Minireview

Agouti and Agouti-related Protein: Analogies and Contrasts*

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The discovery a decade ago of the murine agouti gene was intended to bring scientists a step closer to understanding the complexities of mammalian pigmentation. The first obesity gene was also uncovered in the process. What followed was an explosion of major discoveries in murine as well as human obesity and diabetes research. Recently, a new gene, the agouti-related protein (AGRP), was discovered and found to share a striking similarity in structure and function with agouti, although their patterns of distribution are completely different. Identification of a hypothalamic melanocortin receptor, MC4-R, together with AGRP as central components of feeding behavior and metabolism has helped build a picture, albeit incomplete, of the neuronal pathways involved in energy homeostasis. This review will compare and contrast Agouti and AGRP structure and function and gene regulation and their interaction with melanocortin receptors (MC1-R and MC4-R) and suppressors (mahogany/mahoganoid).

Agouti and Extension, a Brief History

agouti and extension were first described several decades ago (1, 2) as the genetic loci that control the relative amount and distribution of eumelanin (brown/black) and phaeomelanin (red/yellow) pigments in the mammalian coat. extension encodes a member (MC1-R) (3) of the melanocortin receptors, a family of G_α-coupled receptors, of which five isoforms are presently known (reviewed in Ref. 4). MC1-R is the melanocyte-stimulating hormone receptor expressed in melanocytes and has a physiological role in pigmentation (5). MC4-R is expressed mainly in the brain (6, 7) and has been implicated in the regulation of feeding behavior and metabolism (8). MC3-R is found primarily in the hypothalamic and limbic systems (9); however, no definitive function has been assigned to MC3-R as yet. Melanocortins such as α-melanocyte-stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH) are the natural ligands for this family of receptors (α-MSH for MC1-, MC3-, MC4-, and MC5-R and ACTH for MC2-R). Melanocortins are cleaved from a larger polypeptide precursor termed pro-opiomelanocortin that is produced in the pituitary gland, hypothalamus, brainstem, and peripheral sites such as skin.

Agouti is a paracrine-signaling factor that is secreted by dermal papillae cells, adjacent to melanocytes, and acts within the hair follicle microenvironment to block melanocortin action at the MC1-R (10, 11). Binding of α-MSH to the receptor triggers elevation of cAMP levels and activation of tyrosinase, the rate-limiting enzyme of melanogenesis, and results in eumelanin production. In the presence of Agouti the opposite is true; eumelanin synthesis is shut down and the default pathway that has phaeomelanin as the final product is activated. There are now more than 20 dominant, recessive, and pseudogouti alleles that have been identified in rodent, fox, and cattle, with interesting functional variations from species to species. In rodents agouti is expressed in skin only. In humans, however, agouti has a wider pattern of distribution, being expressed in adipose tissue, testis, ovary, heart, and at lower levels in foreskin, kidney, and liver (12, 13). Agouti does not appear to play any role in human pigmentation, and its exact biological function in humans remains unknown. Most of our knowledge of Agouti structure and function comes from studies performed on rodents. This review will therefore focus on murine Agouti and AGRP.

Regulation of Agouti Gene Expression

The genomic organization of the murine agouti gene is complex. It consists of three coding exons designated as 2, 3, and 4, as well as four non-coding exons, 1A, A’, B, and C, located upstream (11, 14). agouti expression is regulated in mice in a regional and temporal manner to create a differential distribution of yellow and black pigment in individual hair shafts and throughout the coat (11). This intricate pattern of gene regulation is achieved through the existence of alternatively spliced agouti transcripts that differ in their 5′-untranslated exons and are controlled by two different sets of control elements. Type I transcripts contain non-coding exons 1B or 1C and are regulated by temporal (hair cycle-specific) elements (14). Gene expression of transcript I is restricted to the midphase of the hair-growing cycle and is associated phenotypically with subapical yellow banded hairs throughout the body (14). In contrast, type II transcripts contain non-coding exons 1A and 1A’ and are under the control of regional (ventral-specific) promoter elements (14). The second class of transcripts is responsible phenotypically for the lighter ventral pigmentation seen in several agouti strains (i.e. white-bellied agouti) (14).

