhibition of both agonist binding and activation at the MSH receptor in the $B_{16}F_{10}$ murine melanoma cell line has been demonstrated over a range of both agonist and agouti concentrations. Control experiments showed that the antagonist effect of agouti is inconsistent with a direct effect on the melanocortin ligand. Furthermore, the inability of agouti to inhibit thyroid-stimulating hormone (TSH)-stimulated cAMP production suggested that agouti acts proximal to adenylate cyclase activation. The results indicate that agouti acts to inhibit melanocortin binding and action at the level of the MSH receptor in the $B_{16}F_{10}$ cell line.

EXPERIMENTAL PROCEDURES

Materials. α-MSH, β-MSH, ACTH(1−39), and (norleucine)^4,(D-phenylalanine)^7-MSH (NDP-α-MSH) were from Bachem. RPMI-1640, penicillin/streptomycin, and HEPES were from Gibco/BRL, fetal bovine serum was from Hyclone, and BSA (Fraction V, protease free) was from Boehringer Mannheim. Forskolin, glycine, and DNAse were from Sigma. Enzymobeads were from Bio-Rad, Na^{125}I was obtained from Amersham, and Sep-Pak C_{18} columns were from Waters. The production of recombinant murine agouti has been previously described (Lu *et al.*, 1994). The protein was at least 85% pure as judged by SDS−PAGE.

Cell Culture. $B_{16}F_{10}$ cells obtained from Dr. David Emerson (Department of Pharmacology, Glaxo Research Institute) were cultured in RPMI-1640 supplemented with 2 mM glutamine, 50 units/mL penicillin, 50 μg/mL streptomycin, and 10% fetal bovine serum in a humidified 5% CO_2/95% air atmosphere. For binding and cAMP accumulation experiments, the cells were plated into 96-well round-bottom tissue culture plates at a density of either 1×10^5 or 2.5×10^4 cells/well 1 or 2 days before use, respectively.

cAMP Measurements. Cell monolayers were washed with RPMI-1640 and 1 mg/mL BSA. Ligands (melanocortin agonists and/or agouti) were diluted in this same buffer with 0.6 mM IBMX. Cells were incubated with the desired ligands in a volume of 200 μL/well at 37 °C. The reaction was allowed to proceed for the desired time (generally 1 h), followed by the addition of 50 μL/well of 50 mM sodium acetate (pH 4) containing 0.1% Triton X-100. The plates were then heated at 90 °C for 5 min to denature protein. Samples were either assayed immediately or stored frozen. The cAMP content of the samples was determined by scintillation proximity assay using a commercially available kit (Amersham).

Preparation and Characterization of [^{125}I]-NDP-α-MSH. Fifty-microliters of 0.2 M sodium phosphate (pH 7.3), 10 μL of a solution of 600 μM NDP-α-MSH in 1 mM HCl, 50 μL of Enzymobeads, and 20 μL of Na^{125}I solution (2 mCi, 1.2 nmol) were mixed in a polypropylene microcentrifuge tube. The reaction was started by the addition of 25 μL of 1% β-D-glucose and was allowed to proceed for 20 min. The reaction was stopped by centrifugation at 1000*g* for 10 min. Purification of the radiolabeled ligand was essentially as described (Tarro & Reichlin, 1987). Briefly, the supernatant was applied to a Sep-Pak C_{18} cartridge equilibrated with 50 mM ammonium acetate (pH 5.8) containing 15% acetonitrile. The [^{125}I]-NDP-α-MSH was eluted from the cartridge at a flow rate of 1.5 mL/min using a gradient of 15−40% acetonitrile in 50 mM ammonium acetate (pH 5.8). Fractions were assayed for total radioactivity and for specific binding using the whole cell binding assay. Twenty-five microliters of 10% BSA was added to each fraction (0.75 mL), and the fractions having a constant specific activity as assessed by the binding assay were stored frozen at −80 °C. The specific activity was determined as previously described (Blanchard *et al.*, 1987) and typically ranged from 500 to 1500 Ci/mmol. For cross-linking experiments, the [^{125}I]-NDP-α-MSH was subjected to an additional HPLC purification step on Hypersil ODS (3 μm, 4.6 × 100 mm), using a gradient of 10−100% acetonitrile in aqueous 0.1% TFA. The radioactive fraction corresponding to monoiodinated product was collected, lyophilized, and redissolved in 1 mM HCl.

