Common Requirements for Melanocortin-4 Receptor Selectivity of Structurally Unrelated Melanocortin Agonist and Endogenous Antagonist, Agouti Protein*

Julia Oosterom‡, Keith M. Garner‡, Wijnand K. den Dekker‡, Wouter A. J. Nijenhuis‡, Willem Hendrik Gispen‡, J. Peter H. Burbach‡, Greg S. Barsh*, and Roger A. H. Adan‡†

From the ‡Department of Medical Pharmacology, Rudolf Magnus Institute for Neurosciences, University Medical Center Utrecht, P. O. Box 85060, 3508 AB Utrecht, The Netherlands and the ¶Howard Hughes Medical Institute, Beckman Center B271A, Stanford University School of Medicine, Stanford, California 94305-5323

The activity of melanocortin receptors (MCR) is regulated by melanocortin peptide agonists and by the endogenous antagonists, Agouti protein and AgRP (Agouti-related protein). To understand how the selectivity for these structurally unrelated agonists and antagonist is achieved, chimeric and mutants MC3R and MC4R were expressed in cell lines and pharmacologically analyzed. A region containing the third extracellular loop, EC3, of MC4R was essential for selective Agouti protein antagonism. In addition, this part of MC4R, when introduced in MC3R, conferred Agouti protein antagonism. Further mutational analysis of this region of MC4R demonstrated that Tyr268 was required for the selective interaction with Agouti protein, because a profound loss of the ability of Agouti protein to inhibit 125I-labeled [Nle4,α-Phe7]-α-melanocyte-stimulating hormone (MSH) binding was observed by the single mutation of Tyr268 to ile. This same residue conferred selectivity for the MC4R selective agonist, [D-Tyr4]MT-II, whereas it inhibited interaction with the MC3R-selective agonist, [Nle4]Lys-γ2-MSH. Conversely, mutation of Ile265 in MC3 (the corresponding residue of Tyr268) to Tyr displayed a gain of affinity for [D-Tyr4]MT-II, but not for Agouti protein, and a loss of affinity for [Nle4]Lys-γ2-MSH as compared with wild-type MC3R. This single amino acid mutation thus confers the selectivity of MC3R toward a pharmacological profile like that observed for MC4R agonists but not for the antagonist, Agouti protein. Thus, selectivity for structurally unrelated ligands with opposite activities is achieved in a similar manner for MC4R but not for MC3R.

Melanocortin (MC) receptors are activated by the POMC (pro-opiomelanocortin)-derived ACTH (adrenocorticotrophic hormone) and MSH (melanocyte-stimulating hormone) peptides. MC3R and MC4R are the main MC receptors in the brain, and MC4R is thought to play a prominent role in the regulation of body weight in both rodents and human (1–3). The identification of the endogenous MC receptor antagonists, Agouti protein and Agouti-related-protein (AgRP), gave rise to an additional level of regulation of MC receptors, in which ligands with opposite activities control MCR activity (4, 5). It is unknown, however, whether the molecular interaction with the receptor of these oppositely acting ligands is achieved in a similar manner.

Mouse Agouti protein is a 131-amino acid protein normally expressed in hair follicles, which acts on MC1R-expressing melanocytes to regulate pigmentation (6, 7). In mice that ec-topically overexpress Agouti protein, chronic MC1R blockade by Agouti protein results in a yellow coat color. In addition, these mice display severe obesity, hyperphagia, and increased plasma levels of the adipose derived satiety factor, leptin (8, 9). In these mice, obesity is thought to be the result of continuous blockade of the hypothalamic MC4R, since recombinant murine Agouti protein has been demonstrated to act as a high affinity antagonist for both mouse MC1R and MC4R in vitro but not for MC3R and MC5R (10–12). This determination is in agreement with the finding that MC4R−/− mice recapitulate the obesity phenotype as observed in yellow obese mice (2).

In wild-type mice, Agouti protein is not expressed in the brain. But its homologue, AgRP, is expressed in the hypothalamus of mice, rats, primates, and humans (13–16). Recombinant human AgRP acts as a high affinity antagonist for the MC3R and MC4R, and to a lesser extent for MC5R, but not for MC1R and MC2R (17). Transgenic mice that overexpress AgRP display an obesity phenotype similar to that found in MC4R−/− mice (4). Thus, pharmacological, histochemical, and genetic studies suggest that hypothalamic AgRP is an important endogenous stimulator of feeding and exerts this function by inhibiting MCR signaling.