Agouti Structure and Function

Despite its genetic complexity the agouti locus encodes a small protein of 131 amino acids. Agouti displays the structural characteristics of a secreted protein having a hydrophobic signal sequence and lacking any transmembrane domains. The prominent structural features of the mature protein are a highly basic N-terminal region, a Pro-rich central domain, and a C-terminal domain rich in Cys residues (11). Biochemical analysis of the Agouti protein shows that it is highly glycosylated and very stable to thermal denaturation (15). The spacing pattern of the 10 Cys residues present in the C terminus is reminiscent of cone snail (conotoxins) and spider toxins (plectoxines), suggesting a conserved three-dimensional motif. Based on this structural similarity it has been postulated that all Cys residues of the Agouti protein are engaged in disulfide bonds (15).

In vitro studies using recombinant mouse Agouti protein prove that Agouti is a potent melanocortin antagonist (nanomolar range) at MC-R subtypes 1 (K_I(app) = 2.6 ± 0.8 nM) and 4 (K_I(app) = 54 ± 18 nM), a relatively weak antagonist at MC3-R (K_I(app) = 190 ± 74 nM), and a very weak antagonist at MC5-R (K_I(app) = 12,000 ± 340 nM) (10, 15, 16). Pharmacological studies of murine Agouti conclude that its mechanism of action is a classical competitive antagonism of melanocortin receptors (10, 15, 16). In addition, a shorter version of Agouti, residues 83–131, is shown to be as potent an antagonist as the full-length protein (15). The Cys-rich C terminus is therefore deemed sufficient for effective antagonism of melanocortin action in vitro as well as in vivo (15, 17). The basic domain, on the other hand, appears to play key roles in Agouti biogenesis (i.e. protein folding, post-translational processing, sorting, and secretion) and/or in facilitating the interaction with the receptor (18).

The mechanism of Agouti action shows interesting variations across species. Functional analysis of recombinant Agouti-signaling protein (ASIP), the human homologue of murine Agouti, indicates a similar pharmacological profile; ASIP is a potent antagonist at human MC1 (K_I(app) = 0.47 ± 0.06 nM) and MC4-R (K_I(app) = 0.14 ± 0.02 nM) and a relatively weak antagonist at MC3 (K_I(app) = 0.06 ± 0.02 nM). ASIP can act as a competitive antagonist at MC1-R in a hair follicle microenvironment and as an antagonist at MC4-R within the hypothalamus, suggesting a differential mechanism of Agouti and ASIP action.
Minireview: Agouti and Agouti-related Proteins

Once the agouti gene was cloned it became possible to address the molecular basis of A", a dominant allele at the agouti locus, and its pleiotropic effects. In doing so scientists were able to reveal a much more complex picture of Agouti function than previously thought. It soon became clear that Agouti and its homologues are part of a general signaling system that extends far beyond the hair follicle and the melanogenesis process. Lethal yellow (A") was identified at the turn of the century (21). Animals heterozygous for the A" allele are not only characterized by a yellow coat color but also by late onset obesity associated with hyperphagia, increased linear growth, and non-insulin-dependent diabetes as well as an increased propensity for developing tumors (reviewed in Ref. 22).

Genetic analysis shows that A" is in fact the result of a chromosomal rearrangement in which the promoter and the first non-coding exon of a closely linked gene, Raly, get spliced to exons of the wild-type agouti gene (23). The agouti gene, now under the control of the relaxed Raly promoter and devoid of temporal and regional restrictions, becomes ectopically expressed. Overexpression of Agouti in multiple tissues is therefore the cause of the A" phenotype. This conclusion is further supported by the ability of agouti, under the control of a β-actin promoter, to recapitulate the A" phenotype in transgenic animals (24).

Pharmacological characterization of murine Agouti makes it now easy to understand why ectopic Agouti expression per se is responsible for the A" phenotype. Chronic antagonism of the cutaneous MC1-R by Agouti results in yellow fur whereas Agouti competition at the hypothalamic MC4-R results in obesity. This conclusion is supported by most of the experimental data available to date. The most compelling evidence are the recent findings that targeted disruption of MC4-R signaling results in knockout mice (MC4-R KO) with a phenotype similar to the MC4-R KO with a phenotype similar to the MC4-R KO (19). By contrast, genetic analysis of fox agouti and extension variants suggests that Agouti functions in this case as a negative antagonist (inverse agonist) of MC1-R rather than a classical competitive antagonist (20). Unlike the fox, in the mouse extension is epistatic to agouti, which means that constitutively active receptors encoded by dominant extension alleles cannot be blocked by Agouti action.