Ligand Binding. Cell monolayers were washed with serum-free RPMI-1640. Ligand and antagonist were diluted in RPMI-1640 containing 50 mM HEPES (pH 7.5) and 1 mg/mL BSA. Cells were incubated with the desired ligand and antagonist concentrations in an assay volume of 100 μL for 2 h at room temperature. The plates were subjected to gentle agitation throughout the incubation period. The plates were cooled on ice, and the incubation medium containing free ligand and antagonist was removed by rapid aspiration. Cells were then washed two times with 125 μL of ice-cold PBS. Scintillation cocktail (125 μL) was added to each well, and radioactive ligand retained by the cells was determined by using a Wallac 1650 Microbeta plate counter.

Chemical Cross-Linking of [^{125}I]-NDP-α-MSH. Covalent cross-linking of [^{125}I]-NDP-α-MSH to melanocortin receptors of intact $B_{16}F_{10}$ cells was essentially as described (Lunec *et al.*, 1992; Shafir *et al.*, 1993). Briefly, ligand binding to cell monolayers in 6-well tissue culture plates was performed as described earlier. The cross-linking agent (1 mM EGS) in phosphate-buffered saline was added following the final wash step, and the monolayers were incubated on ice for 20 min. Glycine (40 mM) was added to quench remaining cross-linking agent. Following an additional 10 min of incubation, the medium was removed by aspiration, and the monolayers were washed four times with 1 mL of ice-cold PBS per well. Fresh PBS was added and the monolayers were scraped with a rubber policeman to release adherent cells. The samples were transferred to polypropylene microfuge tubes, and the cells were pelleted by centrifugation for 10 min at 12000*g*. Supernate was discarded and 60 units of DNAse I (Sigma) in 30 μL of water was added. The samples were incubated for 10 min at room temperature, and the cells were pelleted by centrifugation. Fifteen microliters of 1% SDS and 5 μL of SDS−PAGE sample buffer containing 100 mM DTT were added, and the samples were denatured by heating at 37 °C for 30 min. Samples were run on a 4−20% gradient gel (Novex) using the buffer system described by Laemmeli (1970). Following electrophoresis, the gel was equilibrated in Gel-Dry (Novex) and then dried. Visualization of [^{125}I]-NDP-α-MSH incorporation was accomplished by using a phosphoimager system

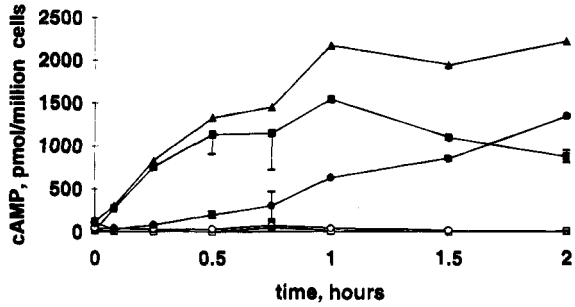

FIGURE 1: Time course of cAMP accumulation. Aliquots (200 μL) of buffer containing the indicated ligands were added to individual wells of a 96-well plate, and the cells were incubated for various times at 37 °C. Medium was removed from the cells and transferred to clean 96-well plates, and 200 μL of fresh medium was added to each well. The reaction was terminated for both the medium and cellular samples by the addition of quench buffer and heating as described in Experimental Procedures. cAMP levels were determined in both cellular samples and medium samples. The total cAMP values were obtained by addition. Plots of cAMP accumulation versus time induced by 1 nM α-MSH are shown for total cAMP accumulation (▲); intracellular cAMP without agouti (■); intracellular cAMP in the presence of 1 nM agouti (□); extracellular cAMP (●); and extracellular cAMP in the presence of 1 nM agouti (○).

(Molecular Dynamics). Further experimental details are given in the legend to Figure 4.

