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Down-regulation of Melanocortin Receptor Signaling Mediated by the Amino Terminus of Agouti Protein in *Xenopus* Melanophores*

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Agouti protein and Agouti-related protein (Agrp) regulate pigmentation and body weight, respectively, by antagonizing melanocortin receptor signaling. A carboxyl-terminal fragment of Agouti protein, Ser^{73}-Cys^{131}, is sufficient for melanocortin receptor antagonism, but Western blot analysis of skin extracts reveals that the electrophoretic mobility of native Agouti protein corresponds to the mature full-length form, His^{23}-Cys^{131}. To investigate the potential role of the amino-terminal residues, we compared the function of full-length and carboxyl-terminal fragments of Agrp and Agouti protein in a sensitive bioassay based on pigment dispersion in *Xenopus* melanophores. We find that carboxyl-terminal Agouti protein, and all forms of Agrp tested, act solely by competitive antagonism of melanocortin action. However, full-length Agouti protein acts by an additional mechanism that is time- and temperature-dependent, depresses maximal levels of pigment dispersion, and is therefore likely to be mediated by receptor down-regulation. Apparent down-regulation is not observed for a mixture of amino-terminal and carboxyl-terminal fragments. We propose that the phenotypic effects of *Agouti in vivo* represent a bipartite mechanism: competitive antagonism of agonist binding by the carboxyl-terminal portion of Agouti protein and down-regulation of melanocortin receptor signaling by an unknown mechanism that requires residues in the amino terminus of the Agouti protein.

Studies of the mouse coat color *Agouti* gene have led to the identification of a novel pair of secreted signaling molecules which regulate mammalian pigmentation and body weight. *Agouti* encodes a 131-amino acid secreted protein that is expressed in the skin, where it induces the production of yellow pigment (pheomelanin) in hair follicle melanocytes (Refs. 1 and 2, and reviewed in Refs. 3 and 4). Agouti protein is secreted but has a small sphere of action; its localized expression is thought to give rise to characteristic white or yellow markings found in many different mammals such as chinchillas and the Doberman breed of domestic dogs (Ref. 5, and reviewed in Ref. 6). In the dominant mutations A^y and A^{vy} or in transgenic mice, ectopic expression of transcripts encoding Agouti protein results in yellow hair, obesity, hyperinsulinemia, and increased body length (Refs. 7–9, and reviewed in Refs. 4 and 10). The nonpigmentary effects of ectopic *Agouti* expression likely reflect the normal function of Agouti-related protein (Agrp),[1] a protein expressed in the hypothalamus and adrenal gland that is similar to Agouti protein in size, sequence, and biochemical activity (11, 12). Ubiquitous expression of *Agrp* transcripts causes obesity and increased body length but does not alter pigmentation (11, 13).

Agouti protein and Agrp act by antagonism of melanocortin receptors, a family of G-protein-coupled receptors responsive to endocrine peptides such as α-melanocyte stimulating hormone (α-MSH) and adrenocorticotrophic hormone (ACTH) (reviewed in Refs. 14 and 15). Genetic and biochemical studies indicate that Agouti protein alters pigmentation by antagonism of the melanocortin 1 receptor (Mc1r) expressed on melanocytes (16, 17), whereas Agrp affects body weight and length by antagonism of the Mc4r and/or Mc3r expressed in the hypothalamus and other regions of the central nervous system (11, 18–21). Most evidence suggests Agouti protein and Agrp act as competitive antagonists of melanocortin receptors (17, 22, 23), meaning that their effects are due solely to their ability to inhibit binding of melanocortin receptor agonists such as α-MSH. In agreement with this model, Agouti protein and α-MSH inhibit the binding of each other to the Mc1r (17, 24, 25). Additional findings, however, have suggested that Agouti protein and possibly Agrp inhibit melanocortin receptor signaling by mechanisms besides simple competitive antagonism of α-MSH binding (26–33). Using a sensitive bioassay based on α-MSH-induced pigment dispersion in *Xenopus* melanophores, we have previously shown that inhibition of melanocortin signaling by Agouti protein is increased significantly by preincubating melanophores in Agouti protein for several hours prior to the addition of α-MSH (25). This observation suggested that Agouti protein induces melanocortin receptor down-regulation in addition to its ability to inhibit α-MSH binding.

Our previous studies utilized a full-length (His^{23}-Cys^{131}) recombinant form of Agouti protein generated in a baculovirus expression system (11). Here we demonstrate that the related cysteine-rich carboxyl-terminal domains of Agouti protein and Agrp are sufficient for competitive antagonism in *Xenopus* melanophores. However, the amino-terminal residues of Agouti protein are required for additional effects likely due to down-regulation of melanocortin receptor signaling. To investigate whether Agouti protein was proteolytically cleaved to smaller forms *in vivo*, extracts of skin were examined by Western blot analysis using antisera that detected epitopes in both the

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[1] The abbreviations used are: Agrp, Agouti-related protein; Mcr, melanocortin receptor; PAGE, polyacrylamide gel electrophoresis; α-MSH, α-melanocyte stimulating hormone; Mcr, melanocortin receptor; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; PIPES, 1,4-piperazinediethanesulfonic acid; NDP, nucleoside diphosphate.

amino and carboxyl termini. We found little, if any, post-translational proteolysis of full-length (His^{23}-Cys^{131}) Agouti protein. These findings demonstrate that Agouti protein alters melanocortin signaling by two mechanisms mediated by distinct domains within the native protein.

EXPERIMENTAL PROCEDURES

Recombinant Proteins—Production and characterization of recombinant mouse Agouti protein and Agrp using the baculovirus system has been previously described (11, 25). In brief, cation followed by anion exchange chromatography resolves mouse Agouti protein to >99% purity as determined by analysis of silver-stained PAGE, mass spectroscopy, and amino-terminal sequencing. Blue-Sepharose (Amersham Pharmacia Biotech) followed by anion exchange chromatography resolves multiple forms of Agrp into two pools. One pool contains form A, a single species processed by signal peptidase cleavage after residue 20, in an approximately equimolar ratio with form B, a mixture of three species processed by cleavage after residues 46, 48, or 50, present in a 15:1:4 ratio, respectively. The second pool contains form C, a mixture of two species processed by cleavage after residues 69 or 71, present in a 1:4 ratio, respectively. Relative purity of Agrp preparations was estimated by scanning densitometry of a 10-μg sample loaded on a 10% Tricine gel stained with ProBlue (Integrated Separation Systems, MA) and was used to calculate 60% purity for forms A+B and 20% purity for form C.

Generation of Anti-mouse Agouti Antibodies—Polyclonal antibodies to full-length (His^{23}-Cys^{131}) mouse Agouti were generated in rabbits by standard procedures (37); 100–200 μg of protein at approximately 90% purity was used for each of five injections; antisera were recovered at day 70.

Following SDS-PAGE carried out under nonreducing conditions and Western blotting, the antiserum detects 1 ng/lane of the immunogen against which it was raised, as well as 1 ng/lane carboxyl-terminal (Ser^{73}-Cys^{131}) Agouti protein. Disulfide bond reduction of the antigen prior to SDS-PAGE slightly reduces sensitivity of the antiserum for detection of the full-length protein (<2-fold) but reduces sensitivity for detection of the carboxyl-terminal fragment by more than 100-fold. These findings suggest that the antiserum contains multiple antibodies: one or more directed at an epitope in the amino terminus and one or more that recognize an epitope in the cysteine-rich carboxyl terminus that requires proper disulfide bonding.