Parallel between Agouti and AGRP

Two lines of evidence suggested the existence in the brain of an “Agouti-like” protein that would block signaling at central melanocortin receptors, MC3-R and MC4-R. First, in vitro pharmacology studies found that Agouti was a highly specific MC4-R antagonist even though it was normally expressed only in hair follicles (10,15,16). Second, central administration of synthetic MC3-R and MC4-R antagonists uncovered a functional role for melanocortin antagonists in vivo, namely stimulation of feeding behavior (25).

The agouti-related protein (AGRP) gene, was isolated in 1997 based on its homology to Agouti (29,30). Like agouti, AGRP contains three coding exons; however, depending on the site of expression (central versus peripheral) AGRP may or may not contain an upstream non-coding exon (29). The genomic organization of the coding exons is similar between agouti and AGRP despite differences in intron/exon junctions (29).

Both Agouti and AGRP are 131-amino acid proteins with putative signal peptide sequences and Cys-rich C-terminal domains (Fig. 1). Unlike Agouti, AGRP lacks the large number of basic residues in the N-terminal region and the Pro-rich central domain (29,30). The strongest homology between the two proteins is within the poly-Cys domain of the C terminus (Fig. 2). Both Agouti and AGRP contain 10 Cys residues, 9 of which are spatially conserved (29,30). Like Agouti, all 10 of the AGRP Cys residues form disulfide bridges (15,31) that are essential for their structural stability and biological function. Biochemical studies indicate also that AGRP is very stable to thermal denaturation (similar to Agouti) as well as acid degradation (32). In addition, the biophysical characterization of Agouti and AGRP shows similar CD spectra for the two proteins, their secondary structure consisting of mainly random coils and β-sheets (15,32).

Both Agouti and AGRP are competitive antagonists of α-MSH action at melanocortin receptors (32–34). Likewise, the C terminus of AGRP, residues 83–131, retains the biological activity of the full-length protein in vitro (34) as well as in vivo (35). In contrast to Agouti, AGRP is equally potent in inhibiting signaling at the central melanocortin receptors, MC3-R and MC4-R (binding affinity of human AGRP close to 1 nM for both receptors), very little inhibition is detected at the MC5-R, and virtually no activity is detected at MC1-R (32–34). Therefore, AGRP is as potent an antagonist at MC4-R as Agouti and a much stronger antagonist at MC3-R.

The tissue distribution of Agouti and AGRP differs greatly. The expression of agouti is normally confined to hair follicles whereas AGRP is expressed primarily in the hypothalamus, adrenal medulla, and at low levels in testis, lung, and kidney (29,30). Unlike Agouti, the localization pattern of human and murine AGRP is strikingly alike (29), indicating similar roles for AGRP in both species. Brain expression of AGRP mRNA is confined to neuronal cell bodies localized in the arcuate nucleus of the hypothalamus (36). These neurons are shown to project to hypothalamic nuclei that receive dense pro-opiomelanocortin innervation and express the two central melanocortin receptors, MC3-R and MC4-R (36). The potency of AGRP action at MC3-R and MC4-R together with regulation of calcium channels by Agouti seems less likely. It is still possible, however, that Agouti binding to MC1-R results in modulation of calcium metabolism via an indirect mechanism.

FIG. 1. Protein sequence alignment of murine Agouti and AGRP. Identical amino acid residues are shown in white letters (lower case) and conserved amino acids are indicated by arrows.

Key points:
- Agouti is a protein that is involved in the regulation of melanogenesis and is expressed in hair follicles.
- Lethal yellow syndrome is caused by the expression of Agouti in multiple tissues due to a genetic defect.
- AGRP is a protein that competes with Agouti for melanocortin receptors, particularly MC4-R.
- Agouti and AGRP share similar structures and functions, with AGRP being more widespread in the body.
- The tissue distribution of Agouti and AGRP differs, with Agouti being more localized and AGRP being more widespread.
their similar distribution pattern suggest that AGRP and not Agouti controls their function in vivo. Homeostatic regulation of the central melanocortin system may well be achieved through changes in antagonist (AGRP) rather than agonist (α-MSH) bioavailability. Thus, AGRP may control the activity of central melanocortin receptors in a manner similar to modulation of MC1-R signaling by Agouti in the skin.