Even though an important role of MC4R in body weight homeostasis is evident, the role of MC3R in the control of body weight and other processes is ill-defined because of the absence of MC3R selective ligands. Knowledge of the interaction between MCR and its ligands at the level of molecular detail would contribute to the design of selective MC3R and MC4R ligands. This is important not only for the understanding of MCR subtype-specific functions, but in addition, ligands that selectively activate or block MC4R may be therapeutically useful in the treatment of obesity (18) and anorexia (19, 20).

To investigate whether MCR agonists and the structurally unrelated antagonist, Agouti protein, are regulated in a similar manner, the aim of this study was to identify which part of the human MC4R is important for selective Agouti protein inter-
action and antagonism. To this end, chimeric and mutant MC3R and MC4R were expressed in cell lines and were tested for their interaction with full-length human Agouti protein, [Nle\textsuperscript{4}]Lys\textsubscript{273}–γ\textsubscript{MC3}-MSH, and [\textsuperscript{[}D-\textsuperscript{Tyr\textsubscript{4}}]MT-II. The results indicate that a single residue in MC4R is required for the selective interaction with Agouti protein. In addition, the results show that selectivity for structurally unrelated agonists and antagonists is achieved in a similar manner.

**MATERIALS AND METHODS**

**Peptides**—NDP-\textsuperscript{α}-MSH ([Nle\textsuperscript{4},\textsuperscript{[}D-Phe\textsuperscript{7}]\textsuperscript{α}-MSH) and \textsuperscript{α}-MSH were purchased from Bachem Feinchemikalien, Bubendorf, Switzerland. [\textsuperscript{[}D-Tyr\textsuperscript{4}]\textsuperscript{MT-II} and [Nle\textsuperscript{4}]Lys\textsubscript{273}–γ\textsubscript{MC3}-MSH were synthesized using solid phase Fmoc chemistry and purified as described previously (21). Full-length human Agouti protein and AgRP were prepared as described previously (17, 22).

**Radioiodination of NDP-\textsuperscript{α}-MSH**—Radioiodination was performed exactly as described previously (23). In short, 4 \mu g of \textsuperscript{125}I-NDP-\textsuperscript{α}-MSH was mixed with 1.2 IU bovine lactoperoxidase (Calbiochem) and 1 \textmu Ci of Na\textsuperscript{125}I in a final volume of 100 \mu l of 0.05 M phosphate buffer (pH 6.5). Then, 5 \mu l of 0.003% H\textsubscript{2}O\textsubscript{2} was added every 60 s. After 4 min, 50 \mu l of 1 M diethanolamine was added to stop the reaction. The sample was purified by high pressure liquid chromatography with a C\textsubscript{18} column, 3.9 \times 300 mm (Waters, Div. of Millipore) by elution with a 22–52% acetonitrile gradient in 10 mM ammonium acetate (pH 5.5) in 40 min. The specific activity of \textsuperscript{125}I-NDP-\textsuperscript{α}-MSH was 2.25 \times 10\textsuperscript{5} Ci/m\textmu l.

**Expression of Chimeric and Mutant Receptors**—All chimeric and MCR mutants used in this study were generated by polymerase chain reaction, and the sequences were verified as described previously (23). In short, the forward primer for the N-terminal domain (corresponding to residues 1–60 of MC4R) was TCTGCAGATCATTCCTCCACCTGTTCTGTGATACATCACCTGCCCCACCACCAACC-3\textsuperscript{′} and the reverse primer was 9\textsuperscript{′}-TTCCTCCACCTGTTCTGTGATACATCACCTGCCCCACCACCAACC-3\textsuperscript{′}.

