Functional properties of an agouti signaling protein variant and characteristics of its cognate radioligand

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Yang, Ying-Kui, Chris Dickinson, Yu-Mei Lai, Ji-Yao Li, and Ira Gantz. Functional properties of an agouti signaling protein variant and characteristics of its cognate radioligand. Am J Physiol Regulatory Integrative Comp Physiol 281: R1877–R1886, 2001.—Agouti signaling protein (ASIP), the human (h) homolog of agouti, is an endogenous melanocortin peptide antagonist. To date, characterization of this protein has been performed with recombinant protein only and without the availability of an ASIP/agouti radioligand. In this report we describe the functional characteristics of a chemically synthesized truncated ASIP variant, ASIP-[90–132 (L89Y)], and the binding characteristics of its cognate radioligand, 125I-ASIP-[90–132 (L89Y)]. Similar to full-length recombinant ASIP/agouti, ASIP-[90–132 (L89Y)] was a potent inhibitor of α-melanocyte-stimulating hormone cAMP generation at the cloned human melanocortin receptor (hMCR) subtypes hMC1R and hMC4R. It also displayed a lesser degree of inhibition at the hMC3R and hMC5R. However, ASIP-[90–132 (L89Y)] was found to be less potent than full-length recombinant ASIP and, surprisingly, only exhibited weak inhibitory activity at the hMC2R. In competition binding assays with the radioligand 125I-ASIP-[90–132 (L89Y)], ASIP-[90–132 (L89Y)] displayed a hierarchy of binding affinity that roughly paralleled its rank order of inhibitory potency at the various MCR subtypes, i.e., hMC1R ≈ hMC4R ≈ hMC3R ≈ hMC5R > hMC2R. Structure-activity studies revealed that ASIP-[90–132 (L89Y)] possessed greater pharmacological potency than either the further truncated ASIP variants ASIP-[116–132] or cyclo(CRFRRSAC). Interestingly, the latter molecules were both weak agonists at the hMC1R. These studies further support the concept that ASIP/agouti inhibits melanocortin action by directly binding to target MCRs and provide additional insight into the structural requirements for maximal inhibitory potency.

AGOUTI is a paracrine signaling molecule whose expression in rodents is normally limited to skin and whose biological function in that tissue is to act at the hair follicle melanocyte to modulate eumelanin (black pigment) synthesis (19). At the hair follicle melanocyte, agouti blocks the actions of the melanocortin peptide α-melanocyte-stimulating hormone (α-MSH) at the seven-transmembrane G protein-coupled melanocortin-1 receptor (MC1R) (18). This physiological antagonism induces the melanocyte to switch from eumelanin to phaeomelanin (yellow pigment) synthesis. Temporal control of agouti expression leads to the production of a banded hair phenotype, the agouti phenotype, whereas regional expression of different agouti gene isoforms is responsible for the differences in dorsal and ventral pigmentation exhibited by some mammals (29). Although there is no obvious human counterpart to banded hairs or regional differences in hair color, a human ortholog of agouti, agouti signaling protein (ASIP), has been cloned and characterized (31). mRNA for ASIP has been reported to be present in a wide range of tissues, including adipose tissue, testis, ovary, heart, and lower levels in foreskin, kidney, and liver (17, 31). However, the physiological function(s) of ASIP in these human tissues presently remains unclear.

Notably, ectopic expression of agouti in the hypothalamus of the Ay mouse with its concomitant antagonism of α-MSH at the melanocortin-4 receptor (MC4R) has also been found to be the genetic basis for the obese phenotype of that mouse (16, 18, 19). This seminal observation suggested the existence of a second endogenous protein that under normal physiological conditions antagonized the actions of α-MSH and functioned within the hypothalamus as an orexigenic agent. Subsequently, a protein with homology to agouti/ASIP, agouti-related protein (AgRP), was isolated and has since been shown by multiple researcher groups to function as a potent orexigenic factor (9, 22, 26).

α-MSH is among a group of peptides that are derived from the pro-opiomelanocortin prohormones referred to as melanocortin peptides or melanocortins. In addition to α-MSH, the melanocortins include β-MSH, γ-MSH, and adrenocorticotropic hormone (ACTH). Importantly, all melanocortins share the common core amino acid sequence HFRW. This peptide sequence has long been recognized to be important for the biological activity and potency of these peptides and has been referred to as the melanocortin “message” sequence.

