Synthesis and Pharmacological Characterization of Adrenocorticotropic Hormone (ACTH 1-24) and C-Terminal Truncated Analogues Identifies the Minimal ACTH N-Terminal Fragment Required for Melanocorton-2 Receptor Activation

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Abstract

The melanocortin system has been implicated in regulating various physiological pathways including skin and hair pigmentation, energy homeostasis, food intake, steroidogenesis, and exocrine gland regulation. Five melanocortin receptors (MCRs) have been identified to date that are activated by agonist peptide ligands derived from the proopiomelanocortin (POMC) prohormone. While the MC1R, MC3R, MC4R, and MC5R can be activated by several of these ligands, the MC2R is unique in that it is only activated by the adrenocorticotropic hormone (ACTH). The aim of the present study was to identify the minimal N-terminal fragment of ACTH that can fully stimulate the MC2R. A series of 12 peptides, representing C- to N-terminal truncated analogs from the starting ACTH(1–24) ligand, were synthesized and pharmacologically characterized in parallel at the five melanocortin receptors. While truncation had minimal effects (≤ 6-fold) at the MC1R, MC3R, MC4R, and MC5R, the basic tetrapeptide Lys-Lys-Arg-Arg sequence corresponding to residues 15–18 of ACTH was identified to be critical for agonist potency at the MC2R. Sequential removal of these residues decreased potency 8-, 114-, 1000-, and > 6500-fold relative to the ACTH(1–24) ligand, with the minimal sequence stimulating the MC2R being ACTH(1–15) (EC_{50} = 1450 nM). These results correlate to the cryo-EM structure reported in 2023 of ACTH-MC2R-MRAP1 which showed these basic ACTH residues make several interactions with MRAP1.

INTRODUCTION

The melanocortin system consists of several endogenous agonists derived from the proopiomelanocortin (POMC) prohormone (Nakanishi et al. 1979), the naturally occurring antagonists agouti and agouti-related protein (AGRP) (Lu et al. 1994; Ollmann et al. 1997; Fong et al. 1997; Shutter et al. 1997), and five melanocortin receptors (MCR) identified to date (Chhajlani and Wikberg 1992; Mountjoy et al. 1992; Chhajlani et al. 1993; Gantz et al. 1993a; Gantz et al. 1993b; Gantz et al. 1994; Griffon et al. 1994; Roselli-Rehfuss et al. 1993). This receptor family and cognate ligands regulate numerous biological pathways, including those involved in skin and hair pigmentation (MC1R) (Robbins et al. 1993), steroidogenesis (MC2R) (Clark et al. 1993), appetite and energy homeostasis (MC3R and MC4R) (Vaisse et al. 1998; Yeo et al. 1998; Lam et al. 2021), and exocrine gland function in rodents (MC5R) (Chen et al. 1997). Due to the variety of physiological processes that are controlled by the melanocortin receptors, the development of potent, selective ligands for receptor subtypes may generate therapeutic leads that bypass off-target effects that result from nonselective activation of the melanocortin receptors. Structure-activity relationship (SAR) studies using classic peptide medicinal chemistry techniques (alanine and D-amino acid scans, cyclization, truncation studies, etc.) on the endogenous ligands may assist in the design of new, potent melanocortin compounds

The POMC prohormone is processed into several different ligands, including α-melanocyte stimulating hormone (α-MSH), β-MSH, γ-MSH, and the adrenocorticotropic hormone (ACTH). The α-MSH peptide is derived from ACTH, composing the 13 N-terminal residues of ACTH (with accompanying N-terminal acetylation and C-terminal amidation relative to ACTH; Table 1). Common to POMC-derived agonists is the tetrapeptide sequence His-Phe-Arg-Trp. Truncation studies have demonstrated that this tetrapeptide, when acetylated at the N-terminal and possessing a C-terminal amidate functionality, is the minimally active all-L amino acid sequence in the *Anolis carolinensis* lizard (Castrucci et al. 1989) and *Rana pipiens* frog (Hruby et al. 1987) skin bioassays, and possesses micromolar agonist activity at the cloned mMC1R, mMC3R and mMC4R and nanomolar potency at the mMC5R (Haskell-Luevano et al. 2001). Such studies are important to identify the minimal pharmacophore required for receptor activation, which can then be utilized for further SAR experiments to optimize potency and/or selectivity.

Table 1

Agonist pharmacology of ACTH(1–24) and truncated peptides at the mMC1R, mMC3R, mMC4R, mMC5R, and hMC2R.[a]