**Model of the human MC4R.** The gray circles indicate a stretch of residues with complete homology between MC3R and MC4R that were replaced. With A being the N-terminal domain that was replaced. The boundaries for construction of the chimeric MC3/MC4 receptors are shown. The large circles indicate residues belonging to the third extracellular loop (EC3, residues 267–282). The black circles with double-residue symbols (for example, Y/I at position 268) indicate that the first residue (Y) is present in MC4R and the second residue (I) is present in MC3R at the corresponding position. The white circles in EC3 are homologous in MC4R and MC3R. The numbering corresponds to the human MC4R amino acid sequence. The N- and C-terminal residues are not shown.
protein displayed high affinity for MC4R. The IC₅₀ of Agouti protein was 10-fold higher for MC3R than for MC4R.²

Next, the antagonistic properties of Agouti protein were analyzed. Agouti protein at a concentration of 40 nM was a potent antagonist for MC4R (Fig. 3) and was able to increase the EC₅₀ of α-MSH almost 20-fold (Table I). In contrast, the same concentration of Agouti protein was not able to significantly alter the EC₅₀ of α-MSH for MC3R.

In addition, it was investigated as to which region of MC4R is required for the antagonistic properties of Agouti protein. Therefore, the ability of Agouti protein to antagonize α-MSH-stimulated adenylate cyclase activity of chimeric MC3/MC4 receptors was analyzed. Agouti protein was able to effectively antagonize α-MSH-stimulated adenylate cyclase activity for all chimeric receptors except chimera 3D, which only has the C-terminal portion of MC4R starting at TM5 (Fig. 3 and Table I).

Furthermore, the reverse chimera, 4D, which is an MC3R with only the C-terminal portion (starting at TM5) of MC4R, was able to confer Agouti protein antagonism to MC3R. Agouti protein was unable to significantly increase the EC₅₀ of α-MSH for chimera 3D as observed for MC3R.

To assess which region of MC4R determined the high affinity of Agouti protein for MC4R, the IC₅₀ values of Agouti protein for chimeric MC3/MC4 receptors was analyzed. Table II summarizes the IC₅₀ values of Agouti protein for all chimeric receptors. Chimerae 3AB, 3B, and 3C all displayed a high affinity for Agouti, which was not significantly different from the IC₅₀ for MC4R. However, chimera 3D (MC4R with the C-terminal portion of MC3R, starting at TM5), displayed a significant reduction in the affinity for Agouti protein. The affinities of Agouti protein for chimera 3D and MC3R were comparable (Fig. 4). Conversely, chimera 4D (the reverse of 3D, MC3R with the C-terminal portion of MC4R) displayed high affinity for

² In this study, the terms “binding” and “affinity” always refer to the ability of a ligand to compete for ¹²⁵I-NDP-α-MSH binding.
TABLE II

Inhibition constants (IC50 in nM) of human Agouti protein for MC3R, MC4R, and chimeric 3AB, 3B, 3C, 3D, and 4D

| Ligand       | MC4 | MC3 | 3AB | 3B | 3C | 3D | 4D |
|--------------|-----|-----|-----|----|----|----|----|
| Agouti protein | 12  | 112 | 38  | 13 | 14 | 123 | 20 |

*Statistically significant different from IC50 of Agouti protein for MC4R (p < 0.01, unpaired t test).

Fig. 4. Affinity of Agouti protein for MC4R, MC3R, and chimerae 3D and 4D. The graph shows competition of 125I-NDP-α-MSH by Agouti protein on MC4R ( ), MC3R ( ), and chimerae 3D ( ) and 4D ( ) expressed in B16G4F cells. Data points represent the mean of duplicate measurements ± S.D. Binding curves were fitted with GraphPad Prism, one-site competition.

**DISCUSSION**

The melanocortin system is unique in the sense that MCR activity is regulated by two structurally unrelated endogenous peptides with opposing activities: on the one hand, POMC-derived melanocortin agonists, and on the other hand, Agouti protein and AgRP, which act as antagonists for MCR. Currently, selective MCR ligands are necessary to obtain a better understanding of the role of brain MC3R and MC4R in processes like the regulation of body weight. Because Agouti protein is a high affinity antagonist for the MC4R, this study was aimed at characterizing the selective interaction of human Agouti protein with the human MC4R. The pharmacological analysis of chimeric and mutant MC3R and MC4R suggested that a single amino acid residue in the first half of the third extracellular loop of MC4R conferred selectivity for Agouti protein.