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There are a total of five seven-transmembrane G protein-coupled MCR subtypes, all of which activate the stimulatory G protein $G_s$. The MC1R is the classical $\alpha$-MSH receptor expressed by the melanocyte. The MC2R is the classical adrenal cortical ACTH receptor. The MC3R and MC4R are receptors expressed primarily in brain, although the mRNA of both has been detected in several peripheral tissues. Notably, both the MC3R and the MC4R have been implicated in the hypothalamic control of appetitive behavior and metabolism (5, 6, 8, 13). The MC5R mRNA has the widest mRNA expression pattern, and its mRNA has been reported to be present in the central nervous system and many peripheral tissues, including the adrenal gland and other glandular tissues.

Agouti/ASIP and AgRP both have a COOH-terminal domain that contains 10 cysteine residues (Fig. 1). Within that domain the two melanocortin antagonists share 44% identity. Pharmacological studies using the cloned MCRs expressed in heterologous cell lines indicate that, at least in vitro, a COOH-terminal portion of murine agouti, mAgouti[Val$^{83}$–Cys$^{131}$], and a COOH-terminal portion of human AgRP (hAgRP), AgRP-(87–132), are equipotent to the corresponding full-length recombinant proteins (30, 36). Outside of this COOH-terminal domain, the two proteins share a lesser degree of homology, with one notable difference being the presence of a highly basic region in the central portion of agouti that is absent in AgRP. In this regard it is noteworthy that transgenic mice lacking the basic domain of agouti have a yellow coat color, like mutant Ay mice, but unlike A$^v$ mice are not obese (20).

NMR studies of AgRP-(87–132) suggest that the COOH-terminal 46 amino acids of this protein are characterized by three large loops stabilized by disulfide bonds (2). Those studies further suggest that the central loop is well structured and that this loop contains the amino acids RFF positioned on an external surface. Several groups have noted that the triplet RFF has biochemical characteristics similar to the melanocortin message sequence HFRW and have postulated that it may mimic this important melanocortin sequence (2, 14, 28). To date, no similar NMR studies of the COOH terminus of agouti/ASIP have been performed, and therefore structural parallels to AgRP-(87–132) must remain conjectural.

Recently it was demonstrated that the mahogany protein functions as a receptor for the NH$_2$-terminal sequence of agouti and functions to increase the local tissue concentration of agouti, although additional mechanisms are possible (7, 12). This observation is potentially relevant to the aforementioned transgenic mouse that lacks the basic agouti domain. Although the NH$_2$ terminus of AgRP does not bind mahogany protein, recently it was shown that the NH$_2$ terminus of AgRP does bind to syndecan 3, a heparan sulfate proteoglycan with an apparently novel role in feeding behavior (12, 24a).

Both agouti/ASIP and AgRP possess different spectrums of pharmacological activity at the MCR subtypes (9, 14, 18, 22, 35, 36). Studies directed toward delineating the pharmacological mechanism of ASIP action at the hMCR subtypes demonstrated that recombinant ASIP displays a surmountable inhibition at the hMC1R, hMC3R, hMC4R, and hMC5R and that its inhibitory potency is greatest at hMC1R $\approx$ hMC4R $>\approx$ hMC3R $\approx$ hMC5R (35). In those studies it was also demonstrated that full-length recombinant ASIP acted as a nonsurmountable antagonist at the hMC2R. In contrast, full-length recombinant AgRP or the truncated variant AgRP-(87–132) was demonstrated to be potent surmountable inhibitors of melanocortin action at the MC3R and MC4R with minimal inhibitory activity at the MC1R or MC2R (36). These studies and others suggest that agouti and AgRP act as competitive antagonists at

**Human Agouti (ASIP) and Human Agouti-related Protein (hAgRP)**

<table>
<thead>
<tr>
<th>hAgRP</th>
<th>hAgouti</th>
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<tr>
<td>MLTA ALLSCALLA LPATRGAQMG IAPMEGIRR</td>
<td></td>
</tr>
<tr>
<td>MVDTRLALLATL LVFLCFFTAN SHLPPEEKLR DDRSLRSNSS</td>
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Fig. 1. hAgouti, human agouti. ASIP, agouti signaling protein. C, cysteine residue. Arrowheads, signal sequence cleavage sites.
the MCRs (1, 30). However, other studies have demonstrated that both agouti and AgRP display inverse agonism, observations that suggest a more complex mechanism by which these proteins and the melanocortin peptides mediate a physiological response via the MCR (21, 27).