Extraction of Agouti Protein from Mouse Skin and Western Blotting—Sections of dorsal and ventral skin from 3-day old a^t/a^t mice were homogenized in ice-cold 1% Nonidet P-40 buffer (25 mM HEPES, pH 7.4, 50 mM NaCl, 10 mM EDTA) or high salt buffer (25 mM HEPES, pH 7.4, 750 mM NaCl, 10 mM EDTA) plus protease inhibitors (0.5 μg/ml leupeptin, 1 mM benzamidine) using a ground glass homogenizer. Following homogenization, samples were centrifuged in a microfuge for 25 min at 4 °C. Samples in 1% Nonidet P-40 buffer and a portion of the high salt extracts were added to an equal volume of 2× SDS loading buffer, boiled 5–10 min, and kept on ice. To concentrate and partially purify Agouti protein by cation exchange, 1 ml of each high salt buffer extract was added to 9 ml of 25 mM HEPES, pH 7.4, to bring the final [NaCl] to 75 mM. The diluted samples were rocked with 50 μl of a cation exchange resin (SP-Sepharose FF, Amersham Pharmacia Biotech) for 30 min at 4 °C. The resin was washed in batch mode with 25 mM HEPES, pH 7.4, 50 mM NaCl, 10 mM EDTA, 0.5 μg/ml leupeptin, 1 mM benzamidine and eluted with 25 mM HEPES, pH 7.4, 750 mM NaCl, 10 mM EDTA. Eluates were added to an equal volume of 2× SDS sample buffer, boiled, and loaded on an 18% polyacrylamide gel. Transfer and incubation with primary antisera at 1:1000 dilution were carried out according to standard methods (37). Secondary immunodetection was accomplished with a goat-anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA) and the ECL detection kit (Amersham Pharmacia Biotech).

Production and Purification of Carboxyl-terminal Agouti Protein (Ser^{73}-Cys^{131})—5 mg of recombinant mouse Agouti protein was dialyzed into 50 mM HEPES, pH 7.1, 50 mM NaCl, 5 mM $CaCl_2$, and incubated at 30 °C for 6 h with 5000 units/mg Kex-2 protease (38, 39), a generous gift from Dr. Michael Kay, Stanford University Department of Biochemistry. To resolve the products of cleavage, the sample was loaded at 800 μl/min onto a 1-ml HiTrap SP cation exchange column, washed with 5 ml of 50 mM Bicine, pH 9.0, 100 mM NaCl and then eluted with a 20-ml NaCl gradient (100–600 mM) in 50 mM Bicine, pH 9.0. Fractions (500 μl) were tested in the melanophore assay for α-MSH-inhibitory activity at a 1:200 dilution, and two peaks of activity were found to correspond to residual uncleaved full-length protein and the Ser^{73}-Cys^{131} fragment at >99% purity. Active fractions were pooled, dialyzed into 20 mM PIPES, pH 6.8, 50 mM NaCl, flash frozen, and stored at −70 °C. The data shown utilized a preparation of carboxyl-terminal Agouti protein that was made by cleaving a full-length hemagglutinin-tagged Agouti protein (25) (the hemagglutinin tag was inserted in the amino terminus of the Agouti protein and is therefore not present in the Ser^{73}-Cys^{131} fragment). Identical results were observed using a preparation of carboxyl-terminal Agouti protein made from recombinant Agouti protein His^{23}-Cys^{131}. To test the effects of the amino terminus in *trans,* a mixture of His^{23}-Arg^{70} or Arg^{72} and Ser^{73}-Cys^{131} was made by digesting recombinant mouse Agouti protein (99% purity) with Kex-2 as above. SDS-PAGE, mass spectroscopy, and amino-terminal sequencing revealed the presence of equimolar amounts of His^{23}-Arg^{70} or Arg^{72} and Ser^{73}-Cys^{131}, along with a minor amount (<2% of total protein) of uncut full-length Agouti protein.

Xenopus Melanophore Culture and Pigment Dispersion Assay—Xenopus melanophores were grown at 27 °C in 50% L-15 medium (Specialty Media, Lavallete, NJ), supplemented with 20% heat-inactivated fetal calf serum, 1 mM L-glutamine, penicillin, and streptomycin; the medium had been previously conditioned using *Xenopus* fibroblasts as described by Potenza and Lerner (48). The pigment dispersion assay developed by Potenza and Lerner (48) is based on the ability of agents that cause a decrease or increase in intracellular cAMP levels to produce a dose-dependent aggregation or dispersion, respectively, of intracellular pigment granules. Because pigment granules are neither fully aggregated nor dispersed in the absence of any drug, pretreatment of the cells with melatonin to aggregate pigment granules increases the range and sensitivity of the assay for detecting agents such as α-MSH that disperse pigment granules. For a typical assay, cells were plated 24–48 h beforehand in 96-well plates at 25,000 cells/well, washed briefly with 250 μl/well assay buffer (70% L-15 medium; 0.1% bovine serum albumin), and 40 μl/well assay buffer was then added, followed by 40 μl/well assay buffer that contained 2 nM melatonin (Sigma) to provide a final melatonin concentration of 1 nM. After a 45-min incubation to aggregate pigment granules, the optical density of each well was measured at 650 nm ($OD_{initial}$) to provide a base-line optical density reading. Test samples (Agouti protein, Agrp, or control buffer) were then added at 40 μl/well, followed by the addition of various concentrations of α-MSH or NDP-MSH at 40 μl/well. All additions were made in assay buffer supplemented with 1 nM melatonin to maintain a constant concentration of melatonin during the assay. Optical density at 650 nm was then determined at multiple time points from 30 to 420 min (OD_{final}). Unless stated otherwise, the entire assay was carried out at 22 °C in triplicate, and assay points represent the mean ± S.E. of the mean. A unitless parameter, degree of pigment dispersion, was calculated as described by Potenza and Lerner (48), $(OD_{final} - OD_{initial})/OD_{final}$, which creates an internal standard for each well ($OD_{initial}$) and scales the maximal degree of pigment dispersion to 1. The effects of 1 mM melatonin occasionally increase during the course of the assay, which gives rise to negative values for the degree of pigment dispersion. Optical density at 650 nM melanophores was measured with a V_{max} kinetic microplate reader (Molecular Devices, Menlo Park, CA) in end point mode, and data were transferred electronically to a Microsoft Excel spreadsheet for analysis. Graphing and curve fitting of dose-response curves were carried out with DeltaGraph (DeltaPoint, Monterey, CA), using a four-parameter logistic equation, $y = a + ((b - a)/(1 + (10^c/10^x)^d))$; where a = minimum, b = maximum, c = half-maximal value, and d = slope.

Schild Analysis of Dose-response Curves (40)—Dose ratios (the amount of α-MSH required for half-maximal response in the presence of Agouti protein/amount of α-MSH required for half-maximal response in the absence of Agouti protein) were determined using the four-parameter logistic equation described above. Within each experiment, individual slopes of the α-MSH dose-response curves were not significantly different and therefore were fixed at the average slope to provide the data for dose-ratio calculations. Schild plots [log(dose ratio − 1)] *versus* log([Ser^{73}-Cys^{131}Agouti or Agrp]) were created and analyzed in DeltaGraph (DeltaPoint, Monterey, CA). Linear curve fitting was used to determine the slope and the correlation coefficient. To estimate K_B, a second linear curve fitting was performed with the slope fixed at 1.