ID	Peptide	Truncation	Structure	mMC1R	mMC3R	mMC4R	mMC5R	hMC2R	Fold Change
				EC_{50} (nM)					
	α-MSH		Ac-SYSMEHFRWGKPV-NH_2	9.0 ± 0.9	18.2 ± 7.0	60 ± 33	16.6 ± 3.7	NA	
1	AT727-1	ACTH(1–24)	SYSMEHFRWGKPVGKKRRPVKVYP	19.6 ± 7.5	18.6 ± 6.5	29.1 ± 10.6	10.2 ± 3.0	1.45 ± 1.02	1
2	AT727-2	ACTH(1–23)	SYSMEHFRWGKPVGKKRRPVKVY	27.4 ± 12.3	17.0 ± 3.8	52.3 ± 15.2	7.8 ± 1.5	8.8 ± 6.0	6
3	AT752-1	ACTH(1–22)	SYSMEHFRWGKPVGKKRRPVKV	22.7 ± 3.8	11.7 ± 3.4	94.3 ± 31.9	9.07 ± 3.22	1.19 ± 0.52	
4	AT752-2	ACTH(1–21)	SYSMEHFRWGKPVGKKRRPVK	38.5 ± 17.9	19.2 ± 8.3	158.9 ± 42.3	26.3 ± 8.7	0.91 ± 0.38	
5	AT760-1	ACTH(1–20)	SYSMEHFRWGKPVGKKRRPV	64.3 ± 34.7	66.1 ± 33.6	187.9 ± 45.1	41.9 ± 17.3	0.28 ± 0.07	-5
6	AT760-2	ACTH(1–19)	SYSMEHFRWGKPVGKKRRP	21.8 ± 8.1	70.0 ± 38.0	142.5 ± 53.3	22.0 ± 9.6	2.39 ± 1.12	
7	AT7100-1	ACTH(1–18)	SYSMEHFRWGKPVGKKRR	38.6 ± 20.5	59.8 ± 33.3	120.0 ± 46.5	37.8 ± 21.1	1.51 ± 0.79	
8	AT7100-2	ACTH(1–17)	SYSMEHFRWGKPVGKKR	22.6 ± 11.3	28.4 ± 16.4	78.3 ± 25.1	7.8 ± 4.2	11.3 ± 0.8	8
9	AT7107-1	ACTH(1–16)	SYSMEHFRWGKPVGKK	8.41 ± 3.58	26.8 ± 19.0	88.2 ± 51.1	18.0 ± 11.0	165 ± 50	114
10	AT7107-2	ACTH(1–15)	SYSMEHFRWGKPVGK	51.3 ± 25.0	70.0 ± 53.8	119 ± 80	13.9 ± 7.8	1450 ± 400	1,000
11	AT7141-1	ACTH(1–14)	SYSMEHFRWGKPVG	25.8 ± 15.6	3.39 ± 1.18	30.1 ± 16.2	3.91 ± 2.04	> 10,000	> 6,500
12	AT7141-2	ACTH(1–13)	SYSMEHFRWGKPV	39.8 ± 20.3	3.75 ± 1.32	80.8 ± 22.0	4.33 ± 1.80	> 10,000	> 6,500

[a]The indicated errors represent the standard error of the mean determined from at least three independent experiments. NA indicates that the compound was not assayed at the indicated receptor. >10,000 indicates that the compound was not activity at concentrations up to 10 μM.

While Ac-His-Phe-Arg-Trp-NH_2 and endogenous α-MSH, β-MSH, γ-MSH, and ACTH peptides stimulate the MC1R, MC3R, MC4R, and MC5R, ACTH is the only POMC-derived ligand that activates the MC2R (Schioth et al. 1996). Although α-MSH is contained in the first 13 amino acids of ACTH, α-MSH cannot displace radiolabeled ^{125}I-ACTH from the MC2R (Schioth et al. 1996), indicating that the extra C-terminal residues found in ACTH are required for MC2R activation. The sequence of ACTH was reported in 1954 as a 39-residue peptide (Bell 1954). Early reported chemical syntheses of biologically active ACTH fragments (Boissonnas et al. 1956; Li et al. 1961; Hofmann et al. 1962a) and ACTH(1–39) (Schwyzer and Sieber 1963) coincide with the advent of solid phase peptide synthesis (Merrifield 1963). Although extensive work has been performed on the ACTH scaffold, as reviewed (Schwyzer 1977; Medzihradszky 1976), early syntheses were done in solution by fragment condensation approaches. Individual analogs were synthesized, assayed in different *in vitro* and *in vivo* assays, and reported. While this created a large collection of fragment activities, the lack of parallel screening of systemically truncated ACTH analogs complicates the identification of the minimal sequence necessary for MC2R activity. The first 24 residues of ACTH [ACTH(1–24)] has been reported to induce the same maximal response and be equipotent to ACTH(1–39) in isolated rat adrenal cortex cells (Schwyzer et al. 1971), indicating that removal of the 15 C-terminal amino acids does not affect potency *in vitro*. A series of truncated analogs corresponding to ACTH(1–10), ACTH(1–14), ACTH(1–15), ACTH(1–16), ACTH(1–17), ACTH(1–24), and ACTH(1–39) has been reported (Chen et al. 2007), but changes relative to the native sequence in these analogs (N-terminal acetylation, replacement of Met4 with Nle) may alter the activity of the fragments in a manner differentially than the endogenous peptide, and may not correspond to the naturally occurring minimal sequence.

An additional protein is also required for ACTH-mediated activation of the MC2R. The melanocortin receptor accessory protein (MRAP) family was identified in patients with familial glucocorticoid deficiency without corresponding mutations in the MC2R and was hypothesized to have a role in trafficking the MC2R to the cell surface (Metherell et al. 2005). In addition to any potential role in surface expression, the 2023 cyro-EM structure of the MC2R with ACTH also demonstrates that MRAP1 is important for ligand binding, creating a "seat-belt" that fastens ACTH within the active pocket of the MC2R (Luo et al. 2023). Within this cryo-EM structure, the first 18 residues of ACTH are observed (Luo et al. 2023), indicating that these residues are structured and not randomly arranged during ligand binding. This supports the observations that residues outside the first 13 amino acids of ACTH are required for receptor activation.

To determine the minimal N-terminal fragment of ACTH required for activation of the MC2R, herein a series of ACTH truncated analogs were synthesized and assayed in parallel. Starting with ACTH(1–24) [equipotent to ACTH(1–39)] (Schwyzer et al. 1971), residues were sequentially removed from the C-terminal. The smallest peptide, ACTH(1–13) (**12**), corresponds to the sequence of α-MSH with different terminal groups (a free amine at the N-terminal and carboxylic acid at the C-terminal relative to α-MSH; Table 1). These fragments were also assayed in parallel at the MC1R, MC3R, MC4R, and MC5R to determine if the truncations altered *in vitro* pharmacological properties at these receptors. Parts of this work have previously been presented as part of conference proceedings (Haskell-Luevano et al. 2004).