Direct binding of AgRP to the MCRs has been demonstrated by conventional and photoemulsion binding studies and more recently by cross-linking of AgRP to the MC4R (7, 36). However, to date, direct agouti/ASIP binding has only been inferred from pharmacological studies that did not use an agouti/ASIP radioligand. Against this background we undertook the present in vitro pharmacological studies to characterize a chemically synthesized truncated agouti variant, ASIP-[90–132 (L89Y)], and its cognate radioligand 125I-ASIP-[90–132 (L89Y)], to determine whether these compounds could serve as useful reagents for studies of melanocortin ligand-receptor interactions.

MATERIALS AND METHODS

Cyclic (cAMP) assays. ASIP-[90–132 (L89Y)] was custom synthesized by Gryphon Sciences (South San Francisco, CA) using methodology previously described for the synthesis of AgRP-[87–132] (36). The sequence of ASIP-[90–132 (L89Y)] is YSAPCVATRN SCKPPAPACC DPCASCQCRF FRSA CRVL SLNC. ASIP-[116–132] was synthesized by Phoenix Pharmaceuticals (Mountain View, CA) and was a kind gift of Dr. J. K. Chang. It eluted as one symmetrical peak by HPLC. Cyclo(CRFFRSAC) was synthesized by Research Genetics and was a kind gift of Merck, and α-MSH and human ACTH [ACTH(1–39)] were purchased from Peninsula Laboratories (Belmont, CA). cAMP generation was measured using a competitive binding assay kit (TRK 432, Amersham, Arlington Heights, IL). Heterologous cell lines stably expressing the hMC1R, hMC3R, hMC4R, and hMC5R. In the cases of the hMC1R, hMC3R, and hMC5R. In the case of the hMC2R, OS3 cell lines were used because this receptor is not functionally expressed in HEK-293 cells. For assays, culture media were removed, and cells were incubated with 0.5 ml Earle’s balanced salt solution that contained 10−6 M ASIP-[90–132 (L89Y)] and melanocortin agonist for 30 min at 37°C in the presence of 10−3 M IBMX. The reaction was stopped by adding ice-cold 100% ethanol (500 μl/well). The cells in each well were scraped and transferred to a 1.5-ml tube and centrifuged for 10 min at 1,900 g, and the supernatant was evaporated in a 55°C water bath with unpurified nitrogen gas. cAMP content was measured according to the instructions accompanying the assay kit. Each experiment was performed a minimum of three times with duplicate wells. The mean values of the dose-response data were fit to a sigmoid curve with a variable slope factor using the nonlinear least squares regression in Graphpad Prism (Graphpad Software, San Diego, CA). All statistical analyses represent the mean ± SE of the data.

Radioiodination. The stable, superpotent melanocortin agonist α-[Nle4, D-Phe7]-MSH (NDP-MSH) was purchased from Peninsula Laboratories (25). AgRP-[87–132] was purchased from Gryphon Sciences. 125I-NDP-MSH and 125I-ASIP-[90–132 (L89Y)] were prepared using chloramine-T (Sigma, St. Louis, MO). The reaction mixture was then diluted in 800 μl of 50 mM ammonium acetate (pH 5.8) and purified by reverse-phase chromatography. One-hundred microliters of a 2% solution of BSA were added to all fractions containing radioactivity.

Binding experiments. After removal of media, the cells were washed twice with MEM and then preincubated with AgRP in 0.5 ml MEM (Life Technologies) containing 0.2% BSA for 30 min before incubation with radioligand. Binding experiments were performed using conditions previously described (35, 36). 125I-NDP-MSH (−20 fmol; 1 × 105 cpm) or 125I-ASIP-[90–132 (L89Y)] (−20 fmol; 1.5 × 105 cpm) were placed into the 0.5-ml volume of MEM. Binding reactions were terminated by removing the media and washing the cells twice with MEM containing 0.2% BSA. The cells were lysed with 0.1 N NaOH, 1% Triton X-100, and the radioactivity in the lysate was quantified in an analytic gamma counter. Nonspecific binding was determined by measuring the amount of 125I label remaining bound in the presence of 10−3 M unlabeled ligand, and specific binding was calculated by subtracting nonspecifically bound radioactivity from total bound radioactivity. Typically, total binding of 125I-ASIP-[90–132 (L89Y)] was 3 × 104 cpm, and nonspecific binding was ~26% of total binding for the hMC1R, 39% of total binding for the hMC2R, 35% of total binding for the hMC3R, 18% of total binding for the hMC4R, and 53% of total binding for the hMC5R.