RESULTS

Agouti Protein Is Present in Mouse Skin in the Full-length Form—The similar genomic structure of *Agouti* and *Agrp* suggests evolution from a common ancestral gene, yet the sequence similarity of the two proteins is confined entirely to the carboxyl-terminal region (Fig. 1). Twenty-one of the final forty-

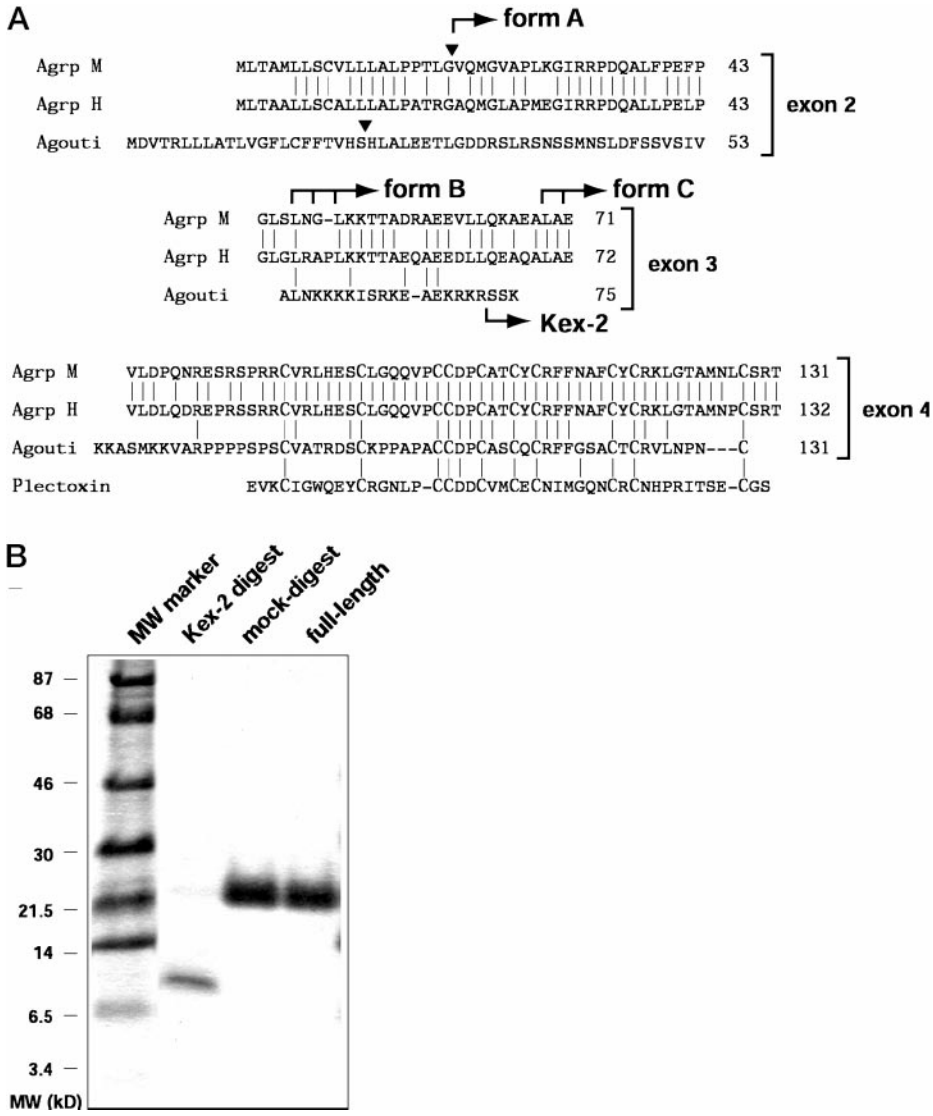

FIG. 1. **Alignment of mouse Agouti protein with mouse (*M*) and human (*H*) Agrp and production of carboxyl-terminal Ser^{73}-Cys^{131} mouse Agouti protein.** *A*, Agouti and Agrp have similar genomic structures, but sequence similarity is confined to the cysteine-rich carboxyl-terminal region; in the same region, plectoxin XI (35) shows a nearly identical pattern of cysteine spacing. *Arrowheads* mark signal sequence cleavage sites, and *arrows* designate peptide fragments used in this study. *Agrp* forms B and C are heterogeneous and likely to result from proteolysis during secretion and/or purification from conditioned media of baculovirus-infected cells as described under "Experimental Procedures." *B*, treatment of full-length Agouti protein with Kex-2 protease (*Kex-2 digest*) yields a mixture of amino-terminal and carboxyl-terminal fragments that comigrate at 7.5 kDa plus a small amount of residual uncleaved full-length protein. The conditions used for Kex-2 digestion are described under "Experimental Procedures"; full-length Agouti protein subjected to the same conditions in the absence of protease addition (*mock-digest*) shows no change in electrophoretic mobility.

seven residues in Agouti protein and Agrp are identical, of which ten residues are cysteines in a spacing similar to that found in conotoxins and plectoxins from the venoms of cone snails (34) and spiders (35), respectively. In addition, the amino terminus of the Agouti protein contains several paired basic residues that could serve as potential proteolytic cleavage sites (Fig. 1*A*). These observations suggested that the Agouti protein might be processed *in vivo* into an active fragment spanning the cysteine-rich region. In support of this hypothesis, proteolytic fragments containing the carboxyl terminus of either protein retain α-MSH-inhibitory activity (11, 23), and a short deletion (Arg^{64}-Lys^{77}) in the amino-terminal half of the mouse Agouti protein does not disrupt activity (36).

Recombinant mouse Agouti protein produced in insect cells is secreted as a mature form with the signal sequence removed (His^{23}-Cys^{131}) (17, 23, 25). However, recombinant proteins secreted by insect cells may undergo altered or incomplete post-translational processing (41, 42). To investigate whether the active form of Agouti protein in mouse skin underwent proteolytic cleavage *in vivo*, we raised polyclonal antibodies against full-length recombinant mouse Agouti protein (His^{23}-Cys^{131}). Western blotting experiments determined that the antiserum detects epitopes in both the amino- and carboxyl-terminal regions of Agouti protein with a sensitivity of <1 ng/lane provided that SDS-PAGE was carried out under nonreducing conditions (see "Experimental Procedures").

We used the anti-Agouti antiserum on nonreducing Western blots of dorsal and ventral skin extracts from 3-day-old black and tan (a^t/a^t) mice. These animals express *Agouti* transcripts in ventral, but not in dorsal skin (43), providing a control for antiserum specificity. As shown in Fig. 2, Agouti protein is detected in ventral, but not dorsal, a^t/a^t skin extracted with 1% Nonidet P-40 or a buffer containing 750 mM NaCl. In addition, we concentrated and partially purified Agouti protein from the 750 mM NaCl extracts using a cation exchange resin known to bind either full-length Agouti protein or the Ser^{73}-Cys^{131} carboxyl-terminal fragment (25). Agouti protein extracted from mouse skin has a mobility of 21.5 kDa on a nonreducing gel, similar to that of the full-length (His^{23}-Cys^{131}) recombinant form. The intensity of the 21.5-kDa band and the lack of any smaller fragments demonstrates that the full-length form predominates in mouse skin and indicates that Agouti protein undergoes little, if any, proteolytic processing *in vivo* after cleavage of the signal sequence.

