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Molecular Determination of Agouti-Related Protein Binding to Human Melanocortin-4 Receptor

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ABSTRACT

Agouti-related protein (AGRP) is an endogenous antagonist of the melanocortin-4 receptor (MC4R) that functions in the hypothalamic control of feeding behavior. Our previous studies have suggested that in addition to exoloops 2 and 3, several transmembrane domains of MC4R may be important for AGRP binding. However, the detailed molecular basis of MC4R domains in AGRP binding is presently unclear. The present studies were designed to determine the specific contribution of MC4R exoloops and transmembrane domains to AGRP binding by using chimeric receptor constructs of the human melanocortin-1 receptor (hMC1R), a receptor that is not inhibited by AGRP, and the human MC4R (hMC4R), a receptor that is potently inhibited by AGRP. Our results indicate that substitutions of the second and third extracellular loops of the MC4R with homologous domains of the MC1R dramatically decreased AGRP 87–132 binding affinity, but did not affect AGRP 110–117 binding affinity. In contrast, cassette substitutions of the third or fourth transmembrane domain of the MC4R with the homologous domain of the MC1R resulted in a substantial decrease of AGRP 87–132 binding affinity and loss of AGRP 110–117 binding affinity. These data suggest that the AGRP fragment 110–117 has no binding sites at exoloops of hMC4R and that transmembrane domains of MC4R may play an important role in AGRP 110–117 binding and function, whereas the exoloops do not. The second and third extracellular loops of MC4R are important for AGRP 87–132 N-terminal binding, whereas the third and fourth transmembrane domains of hMC4R are crucial for AGRP 110–117 binding.

Agouti-related protein (AGRP), transcribed as 132 amino acids in humans and 131 amino acids in mice, is an endogenous antagonist of melanocortin action that functions in the hypothalamic control of feeding behavior (Ollmann et al., 1997). AGRP and neuropeptide Y are coexpressed by the same neurons (Hahn et al., 1998), and AGRP is believed to act as a melanocortin-opposing orexigenic agent mediated by melanocortin receptor (MCR) 4 (Fan et al., 1997; Huszar et al., 1997; Bagnol et al., 1999). In its role as an important mediator of satiety, MC4R has become a prime target for antiobesity drug development (MacNeil et al., 2002).

AGRP was originally identified from its sequence similarity to the agouti protein (Barsh et al., 1999; Wilson et al., 1999). Both AGRP and agouti have a C-terminal cysteine-rich motif. Agouti is a potent antagonist of MC1R, MC2R, MC4R, and MC5R. In contrast, AGRP is a potent antagonist of the MC3R and MC4R (Yang et al., 1999a). AGRP has very little amino acid sequence similarity to melanocortins. In its artificially truncated form, AGRP 87–132 is 46 amino acids and contains 10 cysteine residues capable of forming five disulfide bonds (Fig. 1.1). In contrast, the predominant melanocortin in the hypothalamus, α-melanocyte-stimulating hormone (α-MSH), is only 13 amino acids in length and has no cysteine residues. These observations, therefore, suggest that this agonist-antagonist pair have significantly different tertiary structures despite being ligands for the same receptors. It is also important to note, with respect to their competitive interaction, that α-MSH is capable of activating MCR subtypes 1, 3, 4, and 5, whereas AGRP can only inhibit α-MSH's action at MCR subtypes 3, 4, and 5. In view of their apparent structural dissimilarity and receptor subtype specificity, it is likely that α-MSH and AGRP have receptor binding determinants that are not identical.

Our previous studies have indicated that exoloops 2 and 3 of MC4R are involved in AGRP binding but are not the only ligand-receptor interaction site (Yang et al., 1999b). The transmembrane domains of MC4R may also play an important role in AGRP binding (Yang et al., 1999b). However, the detailed molecular basis of the role for hMC4R in AGRP

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ABBREVIATIONS: AGRP, agouti-related protein; MCR, melanocortin receptor; MC*n*R, melanocortin receptor, where *n* is receptor number; α-MSH, α-melanocyte-stimulating hormone; h, human; NDP-MSH, (Nle^4,D-Phe^7)α-melanocyte stimulating hormone; PCR, polymerase chain reaction; GPCR, G protein-coupled receptor; TM, transmembrane domain; HEK, human embryonic kidney.

A **NDP-MSH** S-Y-S-Nle-E-H-**DF-R-W**-G-K-P-V

AGRP 87-132 CVRLHESCLGQQVPCCDPCATCYC**RFF**NAFCYCRKLGTAMNPCSRT

AGRP 110-117 **CRFF**NAFC

B

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                    N-terminal
hMC1R MAVQGSQRRLLGSLNSTPTAIPQLGLAANQTGARCLEV
hMC4R MVNSTHRGMHTSLHLWNRSSYRLHSNASESLGKGYSDGGCYEQL

                TM1                          loop 1
hMC1R   39  SISDGLFLSL GLVSLVENAL VVATIA   KNRNLH
hMC4R   43  FVSPEVFVTL GVISLLENIL VIVAIA   KNKNLH

                TM2                              loop 2 (1e)
hMC1R   71  SPMYCFICCL ALSDLLVSGT NVLETA   VILLEAGALVARAAVLQQL
hMC4R   77  SPMYFFICSL AVADMLVSVS NGSETI    IITLLNSTDTDAQSFTVNI

                TM3                              loop 3 (2i)
hMC1R  117  DNVIDVITCS SMLSSLCFLG AIAVDRY   ISIFYALRYHSIVTL
hMC4R  122  DNVIDSVICS SLLASICSLL SIAVDRY   FTIFYALQYHNIMTV

                TM4                      loop 4 (2e)
hMC1R  159  PRARQAVAAI WVASVVFSTL FIAY    YDHVAV
hMC4R  164  KRVGIIISCI WAACTVSGIL FITY    SDSSAV

                TM5                              loop 5 (3i)
hMC1R  189  LLCLVVFFLA MLVLMAVLYV HML   ARACQHAQGIARLHKRQRPVHQGFGLKG
hMC4R  194  IICLITMFFT MLALMASLYV HMF    LMARLHIKRIAVLPGTGAIRQGANMKG

                TM6                              loop 6 (3e)
hMC1R  240  AVTLTILLGI FFLCWGPFFL HLTLIVL   CPEHPTCGCIFK
hMC4R  245  AITLTILIGV FVVCWAPFFL HLIFYIS   CPQNPYCVCFMS

                TM7                              C-terminal
hMC1R  279  NFNLFLALII CNAIIDPLIY AFRSQ   ELRRTLKEVLTCSW
hMC4R  283  HFNLYLILIM CNSIIDPLIY ALRSQ   ELRKTFKEIICCYPLGGLCDLSSRY
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Fig. 1. A, sequences of NDP-MSH, AGRP 87–132, and AGRP 110–117. NDP-MSH differs in primary sequence from the native hormone, α-MSH, by the replacement of Met with Nle at position 4, and inversion of chirality of the α-carbon of phenylalanine at position 7 to D-Phe. AGRP 87–132 is the C-terminal portion of full-length AGRP, composed of 46 amino acids. AGRP 110–117 is composed of eight amino acids. B, the amino acid sequences of the hMC1R and hMC4R are shown for comparison. The conserved amino acids between hMC1R and hMC4R are in boldface type.

binding is presently unclear. The present study was designed to determine the contribution of the transmembrane domains and exoloops of hMC4R in AGRP binding by using chimeric receptors from hMC4R (AGRP is a potent antagonist) and hMC1R (AGRP has no effect).