The presence of AGRP-immunoreactive fibers in a subset of hypothalamic nuclei (i.e. arcuate, paraventricular, dorsomedial) strongly suggests a key role for AGRP and the melanocortin system in the regulation of energy homeostasis. This conclusion is supported by multiple findings. First, central administration of AGRP is shown to mimic the effect of synthetic MC3-R and MC4-R antagonists and stimulate feeding (35). In addition, AGRP is able to specifically block the reduction in food intake elicited by administration of α-MSH (25). Second, overexpression of AGRP in transgenic animals results in an obesity phenotype strikingly similar to that of the MC4-R KO or A+ mice (30, 37). In conclusion, melanocortinergic neurons exert a tonic inhibition on feeding behavior and metabolism. This tonic inhibition is relaxed following AGRP antagonism at MC4-R and results in stimulation of caloric intake and energy storage.

**Fine-tuning the Mechanism of Agouti/AGRP Action**

The most accepted mechanism for Agouti/AGRP action is a classical competitive antagonism of melanocortin receptors. However, several additional mechanisms have been proposed, including inverse agonism (20) and direct regulation of calcium channels (26). Recent studies, however, were able to eliminate some of the controversies surrounding the exact biochemical mechanism of Agouti/AGRP action. Ollmann et al. (28) clearly demonstrate that MC1-R is indeed an Agouti receptor and that the presence of a functional MC1-R is absolutely necessary for Agouti function in vivo. Furthermore, AGRP is shown to bind solely to melanocortin receptors in both conventional binding and photoemulsion assays (34). The two studies indicate that AGRP/Agouti and α-MSH bind to melanocortin receptors in a mutually exclusive manner (28, 34). Finally, direct binding of AGRP to the MC4-R has been recently demonstrated by protein cross-linking (38).

It is not clear, however, if the agonist and antagonist compete for the same binding site on the receptor. At first glance, no amino acid sequence similarity between Agouti/AGRP and α-MSH seems obvious. Careful comparison of Agouti and AGRP C-terminal sequences reveals, however, the presence of a conserved RFF motif that resembles the α-MSH pharmacophore HFRW (39). A loop of 8 residues flanked by two Cys residues and including the RFF triplet (AGRP residues 110–117 and Agouti residues 115–122, respectively) is shown to be critical for both Agouti and AGRP antagonism at melanocortin receptors (39). Furthermore, Ala scanning mutagenesis studies indicate that the RFF motif is the most critical in determining antagonist activity (IC₅₀ = 0.5 ± 0.1 nM for AGRP binding to MC4-R whereas IC₅₀ for the three Ala mutants are: 67 ± 46 nM for R111A, 61 ± 35 nM for F112A, and 25 ± 13 nM for F113A, respectively) (39). The octapeptide loop of the antagonist is therefore proposed to mimic the conformation of α-MSH and interact with the receptor through a similar mechanism (39). This model would thus imply that the agonist and antagonist occupy the same binding site on the receptor. An alternative model suggests that the antagonist attaches itself to a different receptor site and blocks ligand binding through an allosteric mechanism. In support of this model it was recently shown that the extracellular loops 2 and 3 of the MC4-R are critical sites for antagonist (AGRP) binding but had little effect on agonist (α-MSH) binding (40).

Although Agouti and AGRP have been shown to share structural and functional similarities, an intriguing difference in their mechanism of action has been recently reported (41). According to this recent report, full-length Agouti, but not AGRP, is characterized by a dual mechanism at the Xenopus MC1-R: competitive antagonism of ligand binding by the C terminus and receptor down-regulation elicited by the N-terminal domain (41). Depression of melanocortin receptor signaling is achieved as a result of Agouti action either by receptor internalization or post-translational modification (41). The results of this study are, however, in disagreement with previous reports that showed identical pharmacological profiles for both full-length and C-terminal Agouti (15). It is very likely that the conflicting results reflect in fact the properties of the different cell culture systems used: Xenopus melanophores (41) and marmalian B16 melanoma cells (15). It may be that the pharmacological properties described for the N-terminal Agouti domain are in fact specific only to the Xenopus MC1-R.