Production and Analysis of an Active Carboxyl-terminal Agouti Protein Fragment—To investigate the potential function of the amino terminus of the Agouti protein, we used the *Xenopus* melanophore pigment dispersion assay to compare the activity of full-length Agouti protein and a carboxyl-terminal Agouti protein fragment. We produced a carboxyl-terminal fragment, Ser^{73}-Cys^{131}, by treating recombinant Agouti protein with Kex-2, a protease that cleaves specifically after paired Lys-Arg

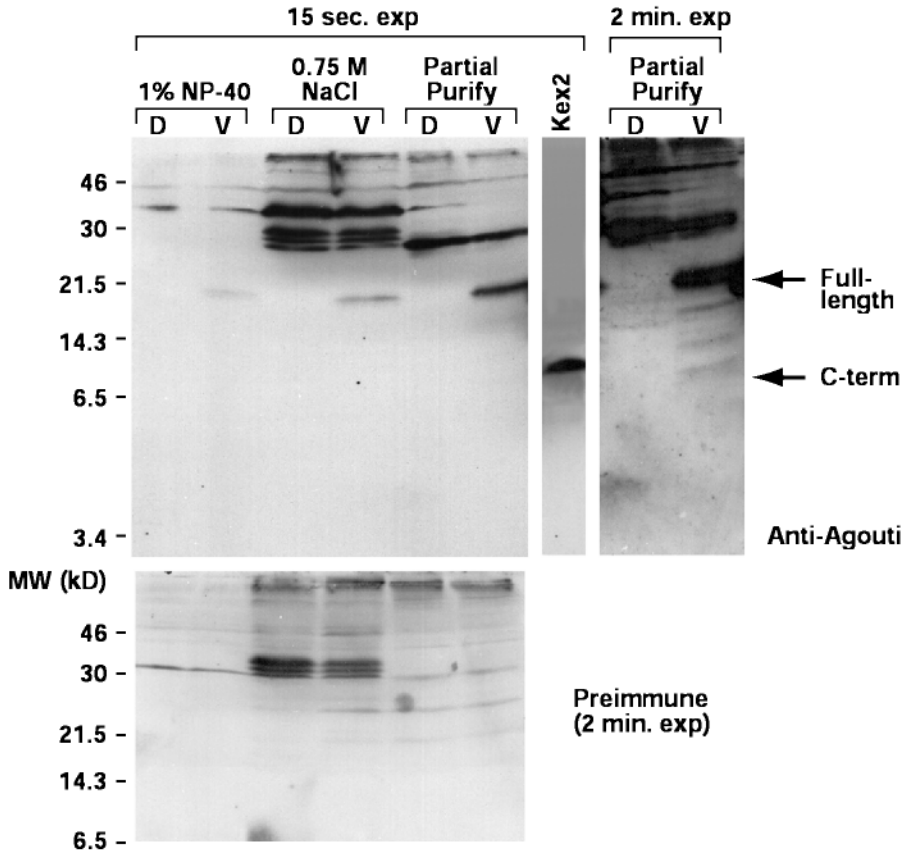

FIG. 2. **Detection of Agouti protein in skin extracts of black and tan (a^t/a^t) mice by Western blotting.** As described under "Experimental Procedures," dorsal and ventral skin samples from a^t/a^t mice were extracted in 1% Nonidet P-40 buffer or 750 mM NaCl buffer; a portion of the 750 mM NaCl extracts were concentrated and partially purified by cation exchange. Samples were separated by SDS-PAGE under nonreducing conditions and transferred to a nylon membrane. Agouti protein was detected using a polyclonal antisera and a chemiluminescent detection system as described under "Experimental Procedures." Exposure time, extraction buffer, and skin source (dorsal (*D*) or ventral (*V*)) are indicated *above each lane*. A 21.5-kDa band is detected in ventral, but not dorsal, skin extracts. Pre-immune serum taken from the rabbit prior to antibody production does not detect this band (*lower panel*). *Arrows* represent the sizes of full-length and carboxyl-terminal (Ser^{73}-Cys^{131}) Agouti protein run on the same gel.

residues (39). Two such sites are present at residues 69 and 70 and at 71 and 72 (Fig. 1*A*). Treatment of Agouti protein with Kex-2 produces a 48- or 50-residue amino-terminal fragment, His^{23}-Arg^{70} or Arg^{72}, and a 59-residue carboxyl-terminal fragment, Ser^{73}-Cys^{131}, that comigrate at 7.5 kDa on SDS-PAGE carried out under reducing conditions (Fig. 1*B*). Cation exchange chromatography resolved the carboxyl-terminal fragment at >99% purity (determined by amino-terminal sequencing, SDS-PAGE, and mass spectrometry) from both the amino-terminal fragment and a small amount of uncleaved full-length protein.

Like full-length Agouti protein, the carboxyl-terminal fragment inhibits α-MSH-induced pigment dispersion in *Xenopus* melanophores but has no effect in the absence of α-MSH or other melanocortin peptides (data not shown). By analyzing the effect of the two Agouti peptides on melanocortin receptor signaling over time, however, we discovered a difference in their activities. Addition of α-MSH to melanophores causes a rapid increase in pigment dispersion that reaches equilibrium in 15–30 min. When full-length Agouti protein is added simultaneously or immediately prior to α-MSH, a gradual inhibition of pigment dispersion ensues that increases for several hours (Fig. 3*A*). By contrast, under the same conditions, the carboxyl-terminal fragment of Agouti protein maximally inhibits the effects of α-MSH within 15–30 min, after which pigment dispersion gradually increases (Fig. 3*A*). As described previously (25) and as shown in Fig. 3*B*, these effects can be studied in more detail by an experimental protocol where melanophores are preincubated with Agouti protein alone for varying periods of time (during which there is no effect on pigment dispersion) and then exposed to α-MSH for a relatively brief and uniform period. Under these conditions, α-MSH dose-response curves display a progressive rightward shift as a function of time preincubated with full-length Agouti protein (Fig. 3*B*). By contrast, the same protocol carried out with Ser^{73}-Cys^{131} yields α-MSH dose-response curves that shift slightly to the left as a function of preincubation time (Fig. 3*B*). Thus, while the effectiveness of full-length Agouti protein increases during preincubation, the effectiveness of the carboxyl-terminal fragment decreases. A quantitative estimate of relative activity can be derived by comparing the α-MSH concentrations required for half-maximal stimulation of pigment dispersion under different sets of experimental conditions. Following a 10-min preincubation in 15 nM full-length Agouti protein, for example, 15 nM α-MSH is required for half-maximal stimulation of pigment dispersion. After a 420-min preincubation in 15 nM full-length Agouti protein, the amount of α-MSH required for half-maximal stimulation is increased 2-fold to 31 nM. A complete analysis for both Agouti peptides at various preincubation intervals is shown in Fig. 3*C* and reveals that a 420-min preincubation increases the effectiveness of full-length Agouti protein 2-fold while decreasing the effectiveness of the carboxyl-terminal fragment 1.5-fold.

The effect of preincubation on full-length Agouti protein and the carboxyl-terminal fragment could result from a change in the specific activity of the peptides in solution over time or from