Materials and Methods

Reagents. NDP-MSH was purchased from Peninsula Laboratories, Inc. (Belmont, CA). AGRP 87–132 was obtained from Graph Science (South San Francisco, CA). The cyclic AGRP 110–117 was obtained from Research Genetics. Their sequences are shown in Fig. 1A.

Construction of Melanocortin Receptor Chimera. The amino acid sequences of the human (h) MC1R and hMC4R were examined by hydrophobicity plot (Genetics Computer Group, Inc., Madison, WI) and by manually comparing their sequences to a previously published alignment of seven transmembrane G-protein coupled receptor α-helices (Baldwin, 1993). The sequence of the wild-type hMC4R used in these studies can be found in GenBank under accession number L08603 (Gantz et al., 1993). The sequence of the wild-type hMC1R used in these studies can be found in GenBank under accession number X65634 (Mountjoy et al., 1992), except that position 163 is Arg and 164 is Gln in the GenBank sequence. A sequence comparison of the hMC1R and hMC4R is shown in Fig. 1B. The chimera used in these studies are schematically diagrammed in Fig. 2. The chimeric receptors were constructed by polymerase chain reaction (PCR) using *Pfu* polymerase (Stratagene, La Jolla, CA) (Yang et al., 1999b). The human MC1R and MC4R served as templates. During an initial round of PCR, partial-length receptor fragments were generated. The sequence of one of the PCR primer oligonucleotides consisted of the transmembrane domain of interest coupled to a portion of the extracellular domain required to form the chimeric receptor. The second oligonucleotide primer consisted of either the 5′- or 3′-end of the MC1R or MC4R. Receptor fragments were separated by agarose gel electrophoresis and used in a second round of PCR in which full-length chimeric receptor constructs were

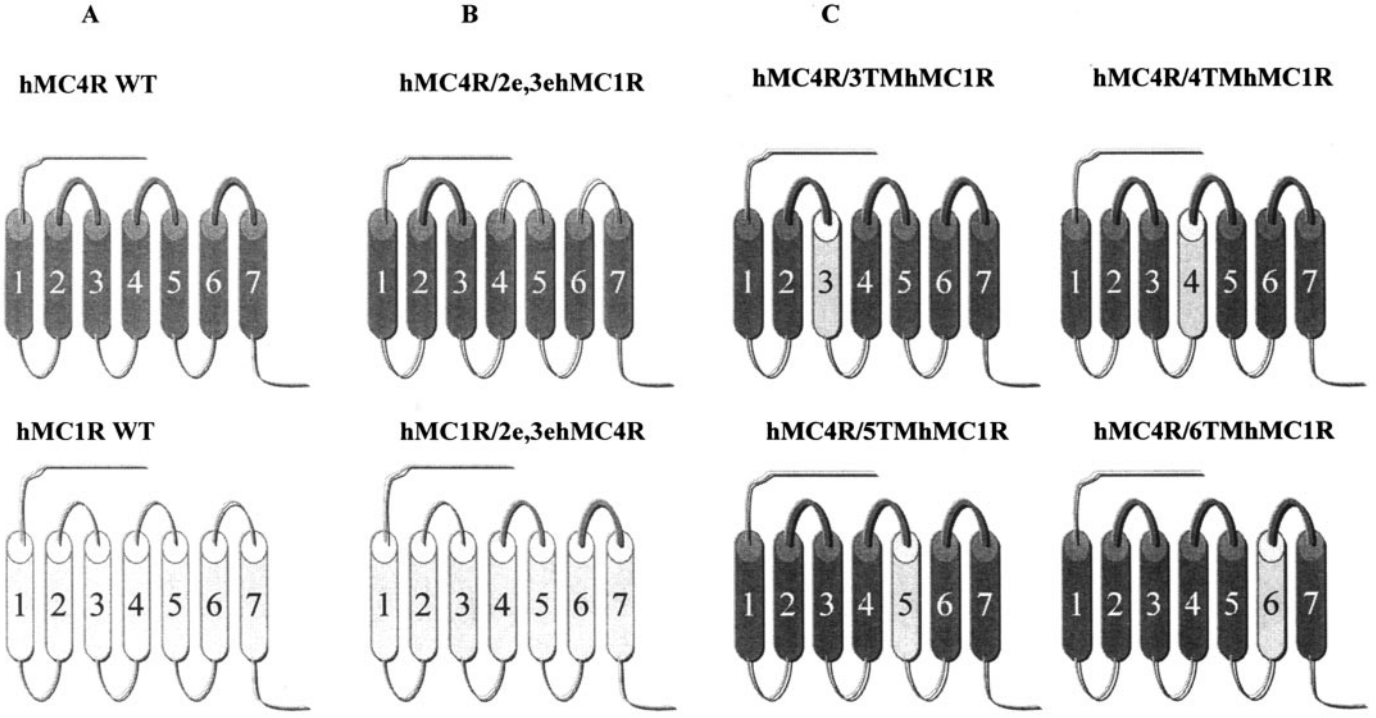

Fig. 2. Schematic representation of the chimeric human melanocortin receptors used in these studies. A, the seven transmembrane structures of the wild-type (WT) MC4R (drawn with heavy lines) and MC1R (drawn with thin lines). B, structure of the chimeric MC4R or MC1R with simultaneous substitutions of the second and third extracytoplasmic loops (2e, 3e) of hMC1R (top) and the chimeric MC1R with the simultaneous substitution of 2e,3e of the hMC4R. C, structure of the chimeric MC4R with the substituted TMs of the MC1R.

assembled by cycling the appropriate fragments together for 10 cycles before adding both 5′ and 3′ receptor primers. The chimeric receptors were subcloned into the eukaryotic expression vector pcDNA 3.1 (Invitrogen, Carlsbad, CA).

Cell Culture and Transfection. The coding regions of the genes for the hMC4R wild-type, hMC1R wild-type, and chimeric receptors were subcloned into the pCDNA3.1. The HEK 293 cell line was used in these experiments. Transfection of cells was accomplished by using LipofectAMINE (Invitrogen), and permanently transfected clonal cell lines were selected by resistance to the neomycin analog G418 (Yang et al., 1997b).