Data Analysis. Data from both the cAMP and binding competition experiments were fit to eq 1, where response-(M,I) is the measured response (either cAMP level or ^{125}I ligand bound) at ligand concentration M and agouti concentration I; b_{max} is the maximal response; K_i is the inhibition constant for agouti; and EC_{50} is the concentration of ligand required to induce a half-maximal response. Note that, for the binding experiments, EC_{50} is just the equilibrium dissociation constant, K_d, for ligand binding. For the cAMP accumulation experiments, however, the EC_{50} is a constant dependent on both the affinity of the agonist for receptor and the efficiency of receptor coupling to the adenylate cyclase system. Nonlinear least-squares fits of the experimental data to eq 1 were obtained using the RS/1 data analysis package (BBN Software).

$$\text{response}(M,I) = \frac{b_{max}}{1 + (EC_{50}/M)(1 + I/K_i)} \quad (1)$$

RESULTS

Time Dependence of cAMP Accumulation and Agouti Action. In many cells, ligand-induced activation of adenylate cyclase leads to both accumulation of intracellular cAMP and increased efflux of cAMP [reviewed in Brunton and Heasley (1988); Fehr *et al.*, 1990). As shown in Figure 1, a significant proportion of the cAMP synthesized by $B_{16}F_{10}$ cells in response to α-MSH is secreted into the extracellular medium. Therefore, the extent of receptor occupancy, as reflected by adenylate cyclase activation, is proportional to the total (extracellular + cellular) cAMP level, *not to cellular cAMP levels alone.* As a result, all further experiments measured the total, rather than cellular, cAMP levels. It should be noted that 0.6 mM IBMX was routinely added to the medium for cAMP accumulation experiments. Interestingly, IBMX addition caused a shift in the ratio of extracellular to intracellular cAMP, but no change in the total (cellular + extracellular) value (not shown).

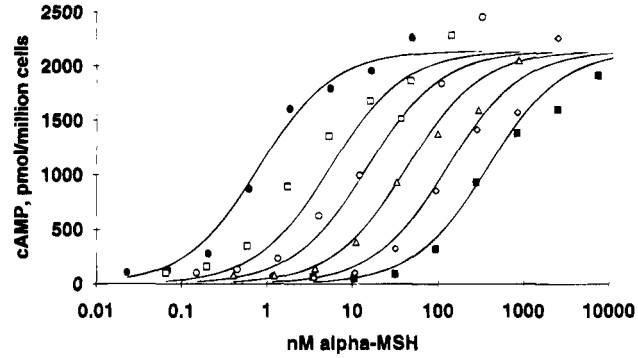

FIGURE 2: Competitive inhibition of α-MSH-induced cAMP accumulation by agouti. Dose–response curves to α-MSH were determined in the presence of increasing concentrations of agouti as described in Experimental Procedures. The individual curves indicate 0 (●), 150 (■), 50 (◇), 16.7 (△), 5.56 (○), and 1.85 nM agouti (□). The solid lines were drawn using the parameters determined by a least-squares fit of the total data set to eq 1. The best fit values obtained in this experiment were b_{max}= 2150 pmol of cAMP/10^6 cells, EC_{50} = 0.9 nM, and K_i = 0.3 nM.

If the mechanism of agouti inhibition is via simple competition, inhibition should not require the preincubation of agouti with receptor. Indeed, Figure 1 demonstrates that the inhibition of cAMP accumulation by 1 nM agouti occurs immediately and that this effect persists over the time course of the experiment. In addition, the dose–response curve for agouti inhibition of cAMP accumulation in the presence of 1 nM α-MSH was unchanged upon preincubation of cells with agouti: an IC_{50} of 9 ± 2 nM was obtained when agouti and α-MSH were added to the cells at the same time, which is in excellent agreement with the value of 7 ± 2 nM obtained if agouti is preincubated with the cells for 15 min before the addition of α-MSH.

Agouti Is a Competitive Inhibitor of cAMP Accumulation. Increasing concentrations of agouti were able to induce a rightward shift in the dose–response curves for α-MSH-induced cAMP production by the $B_{16}F_{10}$ cell line (Figure 2). Consistent with initial observations (Lu *et al.*, 1994), there was no apparent decrease in the maximal level of stimulation observed at any concentration of agouti. A least-squares fit of these data to the simple competitive model given by eq 1 gave b_{max} = 360 pmol of cAMP/well, EC_{50} = 0.9 nM, and K_i = 0.3 nM. In contrast, a satisfactory fit of these data to an alternative, noncompetitive model could not be obtained (not shown). Agouti had no effect on basal cAMP accumulation: in the absence of α-MSH, cAMP production was 1.25 ± 0.19 pmol/10^6 cells, independent of agouti concentration over a range from 0.05 to 100 nM. The level of cAMP observed in the absence of any added agouti was 1.33 pmol/10^6 cells.