RESULTS

Chemical Synthesis and Characterization. Peptides were synthesized manually using standard fluorenylmethyloxycarbonyl (Fmoc) chemistry (Carpino and Han 1970; Carpino and Han 1972) and cleaved in manual reaction vessels or in a peptide synthesizer (Advanced ChemTech 440MOS, Louisville, KY). The peptides were purified using semi-preparative reversed-phase high pressure liquid chromatography (RP-HPLC), purity measured by analytical RP-HPLC in two diverse solvent systems, and exact masses determined by MALDI-TOF mass spectrometry (Protein Core Facility, University of Florida).

Biological results. Compounds were dissolved at stock concentrations of 10^{-2} or 10^{-4} M (depending on ligand potency), and serially diluted (10^{-4} to 10^{-10} M or 10^{-6} to 10^{-12} M, respectively). To determine agonist potency at the mMC1R, mMC3R, mMC4R, and mMC5R, HEK293 stably expressing these receptors were used in a β-galactosidase assay (Chen et al. 1995). To determine agonist potency at the hMC2R, OS3 cells were used with a commercially purchased enzymeimmunoassay (EIA). Due to the inherent errors of the assays, compounds within a 3-fold potency range were considered equipotent.

The non-selective endogenous agonist α-MSH possessed nanomolar activity at the mMC1R, mMC3R, mMC4R, and mMC5R (Table 1). Similar activity was observed for the ACTH(1–24) ligand (**1**) at these receptors. Compared to ACTH(1–24), serial truncation from the C-terminal domain did not alter potency following removal of residues 24 through 14 at the mMC1R. At the mMC3R, three truncated analogs [ACTH(1–20), **5**; ACTH(1–19), **6**; ACTH(1–15), **10**] possessed 4-fold decreased potency, and two truncated analogs [ACTH(1–14), **11**; ACTH(1–13), **12**] possessed 5-fold increased potency compared to **1** [ACTH(1–24)]. None of the truncated analogs were more potent at the mMC4R compared to **1**, and 5 analogs were 4–6 fold less potent [ACTH(1–21), **4**; ACTH(1–20), **5**; ACTH(1–19), **6**; ACTH(1–18), **7**; ACTH(1–15), **10**]. At the mMC5R, two truncated analogs of ACTH [ACTH(1–20), **5**; ACTH(1–18), **7**] were 4-fold less potent than **1**. These results demonstrate that serial C-terminal truncation of ACTH(1–24) down to 13 thirteen residues (the same amino acids as α-MSH with changes at both termini) results in at most a 6-fold change in potency at the mMC1R, mMC3R, mMC4R, and mMC5R.

When assayed at the hMC2R, a greater potency change was observed for truncated analogs of ACTH. Peptide **1** [ACTH(1–24)] possessed single digit nanomolar potency at the hMC2R (Table 1). Serial truncation of residues 24 to 19 resulted in peptides with single digit nanomolar to sub-nanomolar potency at the hMC2R, with ACTH(1–23) (**2**) possessing a 6-fold decrease in potency and ACTH(1–20) (**5**) possessing a 5-fold increase in potency at the hMC2R compared to **1**. Sequential removal of the Lys-Lys-Arg-Arg basic tetrapeptide sequence (residues 15–18) resulted in a serial loss of potency at the hMC2R. Removal of Arg18 [resulting in peptide ACTH(1–17), **8**] resulted in an 8-fold decrease in agonist potency at the hMC2R compared to **1** (11.3 versus 1.45 nM, respectively). Truncation of the next Arg residue [Arg17, ACTH(1–16), **9**] further decreased potency at the hMC2R (165 nM), representing a 114-fold decrease compared to **1**. Eliminating Lys16 resulted in ACTH(1–15) (**10**), which possessed 1450 nM potency at the hMC2R (1,000-fold decrease compared to **1**). Removal of Lys15, generating peptide ACTH(1–14) (**11**), resulted in no observable agonist potency at the hMC2R at concentrations up to 10,000 nM (at least a 6,500-fold decrease compared to **1**). Similarly, ACTH(1–13) (**12**) did not possess agonist potency at the hMC2R at the concentrations assayed (EC_{50} > 10,000 nM). These results demonstrate that the basic tetrapeptide sequence corresponding to residues 15–18 in ACTH is critical for functional activity at the hMC2R.

DISCUSSION

Naturally-derived ACTH is composed of 39 amino acids, with a shorter analogue, ACTH(1–24), being equipotent (Schwyzer et al. 1971). It has been hypothesized that the role of the C-terminus residues in the full length ACTH [ACTH(1–39)] is to protect the bioactive form of ACTH molecule, as reviewed (Schwyzer 1977). Although the first thirteen residues of ACTH and α-MSH are identical, α-MSH possesses agonist activity at the MC1R, MC3R, MC4R, and MC5R, and cannot stimulate the MC2R. Conversely, ACTH is an agonist at all five subtypes of MCR. The differential activity at the MC2R between ACTH and α-MSH would be hypothesized to be due to 11 C-terminal residues in ACTH (residues 14 through 24). The goal of the present study was to synthesize and pharmacologically characterize in parallel ACTH(1–24) and C- to N-terminal truncated analogs to identify the minimal N-terminal fragment of ACTH that can elicit a pharmacological response at the hMC2R. While many truncated forms of ACTH have been synthesized and evaluated, as reviewed (Medzihradszky 1976; Schwyzer 1977), differences in sequences, N- and C-terminal modifications, and assays utilized to analyze ligands complicate the interpretation of the required minimal sequences.

All ACTH peptides (**1**–**12**) possessed full agonist efficacy and nanomolar potency at the MC1R, MC3R, MC4R and MC5R (Table 1). Since both ACTH and α-MSH are full agonists at these receptors, the minimal differences in potency [≤ 6-fold comparing truncated analogs to ACTH(1–24), **1**] may not be surprising. The 11 C-terminal residues of ACTH therefore appear to have a minimal impact on the pharmacology of ACTH at the MC1R, MC3R, MC4R, and MC5R.