Binding Assays. Binding experiments were performed using conditions described previously (Yang et al., 1997a). Briefly, 2×10^5 cpm of ^{125}I-NDP-MSH (Amersham Biosciences, Piscataway, NJ) was used in combination with nonradiolabeled ligands NDP-MSH, AGRP 87–132, or AGRP 110–117. Binding reactions were terminated by removing the media and washing the cells twice with minimal essential medium containing 0.2% bovine serum albumin. The cells were lysed with 0.2 N NaOH, and the radioactivity in the lysate was quantified in an analytical γ-counter. Nonspecific binding was determined by measuring the amount of ^{125}I label bound in the presence of 10^{-6} M unlabeled ligand. Specific binding was calculated by sub-

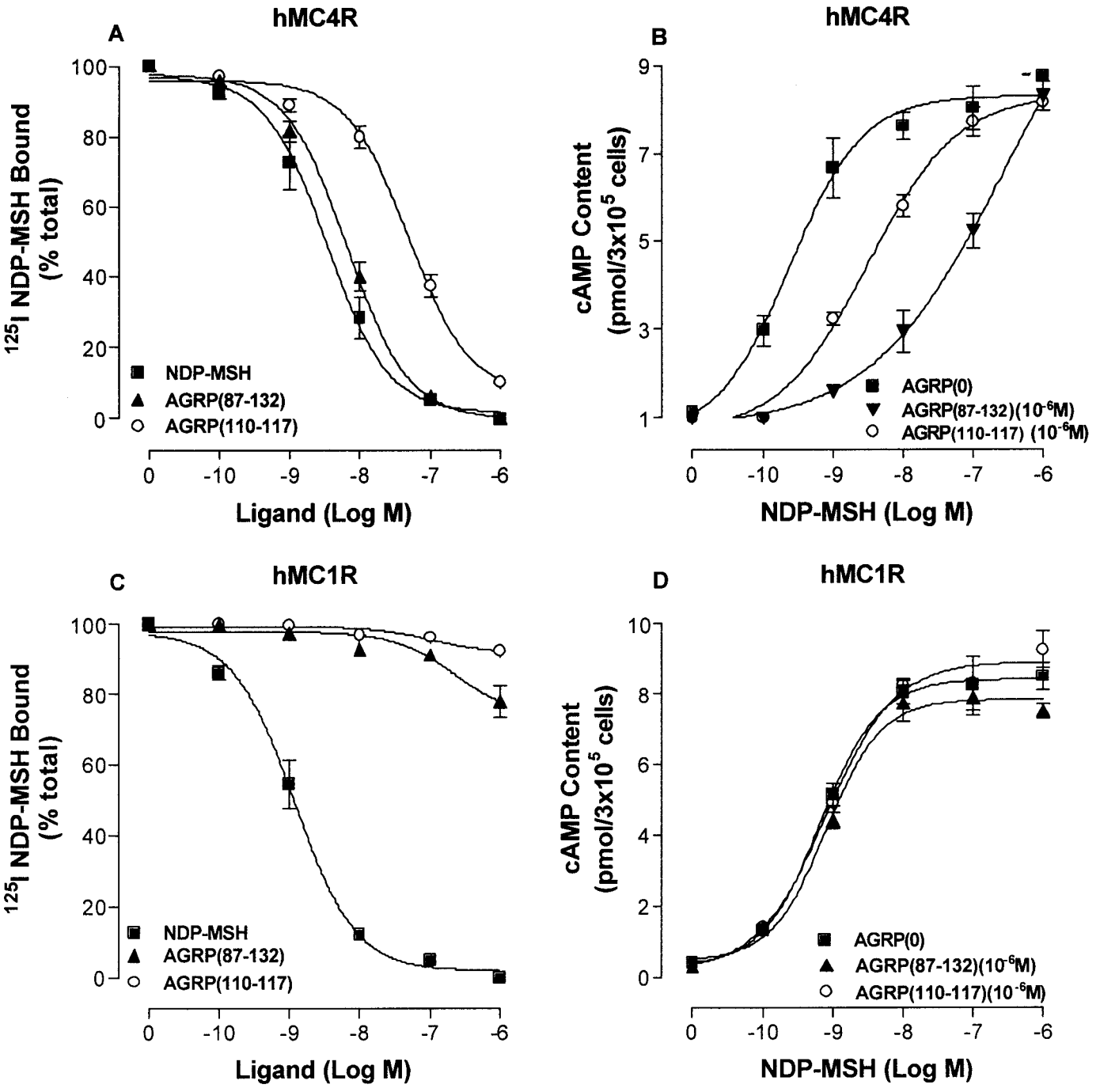

Fig. 3. A, displacement of the radioligand ^{125}I-NDP-MSH from MC4R wild type by NDP-MSH, AGRP 87–132, and AGRP 110–117. B, effect of AGRP fragments on NDP-MSH–stimulated cAMP generation at hMC4R wild type. C, displacement of the radioligand ^{125}I-NDP-MSH from MC1R wild type by NDP-MSH, AGRP 87–132, and AGRP 110–117. D, effect of AGRP fragments on NDP-MSH–stimulated cAMP generation at hMC1R wild type.

tracting nonspecifically bound radioactivity from total bound radioactivity. The binding displacement curves were drawn using Prism (GraphPad Software, San Diego, CA). The maximum binding (B_{max}) was calculated using the equation B_{max} = [NDP-MSH specific binding]/([NDP-MSH]/(K_d + [NDP-MSH]). $K_i = K_d = IC_{50} - [NDP-MSH]$.

cAMP Assay. cAMP generation was measured using a competitive binding assay kit (TRK 432; Amersham Biosciences). Briefly, HEK 293 cell lines stably expressing the human melanocortin receptors were used in these assays (Yang et al., 1997b). Cell culture media was removed, and cells were incubated with 0.5 ml of Earle's balanced salt solution, containing melanocortin agonist NDP-MSH or NDP-MSH with AGRP 87–132 or AGRP 110–117 (10^{-6} M), for 30 min at 37°C in the presence of 10^{-3} M isobutylmethylxanthine. The reaction was stopped by adding ice-cold 100% ethanol (500 μl/well). The cells in each well were scraped, transferred to a 1.5-ml tube, and centrifuged for 10 min at 1900*g*, and the supernatant was evaporated in a 55°C water bath with prepurified nitrogen gas. cAMP content was measured according to instructions accompanying the assay kit. Each experiment was performed a minimum of three times with duplicate wells. The mean value of the dose-response data were fit to a sigmoid curve with a variable slope factor using nonlinear squares regression analysis (GraphPad Prism). Statistical significance of cAMP and radioligand binding assays was determined by analyzing experimental means ± S.E.M. using a paired *t* test with $P < 0.01$ considered significant.