The $B_{16}F_{10}$ melanoma line expresses only a single melanocortin receptor (Solca *et al.*, 1991; Lunec *et al.*, 1993). If agouti inhibition of cAMP accumulation is via direct inhibition of agonist binding to the MSH receptor, the apparent K_i should be independent of the (melanocortin) agonist used to activate adenylate cyclase. This prediction was verified experimentally (Table 1). Inhibition of cAMP accumulation by agouti was competitive with respect to agonist for all melanocortin agonists examined. Furthermore, the K_i's obtained for agouti were essentially independent of the melanocortin ligand used.

The validity of the preceding discussion requires that (a) all ligands used have equal efficacy and (b) the maximal

Table 1: Parameters for Inhibition of cAMP Accumulation by Murine Agouti[a]

ligand	EC_{50} (nM)[b]	K_i (nM)[b]
α-MSH	1.7 ± 0.9	0.9 ± 0.6
desacetyl-MSH	0.9 ± 0.5	1.0 ± 0.2
β-MSH	0.96 ± 1.0	0.8 ± 0.4
ACTH(1−39)	11 ± 1.1	0.6 ± 0.2

[a] Dose−response curves to the indicated ligands were obtained in the presence of varying concentrations of agouti. A minimum of five different concentrations of agouti were tested for each ligand, and a no agouti control was included for each experiment. All concentrations were performed in duplicate. [b] The best fit parameters for each experiment were obtained as described in Experimental Procedures. The parameters given are mean ± standard deviation from a minimum of two experiments per ligand.

Table 2: Melanocortin Ligands Induce Equivalent Maximal Levels of cAMP Accumulation

ligand[a]	b_{max}[b] (pmol of cAMP/10^6 cells)
α-MSH	2150 ± 200
desacetyl-MSH	2050 ± 140
β-MSH	1890 ± 140
ACTH(1−39)	1900 ± 150
forskolin	3550 ± 340

[a] All ligands were tested at a concentration of 100 nM with the exception of forskolin, which was used at a concentration of 100 μM. [b] The mean values ($n = 6$) ± standard deviations are shown.

stimulation of cAMP production achieved by melanocortin agonists is not due to saturation of the adenylate cyclase system in these cells. As shown in Table 2, all ligands tested at 100-fold above their respective K_d values gave essentially identical maximal levels of cAMP accumulation, equivalent to 55% of the forskolin-induced level. This finding is of particular importance, since the observation of no change in b_{max} is a significant indicator of competitiveness *only if* adenylate cyclase activation, as measured by cAMP accumulation, is directly proportional to receptor occupancy. If the maximal levels of cAMP induced by agonist and forskolin were equivalent, it would imply that adenylate cyclase levels were limiting, suggesting a dissociation between receptor occupancy and cyclase activation.

Agouti Inhibits [^{125}I]-NDP-α-MSH Binding. Agouti inhibited binding of [^{125}I]-NDP-α-MSH and cAMP accumulation induced by α-MSH with similar potencies: an IC_{50} of 2.1 nM was obtained for inhibition of [^{125}I]-NDP-α-MSH (0.1 nM) binding, whereas the cAMP accumulation induced by 1 nM α-MSH was inhibited with an IC_{50} of 0.8 nM. A detailed analysis of the inhibition of [^{125}I]-NDP-α-MSH binding by agouti is shown in Figure 3. These data indicated competitive displacement of the radiolabeled melanocortin analog by agouti. Linear least-squares fits of the data from these experiments gave a K_d for [^{125}I]-NDP-α-MSH binding of 0.17 ± 0.03 nM, which is in good agreement with previously published results (Solca *et al.*, 1991). The K_i for agouti in these experiments was 1.9 ± 0.6 nM, which is in excellent agreement with the value of 1 nM obtained from the cAMP accumulation experiments (Figure 2, Table 1).