RESULTS

Biological activity of ASIP-[90–132 (L89Y)]. As our initial step in the characterization of the chemically synthesized ASIP variant ASIP-[90–132 (L89Y)], we sought to assess its biological activity. This activity was assessed using two assays. First, biological activity was assessed by the ability of ASIP-[90–132 (L89Y)] to inhibit melanocortin-stimulated cAMP generation at the five MCR subtypes expressed in heterologous cell lines. As shown in Fig. 2, ASIP-[90–132 (L89Y)] is a potent inhibitor of α-MSH-stimulated cAMP generation at the MC1R and MC4R. A 10−6 M concentration of ASIP-[90–132 (L89Y)] shifted the dose-response curve of α-MSH two logs to the right at the hMC1R and three logs to the right at the hMC4R. ASIP-[90–132 (L89Y)] had a much lesser degree of inhibitory potency at the hMC2R, hMC3R, and hMC5R. At a concentration of 10−6 M ACTH-(1–39), ASIP-[90–132 (L89Y)] only shifted the dose-response curve twofold at the hMC2R (note: ACTH was used in the case of MC2R, or the ACTH receptor, because it is not activated by α-MSH). ASIP-[90–132 (L89Y)] had roughly equal potency at the hMC3R and the hMC5R, shifting the dose-response curve approximately one log to the right. EC50 values are in the legend of Fig. 2.

As shown in Table 1, compared with our previous published determinations of the inhibitory potency of 3 × 10−7 M full-length recombinant ASIP, 3 × 10−7 M ASIP-[90–132 (L89Y)] was approximately one log less potent at inhibiting α-MSH-stimulated cAMP generation at the hMC4R and nearly two logs less potent at...
inhibiting \(\alpha\)-MSH-stimulated cAMP generation at the hMC1R.

As a second means of assessing the biological activity of the synthetic ASIP variant ASIP-[90–132 (L89Y)], we examined its ability to displace 125I-NDP-MSH from the cloned MCRs expressed in competition binding assays using the same cell lines. Figure 3A depicts the ability of NDP-MSH to displace the radioligand 125I-NDP-MSH from the cloned hMCR subtypes hMC1R, hMC3R, hMC4R, and hMC5R (note: the MC2R does not bind 125I-NDP-MSH and is therefore not represented in Fig. 3A). NDP-MSH is noted to have approximately equal affinity for the hMC1R and hMC4R, a somewhat lower affinity for the hMC5R, and possesses an even lower affinity at the hMC3R (IC50 values are in the legend of Fig. 3). In comparison, Fig. 3B shows the ability of ASIP-[90–132 (L89Y)] to displace 125I-NDP-MSH from the cloned hMC1R, hMC3R, hMC4R, and hMC5R subtypes. ASIP-[90–132 (L89Y)] has roughly equal affinity for the hMC1R and hMC4R and a much lower binding affinity for the hMC3R and hMC5R (IC50 values are in the legend of Fig. 3). ASIP-[90–132 (L89Y)] was only able to displace approximately 50 and 65% of the radiolabel from the hMC3R and hMC5R, respectively.

### Table 1. EC50 of \(\alpha\)-MSH in the presence of 3 \(\times\) 10^{-7} M full-length recombinant ASIP or ASIP-[90–132(L89Y)]

<table>
<thead>
<tr>
<th>Recombinant ASIP</th>
<th>ASIP-[90–132(L89Y)]</th>
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<tr>
<td>hMC1R</td>
<td>6.0 (\times) 10^{-6} M</td>
</tr>
<tr>
<td>hMC4R</td>
<td>4.0 (\times) 10^{-6} M</td>
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\(\alpha\)-MSH, \(\alpha\)-melanocyte-stimulating hormone; ASIP, agouti signaling protein; hMC1R and hMC4R, human melanocortin receptor subtypes 1 and 4.