Results

Effect of AGRP 87–132 and AGRP 110–117 on ^{125}I-NDP-MSH Binding and on α-MSH–Stimulated cAMP Formation at hMC1R and hMC4R. Our previous studies have shown that the C terminus of AGRP 87–132 possesses the same binding affinity and potency as the full-length AGRP (Yang et al., 1999a). To determine which AGRP residues are essential for high-affinity binding and potency at the hMC4R, we performed the structure activity studies with the truncated AGRP peptides. We first examined the effect of AGRP 87–132 and AGRP 110–117 (sequences shown in Fig. 1) on hMC4R and hMC1R wild-type function. As shown in Fig. 3A, AGRP 87–132 and AGRP 110–117 dose dependently displace ^{125}I-NDP-MSH binding at hMC4R. AGRP fragment 110–117 still possesses binding affinity at hMC4R, although its binding affinity is less potent than that of AGRP 87–132. In contrast, AGRP 87–132 and AGRP 110–117 have no effect on NDP-MSH binding at hMC1R (Fig. 3C). Consistent with the binding results, both AGRP 87–132 and AGRP 110–117 potently inhibit NDP-MSH stimulated cAMP generation at hMC4R (Fig. 3B), whereas neither AGRP 87–132 nor AGRP 110–117 has an inhibitory effect on hMC1R-mediated cAMP production (Fig. 3D). The K_i and EC_{50} values are listed in Tables 1 and 2.

Effect of AGRP 87–132 and AGRP 110–117 on ^{125}I-NDP-MSH Binding at Exoloop Chimera of hMC1R and hMC4R. We previously identified that exoloops of hMC4R are involved in AGRP 87–132 binding and function. To determine which region of AGRP 87–132 binds to the exoloops of hMC4R, we examined the effect of AGRP 87–132 and AGRP 110–117 binding to those chimeras in which the exoloops of hMC4R were replaced with the same region of hMC1R. We replaced the second and third extracellular loops of hMC4R with the corresponding region of hMC1R (designated hMC4R/2e,3ehMC1R) and replaced the second and third exoloops of hMC1R with the corresponding domains of hMC4R (designated hMC1R/2e,3ehMC4R). As shown in Fig. 4, our result indicates that NDP-MSH dose-dependently displaces ^{125}I-NDP-MSH from hMC4R/2e,3ehMC1R and hMC1R/2e,3ehMC4R (Fig. 4, A and C). In other words, the cassette substitutions of the second or third exoloop of hMC4R or hMC1R in combination with homologous regions of the MC1R or MC4R do not alter ^{125}I-NDP-MSH binding affinity. However, simultaneous substitution of the second and third exoloops of the hMC4R with those of the hMC1R (chimera hMC4R/2e,3ehMC1R) led to a significant decrease in AGRP 87–132 binding affinity compared with hMC4R-WT. However, AGRP 110–117 binding affinity remained the same as that of the hMC4R wild-type (Fig. 4A). In addition, simultaneous substitution of the second and third exoloops of the hMC1R with those of the hMC4R (chimera MC1R/2e,3eMC4R) results in a significant increase in of AGRP 87–132 binding affinity with no change in AGRP 110–117 binding affinity compared with hMC1R wild-type (Fig. 4C). Consistent with binding data, substitution of second and third exoloops of hMC4R did not decrease the ability of AGRP

TABLE 1
Effect of AGRP fragments on ^{125}I-NDP-MSH binding at the HEK cells transfected with exoloop chimera of the hMC4R

	^{125}I-NDP-MSH			
	B_{max}	NDP-MSH (Ki)	AGRP 87–132 (K_i)	AGRP 110–117 (K_i)
	fmol/mg		*nM*	
hMC4RWT	325 ± 5.8	2.16 ± 0.20	8.31 ± 0.31	20.0 ± 2.2
hMC4R/2e,3e hMC1R	284 ± 26.4	1.74 ± 0.20	538 ± 34.1	605 ± 33
hMC1R/2e,3e hMC4R	257 ± 14.9	4.31 ± 0.11	42 ± 8.23	$>10^3$
hMC1RWT	354 ± 6.7	1.32 ± 0.09	$>10^3$	No binding

TABLE 2
Effect of AGRP fragments on NDP-MSH–stimulated cAMP generation at the HEK cells transfected with exoloop chimera of the hMC4R

	EC_{50}		
	NDP-MSH	NDP-MSH + AGRP 87–132 (10^{-6} M)	NDP-MSH + AGRP (110–117) (10^{-6} M)
		nM	
hMC4RWT	0.89 ± 0.11	216 ± 17.2	67.1 ± 7.1
hMC4R/2e,3e hMC1R	2.75 ± 0.21	57.4 ± 12.0	68.3 ± 8.4
hMC1R/2e,3e hMC4R	0.87 ± 0.06	43.1 ± 8.1	2.45 ± 0.3
hMC1RWT	0.63 ± 0.03	0.68 ± 0.1	0.66 ± 0.09

110–117 to inhibit NDP-MSH–stimulated cAMP formation (Fig. 4B). Substitution of second and third exoloops of hMC1R with the same regions of hMC4R did not increase the AGRP 110–117 potency (Fig. 4D). The K_i and EC_{50} values are listed in Tables 1 and 2. These results indicated that the AGRP 110–117 binding site is not located at exoloops 2e or 3e of hMC4R.

Effect of AGRP 87–132 and AGRP 110–117 on ^{125}I-NDP-MSH Binding and α-MSH–Stimulated cAMP Generation with the Substitution of Transmembrane Domains of hMC4R. Results from above experiments indicate that second and third extroloops of hMC4R are not involved in AGRP 110–117 binding. Primary experiments were performed to examine the contribution of hMC4R transmembrane domains in AGRP 110–117 binding. Again, we used a domain-exchange strategy to localize regions of hMC4R responsible for AGRP 110–117 selectivity. Cassette substitutions of the third, fourth, fifth, and sixth transmembrane regions of the hMC4R with the homologous regions of hMC1R were constructed. The first, second, and seventh transmembranes were not chosen for investigation because computer modeling of the ligand receptor interaction and our