While minimal differences in potency were observed at the MC1R, MC3R, MC4R, and MC5R, a much more variable response was observed at the MC2R (Table 1). Peptides **1**–**7**, corresponding to ACTH(1–24) (**1**) and truncated from the C-terminal to ACTH(1–18) (**7**) all possessed single-digit or sub-nanomolar agonist potency at the hMC2R. Sequential removal of the basic tetrapeptide sequence corresponding to residues 15–18 resulted in serially loss of potency at the MC2R, with removal of the residue 15 (Lys) results in no observable activity at the MC2R at concentrations at up to 10 μM concentrations. These observations are similar to another parallel examination of C-terminal truncated ACTH peptides (Chen et al. 2007). Using truncated analogs of ACTH modified by N-terminal acetylation and replacing Met4 with Nle, the authors reported a potency decrease for ACTH(1–17) and ACTH(1–16) compared to ACTH(1–24) (46.8 nM and 567 nM versus 2.8 nM, respectively) (Chen et al. 2007). The fold changes between ACTH(1–17) and ACTH(1–16) to ACTH(1–24) (17- and 202-fold, respectively) are similar in magnitude to the decreased potencies observed in the present study of the corresponding analogs (**8** and **9**) using the natural sequence and no N-terminal modification (8- and 114-fold, respectively), indicating the importance of these residues to maintaining MC2R activity. Minimal agonist efficacy was observed for the prior reported ACTH(1–15) analog at up to 10,000 nM concentrations (Chen et al. 2007), whereas in the present study the equivalent analog (**10**) possessed micromolar potency (Table 1). These differences may be due to experimental conditions or peptide modifications (N-terminal acetylation, Nle replacing Met at position 4).

An additional study examined in parallel ACTH N-terminal fragments possessing a C-terminal amidate functionality (Bajusz and Medzihradszky 1967). It was observed that sequential removal of C-terminal residues decreased activity in an ascorbic acid depletion assay, with reported activities of 27.5, 10.0, 1.4, and 0.2 U/mg for ACTH(1–18), ACTH(1–17), ACTH(1–16), and ACTH(1–15), respectively (Bajusz and Medzihradszky 1967). Although differences in sequence (carboxylic acid vs amidate) and assays preclude direct comparison of values, the serial decrease in activity observed when removing residues from the basic tetrapeptide sequence of ACTH and the minimal activity of ACTH(1–15) support the observations in the present study.

Many prior works on individually synthesized and assayed ACTH truncation ligands also indicate the importance of this basic motif. The hexapeptide ACTH(1–16) was found to minimally active *in vivo* (< 0.1 i.u./mg) (Hofmann et al. 1962b). Elongation by one amino acid, ACTH(1–17), resulted in measurable *in vivo* (1.7 i.u./mg) and *in vitro* (6.4 i.u./mg) activity (Li et al. 1962; Li et al. 1964). The addition of one additional amino acid, ACTH(1–18), was reported to possess 9.35 (*in vitro*) and 17.07 (*in vivo*) USP units/mg (Otsuka et al. 1965). While these analogs were synthesized and pharmacologically characterized in different labs, the apparent loss in activity as basic residues 17 and 18 are removed from ACTH support the present study's findings.

An examination of the cryo-EM (Luo et al. 2023) structure of ACTH with the MC2R and MRAP1 help identify several potentially interactions that might explain why this basic stretch of amino acids is important for maintaining MC2R potency (Fig. 1). Residues beyond position 18 of ACTH are not observed in the cryo-EM structure (Luo et al. 2023), suggesting these residues may be flexible and not contribute to stable interactions required for receptor activation. Removal of residues 19–24 resulted in minimal changes in potency (≤ 6-fold) at the MC2R in the present study, supporting this hypothesis. The cryo-EM structure shows several possible interactions between residues 16–18 of ACTH with the MRAP1 protein, which appear to help properly orient ACTH in the active site of MC2R. An 8-fold decrease in potency was observed when the Arg18 residue of ACTH was removed [resulting in ACTH(1–17), **8**]. In the cryo-EM structure the backbone carbonyl of Arg18 is in close proximity to the Ser12 side chain hydroxyl group of MRAP1, suggesting a potential hydrogen bond which loss might explain the observed decrease in activity. In describing the cryo-EM structure, the authors note that the Arg17 side chain of ACTH appears to form a salt bridge with the Glu15 side chain of MRAP1 (Luo et al. 2023). The loss of this hypothesized salt bridge may explain the 114-

fold decrease in agonist potency of **9** [ACTH(1–16)] versus **1**. The authors also note a potential salt bridge between the Lys16 of ACTH and the Asp19 of MRAP1 (Luo et al. 2023). Another potential interaction, between the sidechain amine of ACTH Lys16 and backbone carbonyl of Tyr20 of MRAP1, may also be observed in the cryo-EM structure. The loss of these interactions supports the observed 1,000-fold decrease in agonist potency between **10** [ACTH(1–15)] and **1**. While the Lys15 residue of ACTH does not appear to make any interaction with MRAP1 or the MC2R, in the truncated ACTH(1–15) peptide the side chain amine of Lys15 may interact with acidic residues of MRAP1 (Glu15, Asp19) that are normally interacting with other ACTH residues (Lys16, Arg17). When Lys15 is removed, resulting in ACTH(1–14) (**11**), there was no observable activity at the MC2R. Thus, the basic tetrapeptide sequence within ACTH is critical for activity at the MC2R, supported by historical evaluation of truncated analogs and by structural insights into ligand-receptor-accessory protein interactions.