The results confirm that human Agouti protein is a high affinity antagonist for MC4R but not for MC3R. In contrast, AgRP does not discriminate between MC3R and MC4R. The selective interaction of Agouti protein with MC4R was studied using chimeric and mutant MC3R and MC4R. Apparently, the lower affinity of Agouti protein for chimera 3D (MC4R with EC3 and surrounding domains of MC3R) and MC3R wild type accounts for the inability of Agouti protein to antagonize α-MSH action on these receptors. Chimera 4D was tested for a gain of function for Agouti binding and antagonism to test whether this region (EC3 and surrounding domains of MC4R)
alone is sufficient to obtain an MC4R-specific pharmacological profile. Indeed, this EC3-containing region of MC4R fully conferred Agouti protein antagonism to MC3R. Thus, this region changes the MC3R pharmacological profile to that of MC4R.

A more detailed analysis of the role of EC3 in Agouti protein binding showed that the first half of EC3, in particular position Tyr268 of MC4R, was critical for high affinity Agouti protein binding because, when it was replaced by Ile, a loss of affinity for Agouti protein occurred. Previously, it was shown that Tyr268 was also required for the selective interaction of the agonist [Nle4]Lys-γ2-MSH with MC4R, whereas it hindered interaction with the MC3R selective agonist [Nle4]Lys-γ2-MSH (21). Thus, Tyr268 is required for selective agonist and antagonist binding of the MC4R. This is a striking observation because there exists no obvious amino acid homology between melanocortins and Agouti protein (Fig. 7).

To test whether Ile265 of MC3R hindered interaction with Agouti protein, Ile265 of MC3R was mutated into Tyr. MC3 (Ile265 → Tyr) did not show a gain of affinity for Agouti protein, indicating that Tyr268, in the context of the MC3R conformation, does not confer selectivity for antagonists. Surprisingly, MC3 (Ile265 → Tyr) displayed increased affinity for [D-Tyr4]MT-II and decreased affinity for [Nle4]Lys-γ2-MSH. Thus, this residue is able to change the pharmacological profile of MC3R toward that of MC4R for agonists but not for antagonists. These data imply that it may not be feasible to design MC3R-selective antagonists based upon MC3R selective agonists (such as γ2-MSH) and MC4R-selective antagonists. This approach, however, may be applicable for MC4R because the structural requirements for selective agonists and antagonists interaction with MC4R is more alike.

In conclusion, through pharmacological analysis of chimeric and mutant MC3R and MC4R, it was demonstrated that Tyr268 in EC3 of MC4R is critical for selective Agouti protein interaction. This study and previous data show that the mutation of MC4R Tyr268 to Ile decreases affinity for MC4R-selective ligands but increases affinity for the MC3R-selective ligands. Moreover, Tyr268 in MC4R determined both agonist and antagonist selectivity. This is the first report describing details of the molecular recognition of the endogenous MCR antagonist, Agouti protein, by MC4R. This report demonstrates that the selectivity of structurally unrelated peptide ligands with opposite activities (melanocortin peptide agonist versus Agouti protein antagonist) is achieved in a similar manner, strongly suggesting that they use the same binding pocket. An understanding of the molecular basis governing selective ligand interaction with MC receptors contributes to the rational design of new selective ligands.