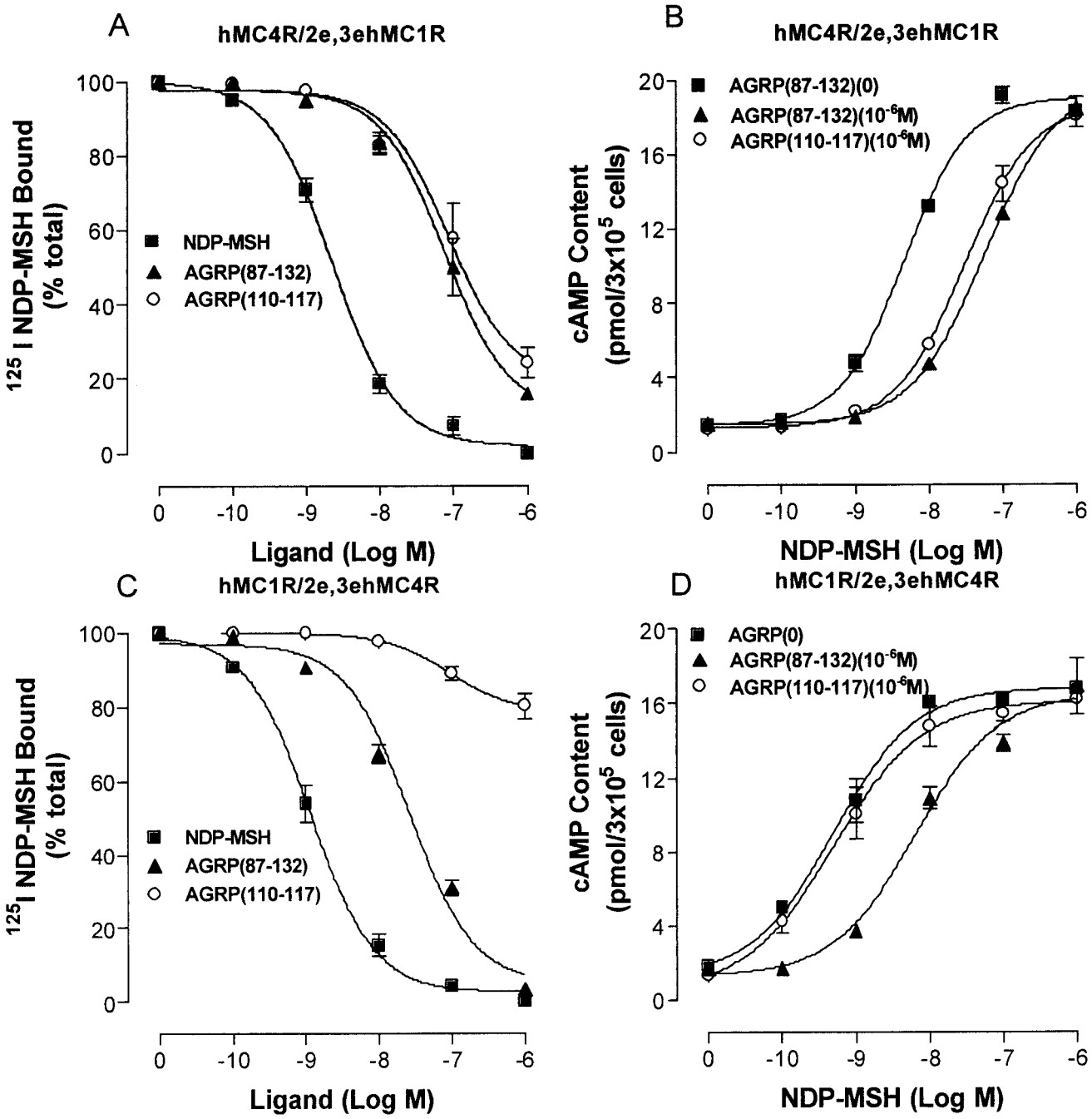

Fig. 4. A depicts the displacement of the radioligand ^{125}I-NDP-MSH from chimeric receptor hMC4R/2e,3e hMC1R by NDP-MSH, AGRP 87–132, and AGRP 110–117. B, effect of AGRP 87–132 and AGRP 110–117 on NDP-MSH–stimulated cAMP generation at the chimeric receptor hMC4R/2e,3e hMC1R. C, displacement of the radioligand ^{125}I-NDP-MSH from hMC1R/2e,3ehMC4R by NDP-MSH, AGRP 87–132, and AGRP 110–117. D, effect of AGRP 87–132 and AGRP 110–117 on NDP-MSH–stimulated cAMP generation at the chimeric receptor hMC1R/2e,3e hMC4R.

previous data suggested that they probably do not play a critical role in NDP-MSH binding but could still be involved in AGRP binding (Haskell-Luevano et al., 1996; Yang et al., 1997a). To assess the binding affinity of AGRP 87–132 and AGRP 110–117 at these chimeric receptors, we performed the displacement experiments of labeled ^{125}I NDP-MSH with unlabeled AGRP 87–132 and AGRP 110–117. The K_i values of NDP-MSH, AGRP 87–132 and AGRP 110–117 were determined and are summarized in Table 3. Our results indicate that each chimeric receptor is functionally expressed and that the expression levels showed no significant variation compared with wild-type receptor expression (Table 3). Figure 5 shows that the cassette substitutions of the third, fourth, fifth, and sixth transmembrane domains of hMC4R with homologous regions of the MC1R did not dramatically alter ^{125}I-NDP-MSH binding affinity and potency compared with hMC4R wild-type (Fig. 5, A and B). Substitution of the fifth or sixth transmembrane domains of the MC4R into the sequence of the MC1R had little effect on the ability of AGRP 87–132 to displace ^{125}I-NDP-MSH binding (Fig. 5, E and F). However, substitution of the third or fourth transmembrane domain of hMC4R into the sequence of the hMC1R decreased AGRP 87–132 binding affinity by approximately 5- and 11-fold, separately, and decreased AGRP 110–117 binding affinity by approximately 100-fold (Fig. 5, C and D, and Table 3).

To study the functional effects of transmembrane substitution of hMC4R in more detail, we examined the ability of AGRP 87–132 and AGRP 110–117 to inhibit NDP-MSH–induced cAMP generation at the MC4R/MC1R chimera (Fig. 6). Consistent with the known effects of AGRP, 10^{-6}M AGRP 87–132 and AGRP 110–117 potently inhibited NDP-MSH action at the chimeric receptor hMC4R/5TMhMC1R and hMC4R/6TMhMC1R (Fig. 6, C and D). However, substitution of the third and fourth transmembrane domains of hMC4R with the corresponding regions of hMC1R causes a 20-fold decrease in the effect of AGRP 87–132 on NDP-MSH–stimulated cAMP generation. Furthermore, these substitutions result in the complete loss of the ability of AGRP 110–117 to inhibit NDP-MSH–stimulated cAMP generation (Fig. 6, A and B, and Tables 4 and 5).