CONCLUSION

The present study reports the synthesis and pharmacological evaluation of ACTH(1–24) and C- to N-terminal serially truncated analogs to identify the minimal N-terminal length of ACTH required for MC2R activation. Minimal differences in potency (≤ 6-fold) were observed when these analogs were assayed at the MC1R, MC3R, MC4R, and MC5R, indicating that the C-terminal residues of ACTH do not play a critical role for these receptors' activation. A broader potency range was observed for truncated analogs at the MC2R. The basic Lys-Lys-Arg-Arg tetrapeptide sequence corresponding to ACTH residues 15–18 was observed to be critical for MC2R activation. Serially truncating these residues resulted in a serial decrease in observed potency. Truncated analogs not containing these residues did not possess agonist potency at the concentrations assayed (up to 10 μM) at the MC2R. These results correlate with the cryo-EM structure of ACTH-MC2R-MRAP1, where this basic tetrapeptide motif of ACTH forms several interactions with MRAP1 that appear necessary for receptor activation. By synthesizing and characterizing peptide analogs in parallel, the minimal N-terminal fragment of ACTH necessary to activate the MC2R [ACTH(1–15)] was identified.

EXPERIMENTAL

Amino acids Fmoc-Trp(Boc), Fmoc-Arg(Pbf), Fmoc-Phe, Fmoc-His(Trt), Fmoc-Tyr(tBu), Fmoc-Val, Fmoc-Lys(Boc), Fmoc-Pro, Fmoc-Gly, Fmoc-Met, Fmoc-Glu(OtBu) and Fmoc-Ser(tBu), resins H-Pro-2-Chlorotrityl (0.68 mequiv/g substitution), Fmoc-Tyr(tBu)-Wang (0.49 mequiv/g substitution), Fmoc-Val-Wang (0.27 mequiv/g substitution), Fmoc-Lys(Boc)-Wang (0.56 mequiv/g substitution), and Fmoc-Gly-Wang (0.60 mequiv/g substitution), and coupling reagents 1-hydroxybenzotriazole (HOBt) and benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) were purchased from Peptides International (Louisville, KY). The Fmoc-Arg(Pbf)-Wang resin (0.60 mequiv/g substitution) was purchased from Advanced ChemTech (Louisville, KY). Dichloromethane (DCM), methanol (MeOH), acetonitrile (ACN) and anhydrous ethyl ether were purchased from Fisher (Fair Lawn, NJ, USA). N,N-Dimethylformamide (DMF) was purchased from Burdick and Jackson (McGaw Park, IL, USA). Trifluoroacetic acid (TFA), pyridine, and piperidine were purchased from Sigma (St. Louis, MO, USA). N,N-Diisopropylethylamine (DIEA), triethylsilane (Et_3SiH), p-cresol and *p*-thiocresol were purchased from Aldrich (Milwaukee, WI, USA). All reagents and chemicals were ACS grade or better and were used without further purification.

Peptide Synthesis

Peptides were synthesized manually using standard Fmoc methodology (Carpino and Han 1970; Carpino and Han 1972). Resin was added to a filtered manual reaction vessel (Peptides International, Louisville, KY) and allowed to swell for 2 h in DMF. Iterative deprotection and coupling cycles were used to synthesize the desired peptides, with DMF washes between steps to remove excess reagents. The N-α-Fmoc group was removed using 20% piperidine in DMF for 2 min, followed by another 20 min 20% piperidine in DMF treatment. Coupling reactions were performed by the addition of an amino acid (3-fold excess), HOBt (3-fold), BOP (3-fold), and DIEA (6-fold) in DMF. The coupling reaction was mixed by bubbling with nitrogen (N_2) gas for two hours. Qualitative Kaiser tests were used to monitor the coupling and deprotection reactions (Kaiser et al. 1970). Following synthesis of the desired sidechain-protected peptide sequence, the peptide-resin was washed in DCM and dried. Removal of the amino acid side chain protecting groups and cleavage of the peptides from the resin was performed in a filtered manual reaction vessel or in parallel using an automated synthesizer (Advanced ChemTech 440MOS, Louisville, KY) by incubation of 5 mL of cleavage cocktail (89.9% TFA, 5% Et_3SiH, 5% H_2O, 0.05% p-cresol, and 0.05% *p*-thiocresol) for 3 h. Resin was washed with an additional 1.5 mL of cleavage cocktail for 5 min and combined with the previous collected solution. Solutions were transferred to 50 mL conical tubes and precipitated with ice-cold (4°C) anhydrous ethyl ether. Precipitated peptides were pelleted by centrifugation (Sorval Super T21 high speed centrifuge using a swinging bucket rotor) at 4000 rpm for 5 min. The solvent was decanted off, peptides were washed with additional ice-cold anhydrous ethyl ether, and pelleted as described above. Crude peptides were dried *in vacuo* for 48 h, purified by RP-HPLC using a Shimadzu chromatography system equipped with a photodiode array detector and a

semipreparative RP-HPLC C_{18}-bonded column (Vydac 218TP1010, 1.0 x 25 cm), and lyophilized. Peptide purity (> 95%) was determined by analytical RP-HPLC (λ = 214 nm) in two solvent systems, and the exact mass of each peptide was determined (University of Florida Protein Core Facility).

β-Galactosidase cAMP Functional Bioassay at the mMC1R, mMC3R, mMC4R, and mMC5R: HEK 293 cells stably expressing the mouse melanocortin receptors were transfected with 4 μg CRE/β-galactosidase reporter gene as previously described (Chen et al. 1995). Briefly, 5,000 to 15,000 post-transfection cells were planted into 96 well Primera plates (Falcon) and incubated overnight. Forty-eight h post-transfection the cells were stimulated with 100 μL peptide (10^{-4} to 10^{-12} M) or forskolin (10^{-4} M) control in assay medium (DMEM containing 0.1 mg/mL BSA and 0.1 mM isobutylmethylxanthine) for 6 h. The assay media was aspirated and 50 μL of lysis buffer (250 mM Tris-HCl, pH = 8.0, and 0.1% Triton X-100) was added. The plates were stored at − 80°C overnight. The plates containing the cell lysates were thawed the following day. Aliquots of 10 μL were taken from each well and transferred to another 96-well plate for relative protein determination. To the cell lysate plates, 40 μL phosphate buffered saline with 0.5% BSA was added to each well. Subsequently, 150 μL substrate buffer (60 mM sodium phosphate, 1 mM $MgCl_2$, 10 mM KCl, 5 mM β-mercaptoethanol, 200 mg/mL ONPG) was added to each well and the plates were incubated at 37°C. The sample absorbance, OD_{405}, was measured using a 96-well plate reader (Molecular Devices). The relative protein was determined by adding 200 μL 1:5 dilution Bio Rad G250 protein dye:water to the 10 μL cell lysate sample taken previously, and the OD_{595} was measured on a 96-well plate reader (Molecular Devices). Data points were normalized both to the relative protein content and non-receptor-dependent forskolin stimulation.