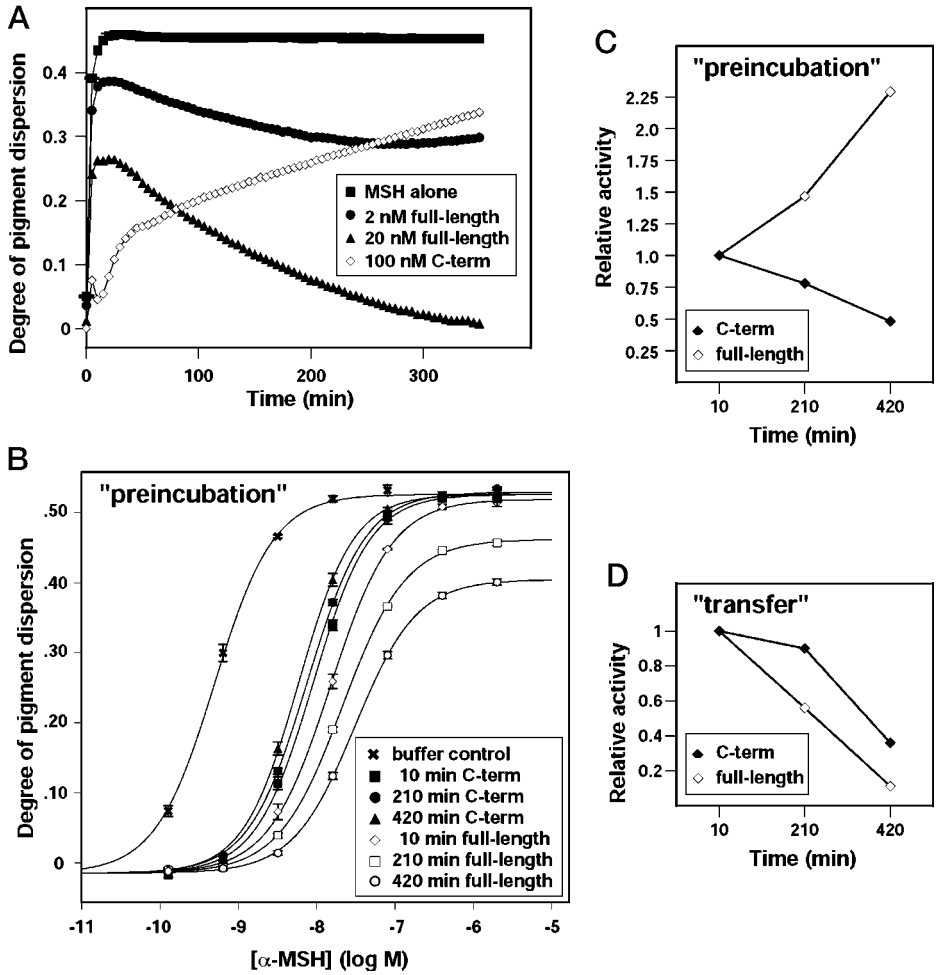

FIG. 3. **Kinetics of α-MSH inhibition by full-length and carboxyl-terminal Agouti protein.** *A*, the indicated concentrations of full-length or Ser^{73}-Cys^{131} Agouti protein were added to melanophores immediately prior to addition of 0.5, 2.0, or 8 nM α-MSH or a buffer control, and pigment dispersion was measured at 5-min intervals for 4 h. For clarity, only the results obtained with 2 nM α-MSH are shown, and *error bars* have been removed; the standard error of all assay points was <5%. Treatment with full-length or carboxyl-terminal Agouti protein in the absence of α-MSH had no effect on pigment dispersion. *B*, full-length or Ser^{73}-Cys^{131} (*C-term*) Agouti protein at a concentration of 15 nM or buffer control was added to melanophores for 10, 210, or 420 min, then α-MSH at the indicated concentrations was added to all assay wells simultaneously. Pigment dispersion was measured at 45–300 min; for clarity only the 50-min time point is shown. Also, different times of preincubation with buffer control had no effect on pigment dispersion, and for clarity, only the 210-min time point is shown. The α-MSH dose-response curves for full-length Agouti protein exhibit a progressive rightward shift and depression of maximal signaling as the time of preincubation with full-length Agouti protein increases; the same effect is not evident for carboxyl-terminal Agouti protein. *C* and *D*, relative activity of carboxyl-terminal or full-length Agouti protein was estimated by first calculating the dose ratio for individual α-MSH dose-response curves, defined as the concentration of α-MSH required for half-maximal pigment dispersion in the presence of Agouti protein, divided by the concentration of α-MSH required for half-maximal pigment dispersion in the absence of Agouti protein. The dose ratios for carboxyl-terminal and full-length Agouti protein after 210 or 420 min of preincubation were then compared with the corresponding dose ratios after 10 min of preincubation. Thus, relative activity corresponds to the degree to which individual α-MSH dose-response curves are shifted; the plot depicted in *panel C* corresponds to the data from *panel B*. In *panel D*, relative activity data are shown for carboxyl-terminal or full-length Agouti protein solutions that were first preincubated, then transferred to, and tested on, new melanophores during a 50-min incubation with various concentrations of α-MSH. This *transfer* data therefore reflects the specific activity of Agouti protein solutions over time, whereas the *preincubation* data reflects a combination of changes in specific activity and melanophore response.

a change in the response of melanophores to α-MSH following prolonged exposure to Agouti peptides. To distinguish between these possibilities, we asked how preincubation of full-length or carboxyl-terminal Agouti protein solutions on melanophores affected the activity of these solutions when they were transferred to and tested on a new plate of melanophores. As above, melanophores in a 96-well plate were preincubated in full-length or carboxyl-terminal Agouti protein for 10, 210, or 420 min. Prior to adding α-MSH, however, the solution in each well was transferred to a new plate of melanophores. Addition of α-MSH to this "transfer" plate revealed that full-length and carboxyl-terminal Agouti peptides in solution exhibit a parallel decline in their relative activity (Fig. 3*D*). In summary, preincubation of melanophores in full-length Agouti protein reduces their responsiveness to α-MSH despite the fact that relative activity of the Agouti protein solution declines. This paradoxical result is not observed with the carboxyl-terminal peptide and implicates amino-terminal residues of the protein in the preincubation effect.

Full-length Agouti Protein and the Carboxyl-terminal Ser^{73}-Cys^{131} Fragment Have Different Effects on the Slope and Maximum of the α-MSH Dose-response Curve in Melanophores—In previous studies (25), we have shown that the effects of full-length Agouti protein on melanophores differ from those of a competitive antagonist because, as above, its effectiveness increases during preincubation and because α-MSH dose-response curves carried out at different Agouti protein concentrations do not exhibit the same slope. Another characteristic of competitive antagonism is the ability of increasing concentrations of agonist to overcome the effects of antagonist regardless