Discussion

AGRP was identified in 1997 by its homology to the more widely studied agouti protein (Ollmann et al., 1997). Both agouti and AGRP are potent MCR antagonists; each selects for a different spectrum of MCR subtypes. Agouti is a potent antagonist for MC1R, MC2R, and MC4R. In contrast, in the brain AGRP antagonizes only MC3R and MC4R (Yang et al., 1999a). Both α-MSH and AGRP are involved in the regulation of food intake via MC4R; however, whether AGRP alone can modulate food intake via MC4R is unclear. The fact that α-MSH and AGRP are expressed in different neurons suggests that AGRP might inhibit the activity of the melanocortin system independently of α-MSH in the brain. Physiological and pharmacological data also suggest that MC4R seems to be controlled primarily by alterations in 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 are relatively modest, 1.5- to 2-fold compared with 10- to 12-fold alterations in AGRP transcript level (Mizuno et al., 1998). Regulation of AGRP expression and function may offer a more precise temporal and spatial control of MC4R function than would be possible by altering levels of circulating melanocortin peptides. Although most evidence suggests that AGRP acts as a competitive antagonist of MC3R and MC4R, meaning that its effect is caused solely by its ability to inhibit binding of melanocortin receptor agonists, such as α-MSH, additional findings have suggested that AGRP inhibit melanocortin receptor signaling by mechanisms in addition to simple competitive antagonism. For instance, AGRP alone could modulate MC4R by acting as an inverse agonist. It has been proposed that G-protein-coupled receptors (GPCRs) naturally oscillate between active and inactive states in the absence of agonist binding (Strange, 2002). In this model, agonists stabilize the active state, whereas certain antagonists may stabilize the inactive state. Among these antagonists, those that stabilize an inactive state can inhibit not only the agonist-induced activation of the receptor but also its constitutive activity. Such antagonists are called inverse agonists. For most constitutively active GPCRs characterized thus far, numerous competitive antagonists have been shown to display inverse agonist activity (Joubert et al., 2002; Casarosa et al., 2003). Such compounds are very useful for the understanding of the possible physiological relevance of GPCR constitutive activity. AGRP has been reported to have inverse agonist activity in which AGRP suppresses basal cAMP generation at the hMC4R (Haskell-Luevano and Monck, 2001; Nijenhuis et al., 2001); however, the mechanism of this action is unknown. Therefore, the molecular determinants of MC4R responsible for AGRP binding should provide insight into the molecular basis of AGRP action as a competitive antagonist as well as an inverse agonist. Both properties may play an important role in the control of feeding behavior.

AGRP was originally identified through the homology of its C-terminal region with the same region of the agouti protein (Dinulescu and Cone, 2000). We have previously shown that the chemically synthesized C-terminal region of AGRP com-

TABLE 3
Effect of AGRP fragments on ^{125}I-NDP-MSH binding at the HEK cells transfected with TM chimera of the hMC4R

	^{125}I-NDP-MSH			
	B_{max}	NDP-MSH (K_i)	AGRP 87–132 (K_i)	AGRP 110–117 (K_i)
	fmol/mg of protein		*nM*	
hMC4RWT	325 ± 5.8	2.16 ± 0.20	8.31 ± 0.31	20.0 ± 2.2
hMC4R/TM3 hMC1R	298 ± 21.5	1.74 ± 0.20	38.1 ± 3.41	$>10^3$
hMC4R/TM4 hMC1R	278 ± 16.4	4.31 ± 0.11	105 ± 8.23	$>10^3$
hMC4R/TM5 hMC1R	301 ± 11.7	1.92 ± 0.32	10.3 ± 2.12	71.3 ± 7.35
hMC4R/TM6 hMC1R	295 ± 22.5	4.70 ± 0.20	14.0 ± 3.40	25.7 ± 4.5
hMC1RWT	354 ± 6.7	1.32 ± 0.09	$>10^3$	No binding