Enzymeimmunoassay cAMP Functional Bioassay at human MC2R: A commercially available enzymeimmunoassay (EIA) kit (RPN225, Amersham Bioscience, Piscataway, NJ) was used according to the manufacturer's instruction modified to a 12-well plate to determine cAMP formation. OS3 cells expressing the hMC2R were maintained in G418/F10 media and 50,000-100,000 cells were plated into 12-well plates. Cells were grown to at least 95% confluence, then stimulated with 900 μL peptide (10^{-5} to 10^{-11} M) in stimulation medium (Hams F10 containing 0.1 mg/mL BSA and 0.1 mM isobutylmethylxanthine) for 1 h at 37°C. The stimulation media was aspirated, 300 μL of Lysis Reagent 1B (0.25% dodecyltrimethylammonium bromide, 0.05 M acetate buffer, 0.02% BSA, 0.01% preservative, pH 5.8; made from Lysis Reagent 1 [NIF1602] in Assay Buffer concentrate [NIF1085] according to manufacturer protocol), and plates were stored at -80°C for up to one week. Plates were thawed and 40 μL aliquots from each well were transferred to a 96-well EIA microtiter plate (anti-rabbit coated; RPN225CW). An additional 60 μL Lysis Reagent 1B was added to each microtiter plate well. For each plate, a standard serial dilution (3200, 1600, 800, 400, 200, 100, 50, 25, 12.5 fmol/well) of cAMP (Non-acetylated standard, RPN225SA prepared in Lysis Reagent 1B according to manufacturer protocol) was plated (100 μL standard/well in duplicate). 100 μL Lysis Reagent 1B was additionally added for wells containing a 0 standard (no cAMP added) and nonspecific binding (NSB, no antiserum) controls. Antiserum (RPN225AS) reconstituted in Lysis Reagent 2B (1:4 dilution of Lysis Reagent 2, NIF1603, proprietary formulation dissolved in Assay Buffer [0.05 M acetate buffer, 0.02% BSA, 0.01% preservative, pH 6.0] per manufacturer protocol) was added to plate wells (100 μL), and the plate was incubated at 4°C for 2 h on a plate rotator (100 rpm). For the NSB wells, 100 μL Lysis Reagent 2B without antiserum was added. After 2 h, 50 μL cAMP Peroxidase conjugate (RPN225HP, dissolved in Assay Buffer for a solution of cAMP-horseradish Peroxidase in 0.05 M Acetate buffer, 0.02% BSA, 0.01% preservative, pH 5.8 as described by manufacturer) was added to each well and incubated 1 h at 4°C. After the second incubation, the plate was washed 5–6 times with 400 μL wash buffer (NIF874, dissolved in distilled water; 0.01M phosphate buffer, 0.05% Tween 20, pH 7.5 as described by the manufacturer). Immediately following the last wash, 150 μL TMB substrate (NIF2008) was added to each well. The sample absorbance, OD_{630}, was measured using a 96-well plate reader (Molecular Devices). Following normalization to the 0 standard and NSB controls, a standard curve was generated from the cAMP serial dilution. This standard curve was used to convert OD_{630} values to fmol/well cAMP for each sample, normalized to percent increase over basal activity, and plotted as a function of concentration to generate pharmacology curves and corresponding EC_{50} values.

Data Analysis

EC_{50} values represent the mean of duplicate replicates performed in three or more independent experiments. EC_{50} estimates and their associated standard errors were determined by fitting the data to a nonlinear least-squares analysis using the PRISM program (v4.0, GraphPad Inc.). The peptides were assayed as TFA salts and are not corrected for peptide content.

Declarations

Author Contribution

AT, MD, and CHL wrote the main manuscript and text. AT synthesized all peptides studied. KG, NS performed all the bioassays at the melanocortin receptors. All authors performed data analysis.