As a second means of assessing the biological activity of the synthetic ASIP variant ASIP-[90–132 (L89Y)], we examined its ability to displace 125I-NDP-MSH from the cloned MCRs expressed in competition binding assays using the same cell lines. Figure 3A depicts the ability of NDP-MSH to displace the radioligand 125I-NDP-MSH from the cloned hMCR subtypes hMC1R, hMC3R, hMC4R, and hMC5R (note: the MC2R does not bind 125I-NDP-MSH and is therefore not represented in Fig. 3A). NDP-MSH is noted to have approximately equal affinity for the hMC1R and hMC4R, a somewhat lower affinity for the hMC5R, and possesses an even lower affinity at the hMC3R (IC50 values are in the legend of Fig. 3). In comparison, Fig. 3B shows the ability of ASIP-[90–132 (L89Y)] to displace 125I-NDP-MSH from the cloned hMC1R, hMC3R, hMC4R, and hMC5R subtypes. ASIP-[90–132 (L89Y)] has roughly equal affinity for the hMC1R and hMC4R and a much lower binding affinity for the hMC3R and hMC5R (IC50 values are in the legend of Fig. 3). ASIP-[90–132 (L89Y)] was only able to displace approximately 50 and 65% of the radiolabel from the hMC3R and hMC5R, respectively. Binding studies were not performed on the hMC2R using an ACTH radiolabel because cAMP assays (Fig. 2) indicated that this MCR...
has a very low affinity for ASIP-[90–132 (L89Y)], and such studies would therefore be uninformative.

ASIP-[90–132 (L89Y)] radioligand. Having demonstrated that ASIP-[90–132 (L89Y)] has a pharmacological profile that reflects important in vivo activities previously reported for endogenous ASIP, i.e., high binding affinity and inhibitory potency at the hMC1R and hMC4R, we sought to determine if this protein could be iodinated and serve as an MCR radioligand. As shown in Fig. 4, we examined the binding characteristics of 125I-ASIP-[90–132 (L89Y)] in competition binding studies with ASIP-[90–132 (L89Y)], AgRP-(87–132), and NDP-MSH at the cloned hMCRs. As shown in Fig. 4A, ASIP-[90–132 (L89Y)] was capable of completely displacing 125I-ASIP-[90–132 (L89Y)] from the hMCR subtypes examined with a hierarchy of displacement: hMC4R ≈ hMC1R > hMC3R ≈ hMC2R > hMC5R (IC50 values are in the legend of Fig. 4). As shown in Fig. 4B, NDP-MSH had a roughly equivalent ability to displace 125I-ASIP-[90–132 (L89Y)] from the hMC1R, hMC3R, and hMC4R. However, NDP-MSH was unable to displace 125I-ASIP-[90–132 (L89Y)] from the hMC5R (IC50 values are in the legend of Fig. 4). As shown in Fig. 4C, AgRP-(87–132) had an equivalent ability to displace 125I-ASIP-[90–132 (L89Y)] from the hMC3R and hMC4R. However, it lacked an ability to completely displace 125I-ASIP-[90–132 (L89Y)] from the hMC1R and hMC5R, and the displacement curves at both receptors were greatly shifted to the right (IC50 values are in the legend of Fig. 4).

Structure-activity studies. Having demonstrated the biological activity of ASIP-[90–132 (L89Y)] and the biological activity of ASIP-[90–132 (L89Y)] and the
ability to serve as a suitable radioligand, we sought to use the novel reagent for structure-activity studies of ASIP at the MCRs. In these studies we compared the binding affinity and inhibitory potency of the further truncated protein ASIP-(116–132), which lacks the NH2-terminal portion of the ASIP cysteine motif, with the binding affinity and inhibitory potency of ASIP-[90–132 (L89Y)]. We also compared these with the binding affinity and potency of the cyclic octapeptide, cyclo(CRFFRSAC), which contains amino acids 116–123 of ASIP, a COOH-terminal portion of ASIP that encompasses two disulfide-linked cysteine residues and the sequence RFF, which has been postulated to mimic the melanocortin message sequence HFRW (2, 14, 28). As depicted in Fig. 5, A and B, both ASIP-(116–132) and cyclo(CRFFRSAC) have a markedly lower affinity for the hMC1R and hMC4R than the larger ASIP-[90–132 (L89Y)]. As depicted in Fig. 6, at high concentrations, ASIP-(116–132) and cyclo(CRFFRSAC) are partial agonists at the hMC1R. However, EC50 values could not be calculated because these responses did not reach a plateau. At the same concentrations, neither peptide displayed agonism at the hMC4R (data not shown). Figure 7 demonstrates that both ASIP-(116–132) and cyclo(CRFFRSAC) are inhibitory at the hMC4R (EC50 values are included in the legend of Fig. 7).