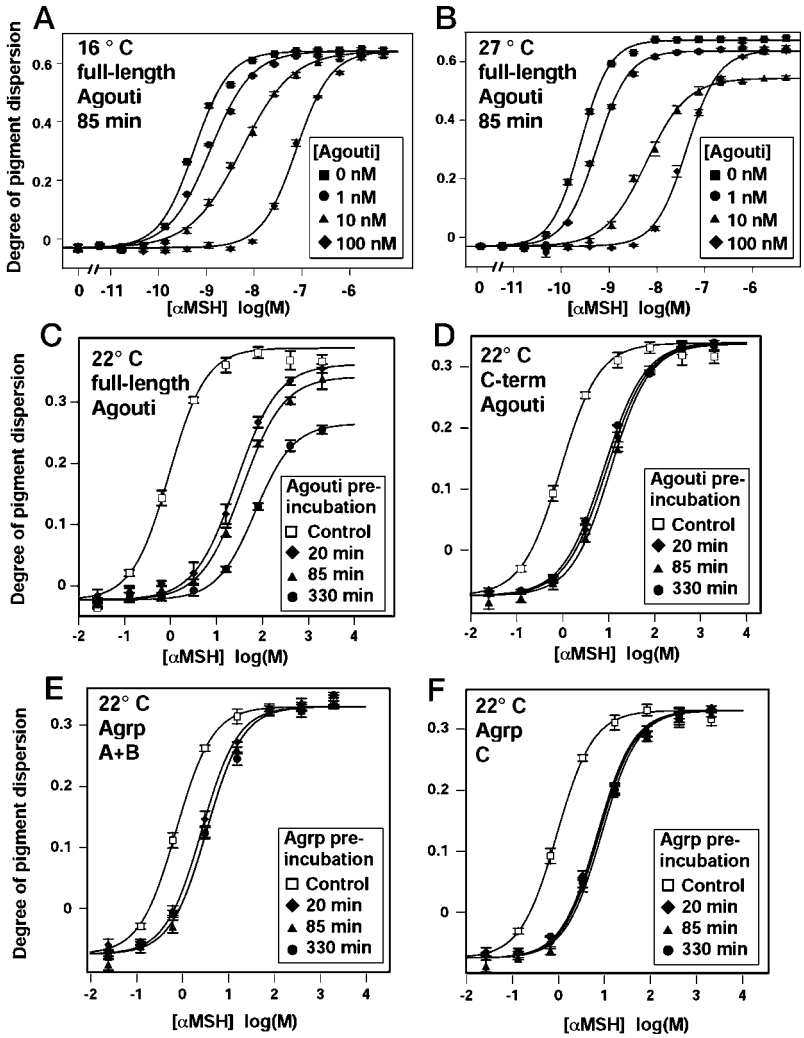

FIG. 4. **Effect of temperature and different forms of Agouti protein and Agrp on depression of α-MSH maximal signaling.** *A* and *B*, assays were carried out in parallel at 16 or 27 °C with the indicated concentrations of full-length Agouti protein added immediately prior to various concentrations of α-MSH, and pigment dispersion was measured from 45 min to 6 h later. For clarity, only results from the 85-min time point are shown, which reveal depression of α-MSH maximal signaling that depends on Agouti protein concentration at 27 °C but not 16 °C. *B*, at 85 min, a greater effect on depression of α-MSH maximal signaling is observed for 10 nM full-length Agouti protein than for 1 or 100 nM; as described under "Results," this effect varies according to the time of incubation and is eventually lost after 6 h. *C–F*, assays were carried out in parallel at 22 °C for full-length Agouti protein, Ser^{73}-Cys^{131} (*C-term*) Agouti protein, Agrp forms A + B, or Agrp form C, all at a concentration of 15 nM; each solution was preincubated for 20, 85, or 330 min, then various concentrations of α-MSH were added, and pigment dispersion was measured after 50 min. A buffer control was also included for each preincubation time point, but there was no effect on pigment dispersion so only the 85-min time point is shown for clarity.

of its concentration, *i.e.* high concentrations of antagonist should have no effect on maximal α-MSH signaling (40, 44). In the conditions used for our previous experiments, full-length Agouti protein did not affect the maximal level of pigment dispersion induced by α-MSH; however, we subsequently found that such an effect can be revealed by carrying out the melanophore assay at higher temperatures.

Fig. 4, *A* and *B*, depicts α-MSH dose-response curves carried out with different concentrations of full-length Agouti protein at 16 and 27 °C, respectively, where Agouti protein is added immediately prior to (<10 min) α-MSH. The slopes of individual dose-response curves vary at both temperatures, but an effect of Agouti protein on the maximal level of α-MSH-induced pigment dispersion is apparent only at 27 °C. A similar experiment with carboxyl-terminal Agouti protein at 27 °C shows no effect on the slope or maximal α-MSH signaling (Fig. 5*A*).

Depression of maximal α-MSH signaling is also observed when melanophores are preincubated with 15 nM full-length Agouti protein (Fig. 4*C*, also Fig. 3*B*), and the degree of depression is proportional to the time of preincubation. By contrast, a parallel experiment carried out with 15 nM carboxyl-terminal Agouti protein shows no effect on maximal α-MSH signaling (Fig. 4*D*). The difference between full-length and carboxyl-terminal Agouti proteins is not because of independent activity of the amino terminus because a mixture of carboxyl-terminal and amino-terminal Agouti protein (after Kex-2 cleavage) behaves identically to the purified carboxyl-terminal protein (data not shown).

Several hours after the addition of α-MSH, the effect of full-length Agouti protein on depression of maximal signaling is lost (data not shown). This raises the possibility that depression of maximal signaling might be because of failure to reach equilibrium between Agouti protein and α-MSH binding, *i.e.* a competitive antagonist with a very slow disassociation rate would fail to come to equilibrium with agonist binding and might effectively block the receptor even in the presence of high agonist concentrations. However, this is unlikely to explain our results because depression of maximal signaling is lost most rapidly at higher Agouti protein concentrations. For example, in the "snapshot" of pigment dispersion at 85 min after α-MSH

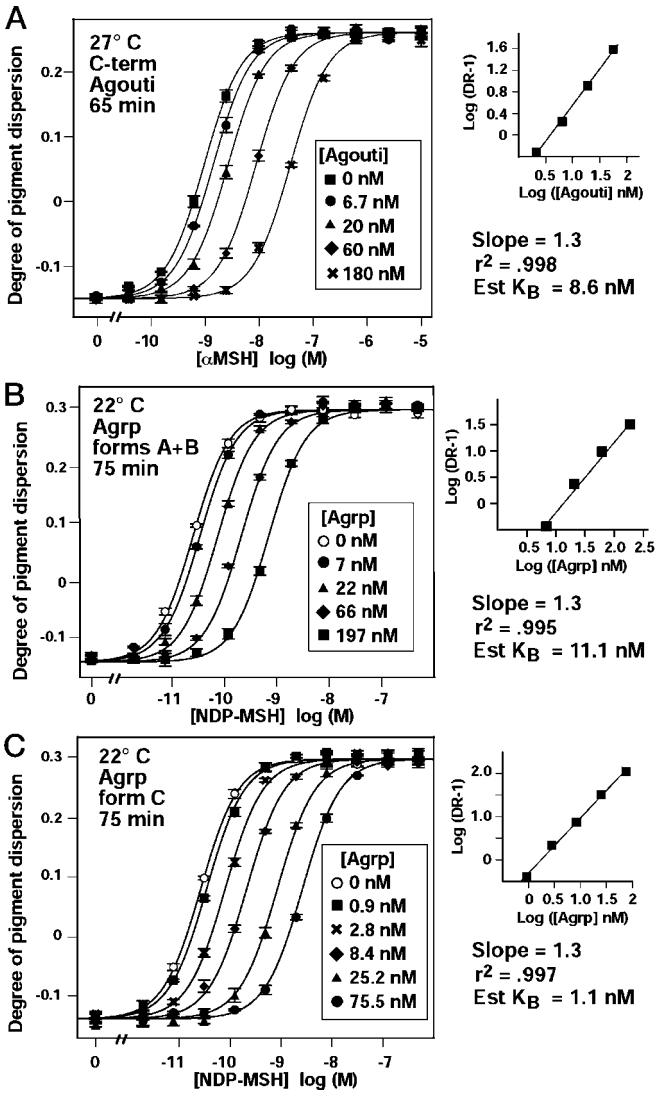

FIG. 5. **Schild analysis of Ser^{73}-Cys^{131} Agouti protein (*A*), Agrp forms A + B (*B*), and Agrp form C (*C*).** In all cases, various concentrations of α-MSH or NDP-MSH were added immediately after the indicated concentrations of antagonist. Data are shown for only a single incubation time as indicated; the effects of varying incubation time are reported in Table I. Note that Schild analysis only yields meaningful results when the shapes of individual agonist dose-response curves at different antagonist concentrations are completely parallel, which applies for the three antagonists shown here but not for full-length Agouti protein.

addition (Fig. 4*B*), depression of maximal α-MSH signaling is greater for 10 nM Agouti than for 100 nM Agouti; however, pigment dispersion measured at earlier times revealed a greater depression of maximal α-MSH signaling for 100 nM Agouti protein than for 10 nM Agouti protein (not shown). These observations indicate that the decrease in maximal α-MSH signaling induced by full-length Agouti protein is not because of a failure to reach equilibrium between agonist and antagonist binding. Instead, these effects are likely explained by an effect of full-length Agouti protein on receptor down-regulation that is saturable and reversible (see "Discussion").