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References

1. Bajusz S, Medzihradszky K (1967) Synthesis and biological activity of corticotropin fragments. Paper presented at the Peptides: Proceedings of the Eighth European Peptide Symposium, Noordwijk, 1966,
2. Bell PH (1954) Purification and structure of β-corticotropin. J Am Chem Soc 76:5565-5567. https://doi.org/10.1021/ja01650a094
3. Boissonnas RA, Guttmann S, Jaquenoud PA, Waller JP (1956) Synthesis of a polypeptide with ACTH-like structure. Experientia 12:446-448. https://doi.org/10.1007/BF02157382
4. Carpino LA, Han GY (1970) 9-Fluorenylmethoxycarbonyl function, a new base-sensitive amino-protecting group. J Am Chem Soc 92:5748-5749. https://doi.org/10.1021/ja00722a043
5. Carpino LA, Han GY (1972) The 9-fluorenylmethoxycarbonyl amino-protecting group. J Org Chem 37:3404-3409. https://doi.org/10.1021/jo00795a005
6. Castrucci AM, Hadley ME, Sawyer TK, Wilkes BC, Al-Obeidi F, Staples DJ, de Vaux AE, Dym O, Hintz MF, Riehm JP, Rao KR, Hruby V (1989) α-Melanotropin: The minimal active sequence in the lizard skin bioassay. Gen Comp Endocrinol 73:157-163. https://doi.org/10.1016/0016-6480(89)90066-x
7. Chen M, Aprahamian CJ, Kesterson RA, Harmon CM, Yang Y (2007) Molecular identification of the human melanocortin-2 receptor responsible for ligand binding and signaling. Biochemistry 46:11389-11397. https://doi.org/10.1021/bi700125e
8. Chen W, Kelly MA, Opitz-Araya X, Thomas RE, Low MJ, Cone RD (1997) Exocrine gland dysfunction in MC5-R-deficient mice: Evidence for coordinated regulation of exocrine gland function by melanocortin peptides. Cell 91:789-798. https://doi.org/10.1016/s0092-8674(00)80467-5
9. Chen WB, Shields TS, Stork PJS, Cone RD (1995) A colorimetric assay for measuring activation of G_s- and G_q-coupled signaling pathways. Anal Biochem 226:349-354. https://doi.org/10.1006/abio.1995.1235
10. Chhajlani V, Muceniece R, Wikberg JE (1993) Molecular cloning of a novel human melanocortin receptor. Biochem Biophys Res Commun 195:866-873. https://doi.org/10.1006/bbrc.1993.2125
11. Chhajlani V, Wikberg JE (1992) Molecular cloning and expression of the human melanocyte stimulating hormone receptor cDNA. FEBS Lett 309:417-420. https://doi.org/10.1016/0014-5793(92)80820-7
12. Clark AJ, McLoughlin L, Grossman A (1993) Familial glucocorticoid deficiency associated with point mutation in the adrenocorticotropin receptor. Lancet 341:461-462. https://doi.org/10.1016/0140-6736(93)90208-x
13. Fong TM, Mao C, MacNeil T, Kalyani R, Smith T, Weinberg D, Tota MR, Van der Ploeg LHT (1997) ART (protein product of agouti-related transcript) as an antagonist of MC-3 and MC-4 receptors. Biochem Biophys Res Commun 237:629-631. https://doi.org/10.1006/bbrc.1997.7200
14. Gantz I, Konda Y, Tashiro T, Shimoto Y, Miwa H, Munzert G, Watson SJ, DelValle J, Yamada T (1993a) Molecular cloning of a novel melanocortin receptor. J Biol Chem 268:8246-8250. https://doi.org/10.1016/S0021-9258(18)53088-X
15. Gantz I, Miwa H, Konda Y, Shimoto Y, Tashiro T, Watson SJ, DelValle J, Yamada T (1993b) Molecular cloning, expression, and gene localization of a fourth melanocortin receptor. J Biol Chem 268:15174-15179. https://doi.org/10.1016/S0021-9258(18)82452-8
16. Gantz I, Shimoto Y, Konda Y, Miwa H, Dickinson CJ, Yamada T (1994) Molecular cloning, expression, and characterization of a fifth melanocortin receptor. Biochem Biophys Res Commun 200:1214-1220. https://doi.org/10.1006/bbrc.1994.1580
17. Griffon N, Mignon V, Facchinetti P, Diaz J, Schwartz JC, Sokoloff P (1994) Molecular cloning and characterization of the rat fifth melanocortin receptor. Biochem Biophys Res Commun 200:1007-1014. https://doi.org/10.1006/bbrc.1994.1550
18. Haskell-Luevano C, Holder JR, Monck EK, Bauzo RM (2001) Characterization of melanocortin NDP-MSH agonist peptide fragments at the mouse central and peripheral melanocortin receptors. J Med Chem 44:2247-2252
19. Haskell-Luevano C, Todorovic A, Gridley K, Sorenson N, Irani B, Xiang Z (2004) The melanocortin pathway: effects of voluntary exercise on the melanocortin-4 receptor knockout mice and ACTH(1-24) ligand structure activity relationships at the melanocortin-2 receptor. Endocr Res 30:591-597. https://doi.org/10.1081/erc-200043759
20. Hofmann K, Liu T-Y, Yajima H, Yanaihara N, Yanaihara C, Humes JL (1962a) Studies on polypeptides. XXII. High adrenocorticotropic activity in the rat and in man of a synthetic eicosapeptide amide. J Am Chem Soc 84:1054-1056. https://doi.org/10.1021/ja00865a039