DISCUSSION

The melanocortin receptors are unique among the family of seven-transmembrane G protein-coupled receptors described to date in that they possess both endogenous agonists and antagonists that appear to act as competing ligands for the same receptor. However, the exact physical nature of this antagonism remains incompletely understood. Several possible mechanisms for this competitive interaction exist. The melanocortin peptides and their antagonists could have identical ligand-receptor interactions (same ligand binding pocket). Alternatively, the two could have overlapping binding epitopes with each possessing some unique ligand-receptor interactions. Yet another possibility is that the antagonists bind allosterically and modify the agonist binding pocket. Relevant to this topic of ligand-receptor interactions is the fact that the overall structure of the melanocortin peptides and their antagonists appears to be quite dissimilar. α-MSH is a linear tridecapeptide, whereas human agouti and AgRP are 112-amino acid proteins (without their signal sequences) with well-defined disulfide linkages in the COOH terminus. This physical distinction would suggest that the agonists and antagonists should have at least some dissimilar receptor binding epitopes. Supporting such an interpretation is the observation that MCR exoloops are important to ASIP/AgRP binding (directly or indirectly), whereas these domains do not appear to be critical to agonist binding (23, 32–34). On the other hand, it is presently thought that certain binding epitopes of the melanocortins and their antagonists may overlap (i.e., the RFF motif of ASIP/AgRP mimicking the HFRW core melanocortin sequence) (2, 14, 28). An important question that arises from these observations is whether there are sufficient differences in the binding epitopes of the melanocortins and their agonists at the MCRs that ASIP/AgRP binding could be interrupted without interfering with agonist peptide binding.

Answering the above question rests in part with defining the exact nature of the interaction of melanocortin peptides and their antagonists at the MCRs. In turn, this is dependent on the availability of appropriate peptides and radioligands for use in the study of those interactions. Although it has been possible to study the interaction of AgRP with the MCRs with the use of a radioligand previously characterized in this laboratory,125I-AgRP-(87–132), studies of agouti/ASIP have been hampered by the lack of a similar radioligand (presently, truncated AgRP variant radioligands are commercially available). This was due in part to the lack of availability of a biologically active chemically synthesized agouti/ASIP molecule suitable for iodination and in part to the absence of a readily iodinatable tyrosine residue within the sequence of agouti/ASIP. The need for a suitable ASIP protein and radioligand is even more important because AgRP does not bind to the MC1R or MC2R so that125I-AgRP-(87–132) cannot be utilized for studies with those MCRs.
subtypes. We therefore designed a truncated agouti/ASIP variant similar to the one we had previously designed for AgRP and for purposes of iodination added a tyrosine residue to its NH2 terminus. Figure 1 can be used to discern that the resultant truncated ASIP variant ASIP-[90–132 (L89Y)] encompasses the entire cysteine motif of this protein. Notably, however, it lacks any basic residues of the basic domain of agouti (see below).

Data depicted in Fig. 2 indicate that the biological activity of the truncated ASIP variant ASIP-[90–132 (L89Y)] is similar in its pattern of inhibition to full-length recombinant agouti/ASIP at the hMC1R, hMC3R, hMC4R, and hMC5R. Like full-length recombinant ASIP, the truncated variant ASIP-[90–132 (L89Y)] possesses its greatest potency and affinity for the hMC1R and hMC4R, with lesser degrees of potency and affinity at the hMC3R and hMC5R. However, in the present studies full-length recombinant ASIP displayed a potent, nonsurmountable pattern of melanocortin antagonism at the hMC2R, which contrasted with the surmountable antagonism observed at all other MCR subtypes (35). One seemingly obvious explanation for the discrepancy between the activity of full-length recombinant ASIP and ASIP-[90–132 (L89Y)] at the hMC2R is the absence of the ASIP NH2 terminus. Under the umbrella of such an explanation, one possibility would be that the MC2R requires additional ASIP/agouti binding determinants not used by other MCR subtypes. In a sense, this could be viewed as analogous to the requirement the hMC2R has for a “larger” melanocortin, i.e., the hMC2R does not bind α-MSH but does bind ACTH-(1–24) [note: desacetyl α-MSH = ACTH-(1–13)]. In this regard it will be of some importance for future studies to determine the minimum agouti/ASIP sequence necessary for high-affinity MC2R binding.