**Mahogany and Mahoganoid-specific Agouti/AGRP Suppressors**

Two autosomal mutations, mahogany (mg) and mahoganoid (md), were identified several decades ago as natural suppressors of Agouti action; both mutations were able to shift melanogenesis from phaeomelanin to eumelanin synthesis (42, 43). It was only in the last year, however, that a clear picture of Mahogany structure and function emerged (44–47). Detailed analysis of the physiological effects of mahogany indicates that in animals carrying dominant agouti mutations (i.e. A⁺) mahogany suppresses not only phaeomelanin synthesis but also the agouti-induced obesity syndrome (45). This evidence suggests that Mahogany is involved in mediating the interaction between Agouti and MC1-R and MC4-R. Furthermore, mahogany was shown to have physiological effects in the absence of A⁺ (45), which strongly argues for its role in facilitating AGRP binding to MC4-R as well. However, a direct interaction between Mahogany and AGRP has not yet been proven. It would be interesting to investigate whether mg mutants are able to respond to endogenous and/or exogenous AGRP treatment.

Mahogany has been recently identified as a single transmembrane domain protein that is widespread in mammals (47). Genetic studies have positioned mahogany at the same level or upstream of melanocortin receptors based on the fact that it cannot suppress the phenotype of either the MC1-R or MC4-R knockout (44, 46).

The interaction between Mahogany and the melanocortin system is illustrated by several potential models (Fig. 3). A first model suggests that Mahogany is a low affinity co-receptor of the MC1-R and MC4-R with the main function of increasing the concentration of Agouti/AGRP antagonist in the immediate vicinity of melanocortin receptors (44, 46, 47). Mahogany action would thus facilitate the antagonist-receptor interaction in two ways: 1) Mahogany binds the antagonist and presents it to the receptor or 2) Mahogany removes the agonist from the immediate vicinity of the receptor, thereby allowing more access of the antagonist to the receptor. There is some doubt, however, as to whether the co-receptor model reflects a likely mode of interaction between Mahogany, receptor, and antagonist. First, the antagonists (Agouti/AGRP) are proposed to bind to Mahogany via their N-terminal domains whereas the C termini are envisioned as interacting with the receptor (47). However, the X-ray structure of Agouti and AGRP show only weak homology with each other. In addition, both mahogany and mahoganoid suppressors appear to have the same phenotype. If Mahogany is indeed the co-receptor of the MC1-R–MC4-R, then what role does mahoganoid play? An alternative model of Mahogany action focuses on the interaction of Mahogany with the receptor rather than the agonist or antagonist. This model suggests that Mahogany is involved in the process of receptor desensitization via post-translational modifications or cellular internalization (44, 47). We would like to propose a third potential model of Mahogany action that takes into account the structural similarities between Mahog-
any and membrane proteins involved in cell adhesion. Our model suggests that Mahogany plays a key role in mediating the proper formation of neuronal architecture necessary for the axonal delivery of agonist/antagonist-containing secretory vesicles to melanocortin receptors. Immunoelectron microscopy studies focused on comparing the distribution of AGRP/Agouti contacts at MC4-R-containing neurons in mg mutants and control animals may help test this model.

Regardless of the actual mode of interaction, absence of Mahogany (i.e., in mg mutants) is presumed to elicit increased signaling at melanocortin receptors (MC1-R and MC4-R). A chronic increase in MC4-R signaling could explain the hypermetabolic behavior of mg animals (45), as recent studies indicate that central administration of melanocortin agonists also results in increased energy expenditure (48).

Concluding Remarks

It is beyond dispute that clear differences exist between the structure and function of Agouti and AGRP. Nevertheless, one cannot help but be amazed at the multitude of similarities displayed by the two proteins. It is uncertain whether Agouti and AGRP are the result of convergent or divergent evolution and what role the two proteins play in melanocortin receptors. It is uncertain whether Agouti and AGRP by binding to the N-terminal secretory vesicles to the melanocortin receptor.

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