Site of Agouti Inhibition of Melanocortin Action in $B_{16}F_{10}$ Cells. As mentioned previously, inhibition of melanocortin action by agouti could be the result of interaction at the level of either (a) the stimulating agonist, (b) the receptor complex, or (c) a "downstream" point in the signal transduction pathway. In contrast to the results obtained for the competition of agouti with melanocortin ligands, our previous study (Lu *et al.*, 1994) reported that agouti has no effect on ligand (TSH)-induced cAMP production by thyroid-stimulating hormone receptors. This clearly suggested that agouti disruption of melanocortin action occurred at a point in the signal transduction pathway prior to the activation of adenylate cyclase. Furthermore, a direct action of agouti on agonist was unlikely, because this mechanism of action would predict that the K_i's for agouti action would differ for different agonists. As shown in Table 1, the K_i for agouti action is independent of the agonist employed. In addition, gel filtration experiments failed to show any interaction of agouti with [^{125}I]-ACTH, and incubation of α-MSH with preparations of agouti had no effect on the integrity of this melanocortin ligand, as assessed by HPLC (data not shown).

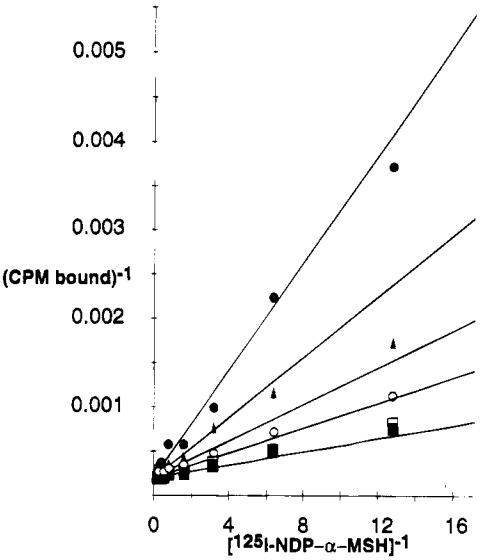

FIGURE 3: Inhibition of [^{125}I]-NDP-α-MSH binding by agouti. Dose−response curves to [^{125}I]-NDP-α-MSH were determined in the presence of increasing amounts of agouti. Individual curves indicate 0 (■), 1.25 (□), 2.5 (○), 5 (▲), and 10 nM agouti (●).

The most likely site of agouti action in $B_{16}F_{10}$ cells, therefore, appears to be at the level of the melanocortin receptor. Further support for this conclusion was obtained from studies in which [^{125}I]-NDP-α-MSH was covalently cross-linked to melanocortin receptors in intact $B_{16}F_{10}$ cells. As shown in Figure 4, covalent cross-linking of [^{125}I]-NDP-α-MSH followed by SDS−PAGE and autoradiography identified a labeled band of ~46 kDa, consistent with results previously reported for MSH receptors (Lunec *et al.*, 1992; Shafir *et al.*, 1993). Formation of the [^{125}I]-NDP-α-MSH−receptor complex was diminished by competition with either unlabeled NDP-α-MSH or agouti protein. The formation of the labeled band was completely inhibited in the presence of 15 nM agouti protein, whereas 1.5 nM agouti gave only partial displacement. Therefore, the ability of agouti to displace [^{125}I]-NDP-α-MSH in the cross-linking experiments was consistent with the observed K_i's of 0.8 and 1.9 nM obtained from the cAMP and competition binding assays, respectively.

DISCUSSION

The data presented here indicate that agouti acts as a competitive antagonist at the level of melanocortin binding to the MSH receptor complex in $B_{16}F_{10}$ cells. This provides a straightforward explanation for the agouti effect seen on