An alternative explanation for the dramatic loss of binding affinity and inhibitory activity of ASIP-[90–132 (L89Y)] at the hMC2R is that an ancillary protein is necessary for high-affinity agouti/ASIP binding. Such an ancillary protein could function either by binding to ASIP within the sequence encompassed by ASIP-[90–132 (L89Y)] or by binding to an auxiliary protein that, in turn, interacts with ASIP to enhance its binding affinity to the MC2R. Regardless of the mechanism, however, it is clear from our studies that the MC2R requires additional ASIP/agouti binding determinants when compared to other MCR subtypes.
radioligand \(^{125}\text{I}\)-ASIP-[\(90–132\) (L89Y)] to perform sev-
all three ligands possess at least some common hMC4R
versely, from the ligand perspective, this suggests that
vations are important in that they suggest that the
natively, despite the homogeneous appearance on
truncated ASIP variants were used, ASIP-(116–132)
truncated ASIP sequence ASIP-(116–123). As might
be further truncated to cyclo(CRFFRSAC), cyclized
binding affinity displayed at the hMC4R (Fig. 5, a and B).
However, unlike their discordant binding affinity displayed at the hMC1R, both ASIP-(116–132) and cyclo(CRFFRSAC) displayed a nearly identical binding affinity at the hMC4R (Fig. 5, A vs. B). Although this observation is consistent with the known affinities of the two receptors for ASIP and AgRP (see above), it also suggests that the COOH-ter
terminal nine amino acids of ASIP add little in terms of
hMC4R binding affinity. That is, ASIP-(116–132) can
be further truncated to cyclo(CRFFRSAC), cyclized
ASIP-(116–123), without loss of binding affinity. This
notion appears to be corroborated by the inhibitory characteristics of these two further truncated mole-
cules in cAMP assays (see Fig. 7 and DISCUSSION). How-
ever, the lack of necessity for a COOH terminus for
high-affinity binding may only be true at the hMC4R
because differential binding of ASIP-(116–132) and
cyclo(CRFFRSAC) was observed at the hMC1R. Alter-
atively, the recent report that it is the NH\(_2\)-terminal
natural affinity for ASIP/agouti and AgRP (i.e., affinity
for AgRP > ASIP/agouti) (9, 14, 35, 36). These ob-
ervation is in that they suggest that the
hMC4R, unlike the other MCRs, has at least some
common binding surfaces for all three ligands. Con-
versely, from the ligand perspective, this suggests that
all three ligands possess at least some common hMC4R
binding epitopes.

In the present studies we used the newly developed
radioligand \(^{125}\text{I}\)-ASIP-[\(90–132\) (L89Y)] to perform sev-
structure-activity studies of agouti/ASIP to gain
more AgRP octapeptide cyclo(CRFFNAFC) have inhib-
atory activity at the hMC4R (28). Interestingly, as
shown in Fig. 7, the present studies demonstrate that
the octapeptide cyclo(CRFFRSAC) is just as potent an
inhibitor of melanocortin action as ASIP-(116–132). This
observation is entirely congruous with the simi-
larity in binding displacement that these molecules
displayed at the hMC4R (Fig. 5B). The importance of
this observation is that it suggests that the COOH-
terminl nine amino acids of ASIP-(87–132) may not be
critical for biological activity. In this regard it is
worthy to note that the NMR structure of AgRP-(87–
132) indicates that the COOH-terminal loop of that
protein, which encompasses an analogous region of
ASIP, is the least well-structured of its three loops, an
observation that suggests a less critical contribution of
that region to ligand-receptor interactions. Therefore,
if one speculates that agouti structure is similar to that
of AgRP, one might anticipate that the COOH-terminal
loop of agouti is also less well structured.

In summary, this report describes the in vitro func-
tional characteristics of the truncated ASIP variant
ASIP-[\(90–132\) (L89Y)] and the binding characteristics of
\(^{125}\text{I}\)-ASIP-[\(90–132\) (L89Y)]. This chemically syn-
thesized molecule and its cognate radioligand should
prove to be of considerable value for future in vivo and
in vitro biological and pharmacological studies. ASIP-
[\(90–132\) (L89Y)] is also likely to be of use in NMR
studies to determine the three-dimensional structure of
the COOH-terminal portion of ASIP. It will be of
substantial scientific importance to compare the NMR
structure of this important regulatory protein with
that already defined for AgRP-(87–132) (1). Finally,
these data provide further evidence that indicate ag-
outi/ASIP directly interacts at a molecular level with the MCRs and provide new insights into the structure-function of that regulatory protein.

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