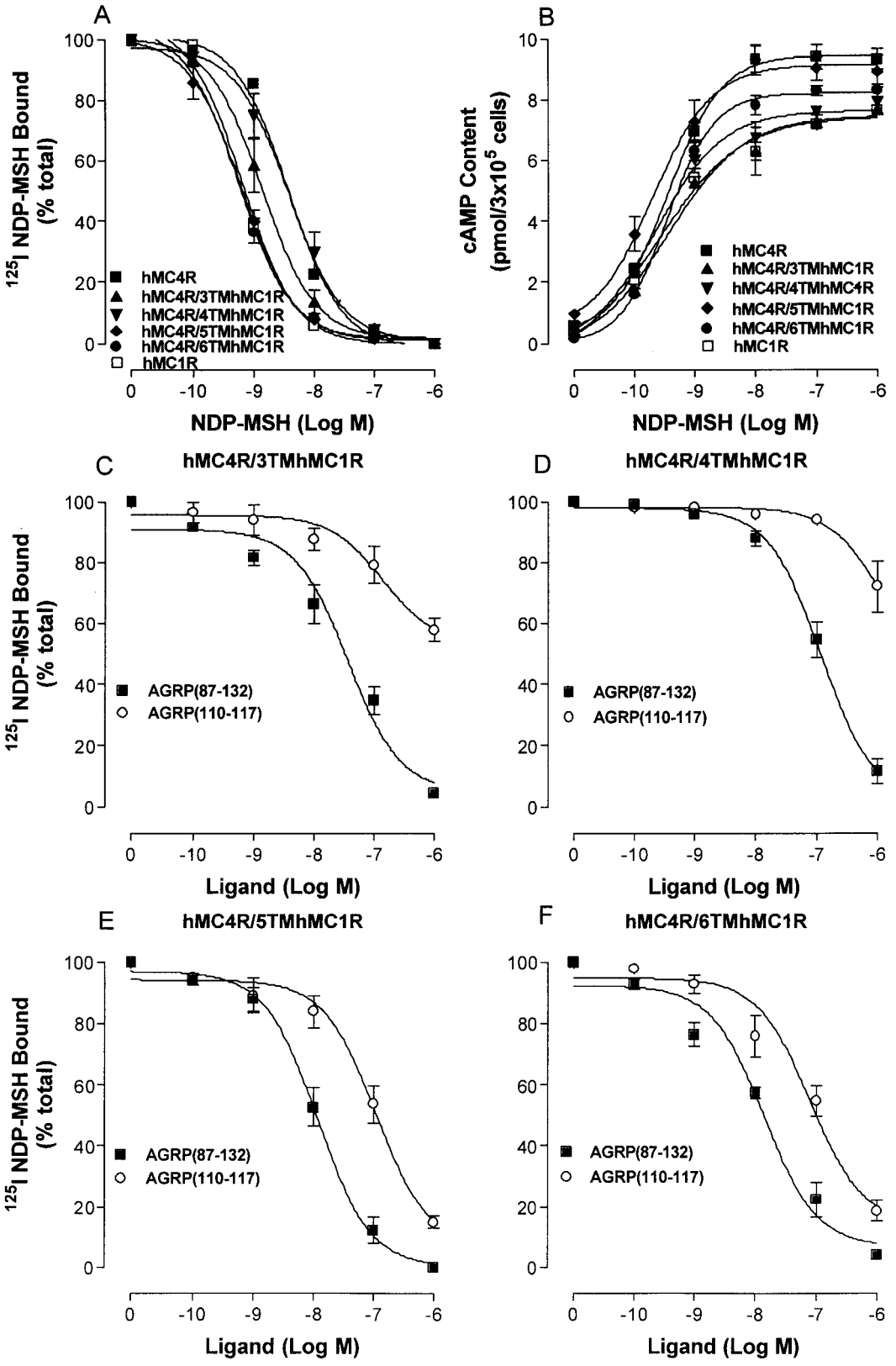

Fig. 5. Effect on AGRP activity of the substitution of the hMC4R transmembrane domain with that of hMC1R. This set of chimeric receptors consists of the basic structure of the MC4R with substitutions of various transmembrane portions of the MC1R: MC4RWT, wild-type MC4R; MC1RWT, "wild-type" MC1R; MC4R/3TMMC1R, MC4R containing TM3 of MC1R; MC4R/4TMMC1R, MC4R containing TM4 of human MC1R; MC4R/5TMMC1R, MC4R containing TM5 of MC1R; MC4R/6TMMC1R, MC4R containing TM6 of MC1R. A, displacement of the radioligand ^{125}I-NDP-MSH from hMC4R/3TMhMC1R, hMC4R/4TMhMC1R, hMC4R/5TMhMC1R, and hMC4R/6TMhMC1R by NDP-MSH. B, effect of NDP-MSH on cAMP generation on these four chimeric receptors. The data indicate the chimeric receptors are functional, and K_i and EC_{50} are shown in Tables 2 and 3. C to F, displacement of the radioligand ^{125}I-NDP-MSH from hMC4R/3TMhMC1R, hMC4R/4TMhMC1R, hMC4R/5TMhMC1R, and hMC4R/6TMhMC1R, respectively, by AGRP 87–132 and AGRP 110–117.

petitively antagonizes α-MSH at melanocortin receptors with potency equal to that of the full-length proteins (Yang et al., 1999a). This is consistent with similar findings for agouti (Yang et al., 2001). In this study, we examine the molecular basis of ligand-receptor interaction between AGRP and hMC4R by pharmacologically studying AGRP fragments and analyzing the function of melanocortin chimeric receptors. We have demonstrated that exoloops two and three of the MC4R are crucial determinants of AGRP 87–132 binding affinity and inhibitory activity, but not AGRP 110–117. TM3 and TM4 of MC4R, however, are crucial for AGRP 110–117 binding. Therefore, it is our expectation that the replacement of TM3 and TM4 of hMC4R into the sequence of the MC1R would most likely lead to the establishment of significant AGRP 100–117 activity at the MC1R.

The three-dimensional structure of AGRP suggests that AGRP 87–132 consists of three major loops that are referred to as the N-terminal loop (residues 87–105), the central loop (residues 106–119), and the C-terminal loop (residues 120–132), which is shown in Fig. 7. The three loops are held together at the base by an apparent scaffold of five disulfide bonds; these bonds stabilize the base of the active loop resi-

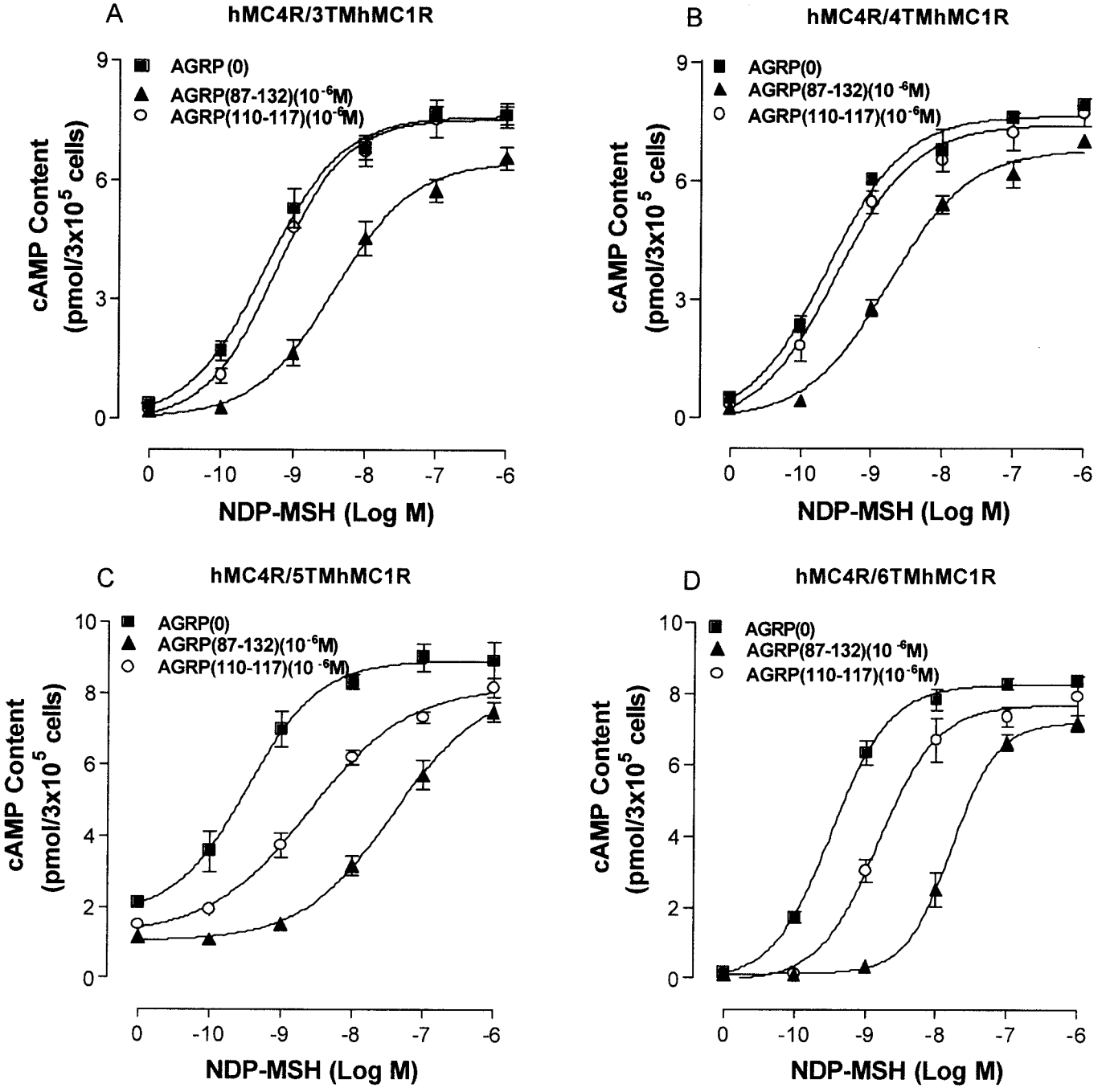

Fig. 6. Comparison of AGRP 87–132 and AGRP 110–117 inhibition of NDP-MSH–stimulated cAMP generation at the chimeric receptor hMC4R/3TMhMC1R (A), chimeric receptor hMC4R/4TMhMC1R (B), chimeric receptor hMC4R/5TMhMC1R (C), and chimeric receptor hMC4R/6TMhMC1R (D).