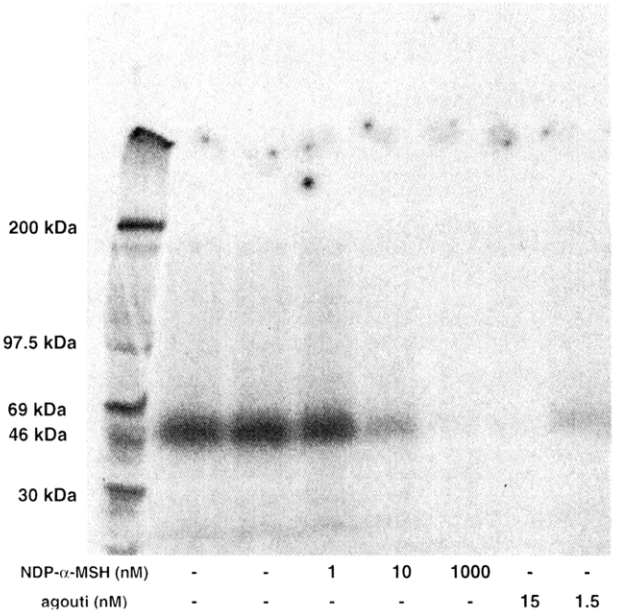

FIGURE 4: Agouti decreases the cross-linking of [^{125}I]-NDP-α-MSH to melanocortin receptors of intact $B_{16}F_{10}$ cells. Cell monolayers in 6-well tissue culture plates (3.5×10^6 cells/well) were incubated with 0.1 nM [^{125}I]-NDP-α-MSH in 1 mL/well of RPMI-1640 medium containing 0.1% BSA for 1 h at 37 °C. The indicated concentration of unlabeled NDP-α-MSH or agouti protein was included in the incubation medium. The monolayers were washed and cross-linking was carried out as described in Experimental Procedures. Both NDP-α-MSH and agouti effected dose-dependent inhibition of the single, diffuse band (~46 kDa) indicative of the melanocortin receptor.

pigmentation in both normal mice and those with ectopic overexpression of agouti (A^{vy}, A^y) (Silvers, 1979; Wolff *et al.*, 1986; Klebig *et al.*, 1994). Indeed, in vivo observations are consistent with a competitive interaction between agouti and α-MSH: injection of α-MSH into the skin of A^{vy} or A^y mice overcomes the effects of agouti overexpression and results in the production of black hair (Shimizu *et al.*, 1988, 1989).

It is possible that agouti antagonism of other members of the melanocortin receptor family may account for the other phenotypic changes observed in the A^{vy} and A^y mice. Identification of the site(s) and/or endogenous ligand(s) antagonized by agouti would allow the design of experimental approaches to test this hypothesis. Recently, novel members of the melanocortin receptor family were identified (Gantz *et al.*, 1993a,b, 1994; Labbe *et al.*, 1994). Their functions are, however, unclear, and these receptors exhibit tissue distributions distinct from those of the MSH and ACTH receptors. We have shown that agouti can antagonize multiple members of the melanocortin receptor family (Lu *et al.*, 1994), although the mechanisms and relative affinities of agouti for these receptors were not established. On the basis of the findings reported here, it appears reasonable to hypothesize that competition between agouti and melanocortin peptide may be a common feature for all members of the melanocortin receptor family. The observation of competitive antagonism suggests that the phenotypic changes resulting from agouti overexpression might be modulated by experimental manipulation of the relative concentrations of agouti (antagonist) and melanocortin (agonist). Importantly, competition between agouti and melanocortin predicts that the phenotypic changes observed in A^{vy} and A^y mice could be reversed either by (a) decreasing the effective concentration of agouti protein, e.g., with neutralizing antibodies, or (b) increasing the effective concentration of agonist by the administration of exogenous melanocortin peptide.

It should be noted that some melanocortin peptides, e.g., β-LPH, have been implicated in the activation of lipolysis in vitro (Chrétien, 1974). The physiological role of novel melanocortin receptors in energy metabolism, however, has not been established. The discovery of melanocortin analogs selective for particular members of the melanocortin receptor family, coupled with an analysis of their ability to modulate the phenotypic changes observed in A^{vy} and A^y mice, could, however, provide insight in this regard.

The current work indicates that melanocortin peptide and agouti bind to the MSH receptor in an apparently competitive manner. The lack of primary sequence similarity between agouti and the melanocortins makes it seem unlikely that both ligands occupy a common binding site. Nevertheless, the possibility of a common, or overlapping, binding site remains a formal possibility. Complicated models of receptor antagonism have not been considered since a simple, competitive model clearly accounts for the observed data, in that (a) the formation of significant levels of a ternary melanocortin−receptor−agouti complex was not observed and (b) agouti inhibition of both melanocortin binding and activity can be overcome by increasing the concentration of agonist used.

In summary, these findings indicate mutually exclusive occupation of agouti and melanocortin for their respective binding site(s) on $B_{16}F_{10}$ cells. The current work, however, does not allow one to distinguish between agouti binding directly to the 46 kDa MSH receptor polypeptide or to another, as yet unidentified, protein that constitutes part of a functional MSH receptor complex. Direct identification of the agouti binding site using labeled agouti will be necessary to differentiate between these two possibilities.

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