The Ser^{73}-Cys^{131} Agouti Protein Fragment Acts as a Competitive Antagonist of Melanocortin Receptors in Xenopus Melanophores—As described above, the characteristics of full-length Agouti protein differ in several ways from those predicted for a competitive antagonist. However, the carboxyl-terminal fragment of Agouti protein meets all expectations for competitive antagonism. As depicted in Fig. 5*A*, increasing concentrations of carboxyl-terminal Agouti protein cause a progressive rightward shift in the α-MSH dose-response curve without altering the slopes of individual curves (*e.g.* compare with Fig. 4, *A* or *B*). An additional prediction for a competitive antagonist is that displacement of the agonist dose-response curve should be proportional to changes in antagonist concentration (40). These criteria are commonly analyzed by a Schild regression plot, log(dose ratio − 1) *versus* log[antagonist], which should yield a straight line with a slope near unity for competitive antagonists, and allows an estimation of K_B, the antagonist dissociation constant. A Schild regression for the data depicted in Fig. 5*A* is linear ($r^2 = 0.998$) with a slope = 1.3 and an estimated K_B of 8.6 nM.

Effects of Agrp on Melanophores—In parallel with our studies of full-length and carboxyl-terminal Agouti protein, we examined different forms of recombinant Agouti-related protein (Agrp). Agrp can be partially purified into two pools (11) (Fig. 1*A*). The first pool contains full-length Agrp with the signal sequence removed (Fig. 1*A*, *form A*) along with three fragments cleaved after residues 46, 48, and 50 (referred to collectively as "*form B*"). The second pool contains carboxyl-terminal fragments cleaved after residues 69 and 71 (referred to collectively as "*form C*"). We first investigated the kinetics of Agrp using a preincubation protocol as described above for Agouti protein. As shown in Fig. 4, *E* and *F*, preincubation does not increase the apparent effectiveness of 15 nM Agrp forms A + B or form C. Maximal α-MSH-induced pigment dispersion is also unchanged by treatment with Agrp. Thus, the different pools of Agrp, even the full-length protein "form A," exhibit pharmacologic characteristics consistent with competitive antagonism, similar to those displayed by carboxyl-terminal Agouti protein.

In previous studies with Agrp forms A + B, we found its effects on melanophores were consistent with competitive antagonism of melanocortin receptors, with Schild plots providing an estimated K_B of 6.9 nM for Agrp forms A + B. Fig. 5, *B* and *C*, depict similar experiments carried out with Agrp forms A + B, Agrp form C, and the potent melanocortin analog [Nle^4, D-Phe^7] α-MSH (NDP-MSH). At equilibrium, a competitive antagonist should yield similar Schild plots for agonists with similar binding sites, such as α-MSH and NDP-MSH. To assure analysis at equilibrium, data was obtained at several time points from 1–4 h after the addition of Agrp and α-MSH or NDP-MSH. Increased incubation time has little or no effect on the Schild plot and estimated K_B (Table I). The slope of the Schild plot remains slightly greater than unity in experiments carried out with NDP-MSH, possibly because this melanocortin analog dissociates very slowly from the receptor and may prevent complete equilibrium between agonist and antagonist binding. Regardless, the effects of all forms of Agrp conform to predictions for a competitive antagonist yielding estimated dissociation constants of 10.8 ± 1.5 nM for Agrp forms A + B and 1.1 ± .09 nM for Agrp form C (Table I).

DISCUSSION

The molecular mechanism by which Agouti protein and Agrp inhibit melanocortin signaling has been controversial. Many studies support competitive antagonism (17, 22, 23) in which all of the effects of Agouti protein and Agrp result from inhibition of agonist binding, whereas other findings suggest more complicated interactions of Agouti protein/Agrp and melanocortin receptors (25–28, 32, 33, 45, 46). Our studies in *Xenopus* melanophores indicate that Agouti protein has effects that appear to be a combination of two mechanisms: competitive antagonism of melanocortin receptors and down-regulation of melanocortin signaling. Removal of amino-terminal residues from Agouti protein, however, yields a peptide (Ser^{73}-Cys^{131}) that acts solely as a competitive antagonist of melanocortin

TABLE I
Schild analysis of Agrp[a]

Experiment	Antagonist	Agonist	Time	Slope[b]	K_B[c]
			min		*nM*
1	Agouti C-term	α-MSH	65	1.30	8.6
	Agouti C-term	α-MSH	120	1.20	8.6
2	Agrp A+B	α-MSH	95	1.02	6.9
	Agrp C	α-MSH	95	1.14	1.0
	Agrp C	α-MSH	195	1.06	0.9
3	Agrp A+B	NDP-MSH	75	1.30	11.1
	Agrp A+B	NDP-MSH	190	1.39	14.5
	Agrp C	NDP-MSH	75	1.27	1.1
	Agrp C	NDP-MSH	190	1.31	1.4
4	Agrp A+B	α-MSH	60	1.18	8.1
	Agrp A+B	α-MSH	240	1.09	6.9
	Agrp C	α-MSH	60	1.15	0.8
	Agrp C	α-MSH	130	1.10	0.8

[a] Assays were carried out at 22 °C except for experiment 1 which was carried out at 27 °C. Antagonist was added immediately prior to agonist, and pigment dispersion was measured at the indicated times. Data from experiment 1 at 65 min are depicted in Fig. 6*A*; data from experiment 3 at 75 min are depicted in Fig. 6, *B* and *C*). Dose-response curve at different antagonist concentrations were fitted individually to a sigmoid logistic; in each case, the minima, maxima, and slopes of individual dose-response curves were identical.
[b] In each case, a linear curve fit of the Schild regression plot, (log (Dose Ratio − 1)/log ([antagonist]), yielded a correlation coefficient, $r^2 > 0.99$.
[c] Values for mean estimated $K_B \pm$ S.E. are 10.8 ± 1.7 nM and 1.1 ± 0.09 nM for Agrp forms A+B and form C, respectively.

receptors, without the time- and temperature-dependent down-regulation of melanocortin signaling observed with full-length Agouti protein. Agrp also acts as a competitive antagonist of melanocortin receptors in the melanophore assay. Conservation between Agrp and Agouti protein is confined to the cysteine-rich regions in their carboxyl termini (11, 12), providing further evidence that this region is sufficient for competitive antagonism of melanocortin receptors, whereas residues unique to the amino terminus of Agouti protein are required for down-regulation of melanocortin receptor signaling in melanophores.

Schild Analysis and the Xenopus Melanophore Assay—Although there is no sequence similarity between Agouti protein and the 13-residue peptide α-MSH, each inhibits the binding of the other to melanocortin receptors (17, 25). To help distinguish between competitive antagonism (interaction with identical or neighboring sites such that binding of one ligand physically blocks binding of the other) and allosteric antagonism (interaction with nonoverlapping sites that induces reciprocal conformational changes in the receptor), we used Schild analysis to examine agonist dose-response curves carried out at different antagonist concentrations (40, 44). For a competitive antagonist, these curves should remain completely parallel and, in addition, should exhibit a progressive rightward displacement proportional to the change in antagonist concentration. Both criteria were fulfilled for the Ser^{73}-Cys^{131} fragment of Agouti protein and for all forms of Agrp tested, which suggests that the carboxyl-terminal fragments of these proteins contains a domain or subdomains that physically block α-MSH binding. A more detailed understanding of these interactions will probably require three-dimensional structural studies, but given the differences in size and probable tertiary structure between Agouti protein and α-MSH, individual subdomains within the cysteine-rich regions of Agouti protein and Agrp may mediate receptor binding separately from α-MSH competition. In this regard, Wilkison and colleagues (47) have recently demonstrated that substitution of alanine for Arg^{116} and Phe^{118} causes large decreases in the ability of Agouti protein to inhibit α-MSH binding, and it will be interesting to determine the effect of these substitutions on binding of Agouti protein to melanocortin receptors.