REFERENCES

1. Kistler-Heer, V., Lauber, M. E. & Lichtensteiger, W. (1998) J. Neuroendocrinol. 10, 133–146
2. Huszar, D., Lynch, C. A., Fairchild-Huntress, V., Dunmore, J. H., Fang, Q., Berkeemeier, L. R., Gu, W., Kesterson, R. A., Boston, B. A., Cone, R. D., Smith, F. J., Campfield, L. A., Burn, P. & Lee, F. (1997) Cell 88, 131–141
3. Vaisse, C., Clement, K., Guy-Bragon, B. & Praguel, P. (1998) Nat. Genet. 20, 113–114
4. Ollmann, M. M., Wilson, B. D., Yang, Y. K., Kerns, J. A., Chen, Y., Ganz, I. & Barash, G. S. (1997) Science 278, 135–138
5. Shuttle, J. R., Graham, M., Kinsey, A. C., Scully, S., Luthy, R. & Stark, K. L. (1997) Genes Dev. 11, 583–602
6. Bhutiman, S. J., Michaela, E. & Woychik, R. P. (1992) Cell 71, 1195–1204
7. Miller, M. W., Duhl, D. M., Vrieling, H., Corder, S. P., Ollmann, M. M., Winkes, B. M. & Barash, G. S. (1993) Genes Dev. 7, 454–467
8. Wolff, G. L., Roberts, D. W. & Galbraith, D. B. (1990) J. Hered. 71, 151–158
9. Miltenberger, R. J., Mynatt, R. L., Wilkinson, J. E. & Woychik, R. P. (1997) J. Nutr. 127, 1992–1997
10. Lu, D., Willard, D., Patel, I. R., Kadwell, S., Overton, L., Kost, T., Luther, M., Chen, W., Woychik, R. P. & Wilkinson, W. O. (1994) Nature 371, 799–802
11. Kiefer, L. L., Ittoo, O. R., Bunc, K., Truesdale, A. T., Willard, D. H., Nichols, J. S., Blanchard, S. G., Montjoy, K., Chen, W. J. & Wilkinson, W. O. (1997) Biochemistry 36, 2084–2090
12. Montjoy, K. G., Willard, D. H. & Wilkinson, W. O. (1999) Endocrinology 140, 2167–2172
13. Haskell-Luevano, C., Chen, P., Li, C., Chang, K., Smith, M. S., Cameron, J. L. & Cone, R. D. (1999) Endocrinology 140, 1408–1415
14. Elias, C. F., Saper, C. B., Maratos-Flier, E., Tritos, N. A., Lee, C., Kelly, J., Tatro, J. B., Hoffman, G. E., Ollmann, M. M., Barash, G. S., Sakuraz, T., Yanagisawa, M. & Elmgrist, J. K. (1998) J. Comp. Neurol. 402, 442–459
15. Broberger, C., De Lececa, L., Sutcliffe, J. G. & Hokfelt, T. (1996) J. Comp. Neurol. 402, 460–474
16. Broberger, C., Johansen, J., Johansson, C., Schalling, M. & Hokfelt, T. Proc.
Melanocortin-4 Receptor Selectivity

17. Yang, Y. K., Thompson, D. A., Dickinson, C. J., Wilken, J., Barsh, G. S., Kent, S. B. & Gantz, I. (1999) Mol. Endocrinol. 13, 148–155
18. Bray, G. A. & Tartaglia, L. A. (2000) Nature 404, 672–677
19. Huang, Q. H., Hruby V. J. & Tatro J. B. (1999) Am. J. Physiol. 276, R864–871
20. Vergoni, A. V., Bertolini, A., Wikberg, J. E. & Schioth, H. B. (1999) Eur. J. Pharmacol. 369, 11–15
21. Schaaper, W. M., Adan, R. A., Posthuma, T. A., Oosterom, J., Gispen, W. H. & Meloen, R. H. (1998) Lett. Peptide Sci. 5, 1–4
22. Yang, Y. K., Ollmann, M. M., Wilson, B. D., Dickinson, C., Yamada, T., Barsh, G. S. & Gantz, I. (1997) Mol. Endocrinol. 11, 274–280
23. Oosterom, J., Nijenhuis, W. A., Schaaper, W. M., Slootstra, J., Meloen, R. H., Gispen, W. H., Burbach, J. P. & Adan, R. A. (1999) J. Biol. Chem. 274, 16853–16860
24. Roselli-Rehfuss, L., Mountjoy, K. G., Robbins, L. S., Morrud, M. T., Low, M. J., Tatro, J. B., Entwistle, M. L., Simerly, R., B. & Cone, R. D. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8856–8860
25. Gantz, I., Miwa, H., Konda, Y., Shimoto, Y., Tashiro, T., Watson, S. J., DelValle, J. & Yamada, T. (1993) J. Biol. Chem. 268, 15174–15179
26. Salmon, Y., Londes, C. & Rodbell, M. (1974) Anal. Biochem. 58, 549–558
27. Kiefer, L. L., Veal, J. M., Mountjoy, K. G. & Wilkison, W. O. (1998) Biochemistry 37, 991–997