dues 110 to 117 that present the Arg-Phe-Phe triple on the protein surface and are exposed to the surrounding solvent (McNulty et al., 2001). The central loop AGRP 106–119 containing Arg-Phe-Phe may be critical for AGRP function, whereas the N- and C- terminal loops of AGRP 87–132 may confer receptor subtype specificity (Bolin et al., 1999). The Arg-Phe-Phe in AGRP may resemble the properties of Phe-Arg-Trp in α-MSH, His-(*D*-Phe)-Arg-Trp in MT-II (an analogue of α-MSH), or His-(*D*-Nal)-Arg-Trp in SHU9119 (an antagonist of MC4R) (Hruby et al., 1995; Tota et al., 1999). Therefore, AGRP 110–117 is particularly interesting because it is a relatively small, simple peptide consisting of only seven amino acids in the C-terminal of AGRP, AGRP 110–117. To examine whether or not AGRP 110–117 has a binding site at transmembrane domains of hMC4R and which transmembrane domains might be involved in AGRP 110–117 activity, we used chimeric receptors to determine the role of transmembrane domains of hMC4R in AGRP 110–117 binding. Our results indicate that AGRP 110–117 still possesses antagonist activity at MC4R wild type, although its activity is less potent than that of AGRP 87–132. Our results also suggest that this octapeptide is essential for AGRP binding and inhibitory activity. Our results from MCR chimeric receptors demonstrate that AGRP 110–117 has no binding site at exoloops of hMC4R but TM3 and TM4 of MC4R are required for its specific binding and support the hypothesis that the central loop AGRP 110–117 containing Arg-Phe-Phe may be critical for AGRP function, whereas the AGRP loop 87–105 and loop 120–132 may confer receptor subtype specificity.

TABLE 4
Effect of AGRP 87–132 on NDP-MSH–stimulated cAMP formation at the HEK cells transfected with TM chimera of the hMC4R

	EC_{50}		NDP-MSH + AGR 87–132
	NDP-MSH	NDP-MSH + AGRP 87–132 (10^{-6} M)	NDP-MSH
	nM		
hMC4RWT	0.89 ± 0.11	216 ± 17.2	242.7
hMC4R/TM3hMC1R	2.45 ± 0.85	30.1 ± 5.42	12.3
hMC4R/TM4hMC1R	4.30 ± 6.24	57.6 ± 6.5	13.4
hMC4R/TM5hMC1R	1.59 ± 0.42	182 ± 12.4	114.8
hMC4R/TM6hMC1R	1.80 ± 0.21	195 ± 13.7	108.3
hMC1RWT	0.63 ± 0.03	0.67 ± 0.1	1.1

TABLE 5
Effect of AGRP 110–117 on NDP-MSH–stimulated cAMP formation at the HEK cells transfected with TM chimera of the hMC4R

	EC_{50}		NDP-MSH + AGRP 110-117
	NDP-MSH	NDP-MSH + AGRP 110–117 (10^{-6}M)	NDP-MSH
	nM		
hMC4RWT	0.89 ± 0.11	67.1 ± 7.1	75.4
hMC4R/TM3hMC1R	2.45 ± 0.85	2.95 ± 0.75	1.2
hMC4R/TM4hMC1R	4.3 ± 0.42	5.7 ± 0.3	1.3
hMC4R/TM5hMC1R	1.59 ± 0.42	71.5 ± 4.2	44.9
hMC4R/TM6hMC1R	1.80 ± 0.21	79.7 ± 1.5	44.3
hMC1RWT	0.63 ± 0.03	0.66 ± 0.1	1.0

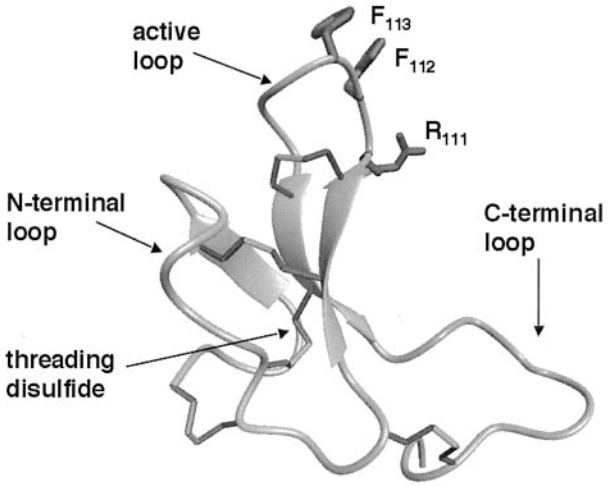

Fig. 7. Ribbon diagram of AGRP 87-132 structure. Backbone arrows represent the assigned secondary structure of AGRP (87-132). The N-terminal loop represents amino acids 87 to 105 of AGRP 87-132. The active loop represents amino acids 106 to 119 of AGRP 87-132, and the C-terminal loop represents amino acids 120–132 of AGRP 87-132. The active loop containing the Arg-Phe-Phe triplet is believed to be critical for AGRP antagonist activity. [Adapted and modified from McNulty JC, Thompson DA, Bolin KA, Wilken J, Barsh GH, and Millhauser GL (2001) High-resolution NMR structure of the chemically-synthesized melanocortin receptor binding domain AGRP(87-132) of the agouti-related protein. *Biochemistry* **40:**15520–15527. © Copyright 2001 American Chemical Society. Used with permission.]

It is possible that the pharmacological differences observed simply result from an alteration in chimeric receptor structure and are unrelated to the pharmacophore-binding pocket that, although uncharacteristic of the wild-type receptor on which they were based, permitted or discouraged AGRP binding. However, several observations favor an interpretation that specific sites of ligand-receptor interaction have been identified in these studies. First, all chimeras retained a ^{125}I-NDP-MSH binding affinity similar to that observed at wild-type MCRs. Secondly, all chimeras were activated by nanomolar concentrations of NDP-MSH. If the tertiary structure of the chimeric receptors had been drastically altered, one might expect that the ability of NDP-MSH to bind and activate these receptors should have been altered.

In summary, the present data have identified that a minimal fragment of AGRP 110–117 is able to bind to hMC4R and function as an antagonist. Chimeric receptor studies indicate that hMC4R has at least two binding regions for AGRP. Exoloops 2 and 3 of hMC4R are important for N-terminal AGRP 87–132 binding and function, and both TM3 and TM4 of hMC4R are important for C-terminal binding and function of AGRP 87–132, especially for AGRP 110–117 binding. These results also suggest that AGRP has a different binding site than that of NDP-MSH because TM4 of MC4R affects AGRP binding but not NDP-MSH. It will be important in the future to determine which amino acid residue of TM3 and TM4 are involved in AGRP specific binding and to define the importance of those residues in receptor binding and antagonism.

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