Our results are based on the endogenous melanocortin receptor of *Xenopus* melanophores, which exhibits agonist and antagonist profiles in between those of the mammalian Mc1r and Mc4r (48, 49), but whose sequence is not known. Compared with most assays for G_s-coupled receptors in mammalian cells, a principle advantage of the melanophore assay is the ability to examine multiple time points to assure that our analyses are performed at equilibrium. In addition, because hundreds of individual assays can be carried out simultaneously, the shapes of individual dose-response curves can be determined and compared with a precision typically not approached by most mammalian assay systems.

A Unique Role for the Amino Terminus of Agouti Protein—Unlike the Ser^{73}-Cys^{131} fragment, full-length Agouti protein exhibits several pharmacologic characteristics that are inconsistent with competitive antagonism, including a time- and temperature-dependent potentiation of α-MSH inhibition, agonist dose-response curves that exhibit different slopes depending on antagonist concentration, and depression of maximal α-MSH signaling. Although depression of maximal α-MSH signaling might be explained by a very slow antagonist disassociation rate in some systems, it is unlikely to account for our results because it was only observed at temperatures 22 °C or above, and is eventually lost at a rate proportional to Agouti protein concentration. Taken together, these observations suggest that full-length Agouti protein has a direct effect on melanocortin receptor down-regulation distinct from of its ability to block α-MSH binding. By analogy to mammalian systems, down-regulation could be mediated by receptor internalization or post-translational modifications such as phosphorylation or palmitoylation that alter the ability of the receptor to couple to second messenger systems (reviewed in Ref. 50). Both types of mechanism are likely to be temperature-sensitive and occur over a time course longer than that required for antagonist binding. In this regard, Siegrist *et al.* (27) have reported that treatment of B16 melanoma cells with Agouti protein causes a loss of α-MSH binding sites on the cell surface. Regardless of the exact mechanism, the differences we observe between full-length and carboxyl-terminal Agouti protein demonstrate a unique role for a domain or domains in the amino terminus of Agouti protein. Furthermore, arguments based on evolutionary conservation, genomic structure, and secondary structure predictions suggest that the amino terminus and carboxyl terminus of Agouti protein constitute distinct protein modules. However, the amino terminus of Agouti protein cannot act in *trans*, *i.e.* full-length Agouti protein digested with Kex-2 protease behaves identically to the purified carboxyl-terminal fragment (data not shown); therefore effects of the amino terminus are likely to require high affinity binding to melanocortin receptors

mediated by a domain or domains in the carboxyl-terminal region.

Results from other groups also support a role for the amino terminus of Agouti protein. Kiefer *et al.* (52) have shown that deletion of Lys^{57}-Arg^{85} impairs the ability of Agouti protein to inhibit α-MSH binding and adenylate cyclase activation (a 5–15-fold increase in apparent K_i). Site-directed mutagenesis has also revealed amino acids outside of the cysteine-rich region which are important for Agouti protein activity, including Val^{83}, Arg^{85}, Pro^{86}, Pro^{89}, and the glycosylation site Asn^{39} (36, 52). None of these residues are conserved in Agrp, which suggests that they are not crucial for antagonist binding and instead may play a role in protein folding and/or stability or, as described here, a signaling role distinct from inhibition of α-MSH binding.

In pharmacologic studies apparently at odds with those described here, Willard *et al.* (23) reported that a Val^{83}-Cys^{131} carboxyl-terminal Agouti protein fragment was identical to full-length Agouti protein in its ability to inhibit melanocortin binding and adenylate cyclase activation in B16 melanoma cells. In addition, dose-response analysis of full-length Agouti protein in melanoma cells was consistent with competitive antagonism (22). It is possible that the noncompetitive actions of full-length Agouti protein we have described are unique to melanophores; alternatively, the increased accuracy and sensitivity of the melanophore assay may reveal aspects of Agouti protein function that are difficult to detect in some mammalian cell culture systems. *In vivo*, the pigmentary effects of Agouti protein differ from those caused by deficiency for the Mc1r (25), which points to a mechanism or mechanisms of action other than simple competitive antagonism. Regardless, our results demonstrate that Agouti protein normally produced *in vivo* contains the amino-terminal portion largely intact and highlights a potential role for these residues in its biologic function.

Agouti Protein and Agrp Similarities and Differences—The pharmacologic behavior of Ser^{73}-Cys^{131} Agouti protein is very similar to that displayed by a carboxyl-terminal fragment of Agrp (form C); both ligands act as simple competitive antagonists. Although Agrp form C is approximately 10-fold more potent than Ser^{73}-Cys^{131} Agouti protein in the melanophore assay, estimated antagonist dissociation constants for both ligands are in the nanomolar range, which lends further support to the notion that the domain or domains required for melanocortin receptor binding are contained entirely within the cysteine-rich carboxyl-terminal fragments of Agouti protein or Agrp and implies that these domains have been conserved since divergence from a common ancestor. Orthologs for Agouti protein and Agrp have been found in all mammals examined (12, 33, 53), but there is, as yet, no molecular evidence for endogenous melanocortin receptor antagonists in nonmammalian vertebrates. Primary sequence similarity at the level of cysteine spacing between Agouti protein and Agrp is also shared by several of the plectoxins (Fig. 1*A*) and, to a lesser extent, ω-conotoxins, which suggests the possibility of a more distantly related common ancestor. Calcium channels are the direct targets for most of the ω-conotoxins (reviewed in Ref. 34), but the same is unlikely to be true for Agouti protein because it binds the Mc1r and its pigmentary effects are completely blocked by deficiency for the Mc1r (25). Nonetheless, an ancestral pattern of protein folding common to the endogenous melanocortin receptor antagonists and invertebrate toxins may serve as a scaffold for interaction with a diverse group of cell surface proteins, and binding of the Mc1r by Agouti protein may lead indirectly to changes in calcium metabolism that have been reported in certain cell culture systems.

In melanophores, longer forms of Agrp (A + B) are approximately 10-fold less potent than form C but, unlike full-length Agouti protein, still display pharmacologic behavior expected for a competitive antagonist. This difference is unlikely to be because of impurities in the preparations because full-length Agouti protein is >99% pure. Effects of the Agrp amino terminus on α-MSH signaling analogous to those displayed by Agouti protein might be apparent in other assay systems and are consistent with our earlier studies (25) in which Agrp forms A+B were found to depress basal levels of intracellular cAMP and α-MSH maximal signaling in Mc4r-expressing cells.

Concluding Remarks—The Mc1r and Mc4r play important roles in the regulation of pigmentation and body weight, respectively (16, 19), but signaling through these receptors seems to be controlled primarily by alterations in Agouti protein or Agrp rather than melanocortin peptides. For example, in altered metabolic states induced by fasting or leptin deficiency, changes in the levels of transcripts encoding α-MSH (Pomc) are relatively modest, 1.5- to 2-fold (54, 55) compared with 10- to 12-fold alterations in Agrp (11, 12). Similarly, altered expression of Agouti protein rather than Pomc is the primary determinant of the balance between pheomelanin and eumelanin synthesis in pigment cells (56, 57). In part, regulation of melanocortin receptor signaling by Agouti or Agrp offers more precise temporal and spatial control than would be possible by altering levels of circulating melanocortin peptides. In addition, the ability of Agouti protein to signal via two separate mechanisms may allow a wider phenotypic range than would be possible by varying levels of α-MSH. As described here, these mechanisms require separate regions of the Agouti protein: the similar cysteine-rich regions of Agouti protein and Agrp are sufficient for competitive antagonism, while amino-terminal residues in Agouti protein are necessary for the time- and temperature-dependent down-regulation of melanocortin receptor signaling we have observed. We anticipate that studies examining receptor trafficking and post-translational receptor modifications will provide additional insight into the underlying biochemical and cell biologic mechanisms and may lead to a deeper understanding of Agouti protein and Agrp signaling in several physiologic processes.

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