21. Hofmann K, Yanaihara N, Lande S, Yajima H (1962b) Studies on polypeptides. XXIII. Synthesis and biological activity of a hexadecapeptide corresponding to the N-terminal sequence of the corticotropins. J Am Chem Soc 84:4470-4474. https://doi.org/10.1021/ja00882a021
22. Hruby VJ, Wilkes BC, Hadley ME, Al-Obeidi F, Sawyer TK, Staples DJ, Devaux AE, Dym O, Castrucci AMD, Hintz MF, Riehm JP, Rao KR (1987) α-Melanotropin: The minimal active sequence in the frog-skin bioassay. J Med Chem 30:2126-2130. https://doi.org/10.1021/jm00394a033
23. Kaiser E, Colescott RL, Bossinger CD, Cook PI (1970) Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. Anal Biochem 34:595-598. https://doi.org/10.1016/0003-2697(70)90146-6
24. Lam BYH, Williamson A, Finer S, Day FR, Tadross JA, Goncalves Soares A, Wade K, Sweeney P, Bedenbaugh MN, Porter DT, Melvin A, Ellacott KLJ, Lippert RN, Buller S, Rosmaninho-Salgado J, Dowsett GKC, Ridley KE, Xu Z, Cimino I, Rimmington D, Rainbow K, Duckett K, Holmqvist S, Khan A, Dai X, Bochukova EG, Genes, Health Research T, Trembath RC, Martin HC, Coll AP, Rowitch DH, Wareham NJ, van Heel DA, Timpson N, Simerly RB, Ong KK, Cone RD, Langenberg C, Perry JRB, Yeo GS, O'Rahilly S (2021) MC3R links nutritional state to childhood growth and the timing of puberty. Nature 599:436-441. https://doi.org/10.1038/s41586-021-04088-9
25. Li CH, Chung D, Ramachandran J, Gorup B (1962) The synthesis of a heptadecapeptide possessing adrenocorticotropic, melanotropic and lipolytic activities. J Am Chem Soc 84:2460-2462. https://doi.org/10.1021/ja00871a046
26. Li CH, Meienhofer J, Schnabel E, Chung D, Lo T-B, Ramachandran J (1961) Synthesis of a biologically active nonadecapeptide corresponding to the first nineteen amino acid residues of adrenocorticotropins. J Am Chem Soc 83:4449-4457. https://doi.org/10.1021/ja01482a035
27. Li CH, Ramachandran J, Chung D, Gorup B (1964) Synthesis of a biologically active heptadecapeptide related to adrenocorticotropin. J Am Chem Soc 86:2703-2710. https://doi.org/10.1021/ja01067a038
28. Lu D, Willard D, Patel IR, Kadwell S, Overton L, Kost T, Luther M, Chen W, Woychik RP, Wilkison WO, Cone RD (1994) Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. Nature 371:799-802. https://doi.org/10.1038/371799a0
29. Luo P, Feng W, Ma S, Dai A, Wu K, Chen X, Yuan Q, Cai X, Yang D, Wang MW, Eric Xu H, Jiang Y (2023) Structural basis of signaling regulation of the human melanocortin-2 receptor by MRAP1. Cell Res 33:46-54. https://doi.org/10.1038/s41422-022-00751-6
30. Medzihradszky K (1976) Synthesis and biological activity of adrenocorticotropic and melanotropic hormones. In: Bognar R, Bruckner V, Szantay C (eds) Recent developments in the chemistry of nautral carbon compounds, vol VII. Akademiai Kiado, Budapest, Hungary, pp 119-250
31. Merrifield RB (1963) Solid phase peptide synthesis .1. Synthesis of a tetrapeptide. J Am Chem Soc 85:2149-2154. https://doi.org/10.1021/ja00897a025
32. Metherell LA, Chapple JP, Cooray S, David A, Becker C, Ruschendorf F, Naville D, Begeot M, Khoo B, Nurnberg P, Huebner A, Cheetham ME, Clark AJ (2005) Mutations in MRAP, encoding a new interacting partner of the ACTH receptor, cause familial glucocorticoid deficiency type 2. Nat Genet 37:166-170. https://doi.org/10.1038/ng1501
33. Mountjoy KG, Robbins LS, Mortrud MT, Cone RD (1992) The cloning of a family of genes that encode the melanocortin receptors. Science 257:1248-1251. https://doi.org/10.1126/science.1325670
34. Nakanishi S, Inoue A, Kita T, Nakamura M, Chang AC, Cohen SN, Numa S (1979) Nucleotide sequence of cloned cDNA for bovine corticotropin-β-lipotropin precursor. Nature 278:423-427. https://doi.org/10.1038/278423a0
35. Ollmann MM, Wilson BD, Yang YK, Kerns JA, Chen YR, Gantz I, Barsh GS (1997) Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. Science 278:135-138. https://doi.org/10.1126/science.278.5335.135
36. Otsuka H, Inouye K, Shinozaki F, Kanayama M (1965) Synthesis of an octadecapeptide and its 18-amide analog corresponding to the first eighteen amino acid residues of corticotropin (ACTH), and their biological activities. J Biochem 58:512-514. https://doi.org/10.1093/oxfordjournals.jbchem.a128234
37. Robbins LS, Nadeau JH, Johnson KR, Kelly MA, Roselli-Rehfuss L, Baack E, Mountjoy KG, Cone RD (1993) Pigmentation phenotypes of variant extension locus alleles result from point mutations that alter MSH receptor function. Cell 72:827-834. https://doi.org/10.1016/0092-8674(93)90572-8
38. Roselli-Rehfuss L, Mountjoy KG, Robbins LS, Mortrud MT, Low MJ, Tatro JB, Entwistle ML, Simerly RB, Cone RD (1993) Identification of a receptor for γ melanotropin and other proopiomelanocortin peptides in the hypothalamus and limbic system. Proc Natl Acad Sci U S A 90:8856-8860. https://doi.org/10.1073/pnas.90.19.8856
39. Schioth HB, Chhajlani V, Muceniece R, Klusa V, Wikberg JE (1996) Major pharmacological distinction of the ACTH receptor from other melanocortin receptors. Life Sci 59:797-801. https://doi.org/10.1016/0024-3205(96)00370-0

40. Schwyzer R (1977) ACTH: a short introductory review. Ann N Y Acad Sci 297:3-26. https://doi.org/10.1111/j.1749-6632.1977.tb41843.x
41. Schwyzer R, Schiller P, Seelig S, Sayers G (1971) Isolated adrenal cells: Log dose response curves for steroidogenesis induced by ACTH1–24, ACTH1–10, ACTH4–10 and ACTH5–10. FEBS Lett 19:229-231. https://doi.org/https://doi.org/10.1016/0014-5793(71)80520-3
42. Schwyzer R, Sieber P (1963) Total synthesis of adrenocorticotrophic hormone. Nature 199:172-174. https://doi.org/10.1038/199172b0
43. Shutter JR, Graham M, Kinsey AC, Scully S, Luthy R, Stark KL (1997) Hypothalamic expression of ART, a novel gene related to agouti, is up-regulated in obese and diabetic mutant mice. Genes Dev 11:593-602. https://doi.org/10.1101/gad.11.5.593
44. Vaisse C, Clement K, Guy-Grand B, Froguel P (1998) A frameshift mutation in human MC4R is associated with a dominant form of obesity. Nat Genet 20:113-114. https://doi.org/10.1038/2407
45. Yeo GS, Farooqi IS, Aminian S, Halsall DJ, Stanhope RG, O'Rahilly S (1998) A frameshift mutation in MC4R associated with dominantly inherited human obesity. Nat Genet 20:111-112. https://doi.org/10.1038/2404

Figures

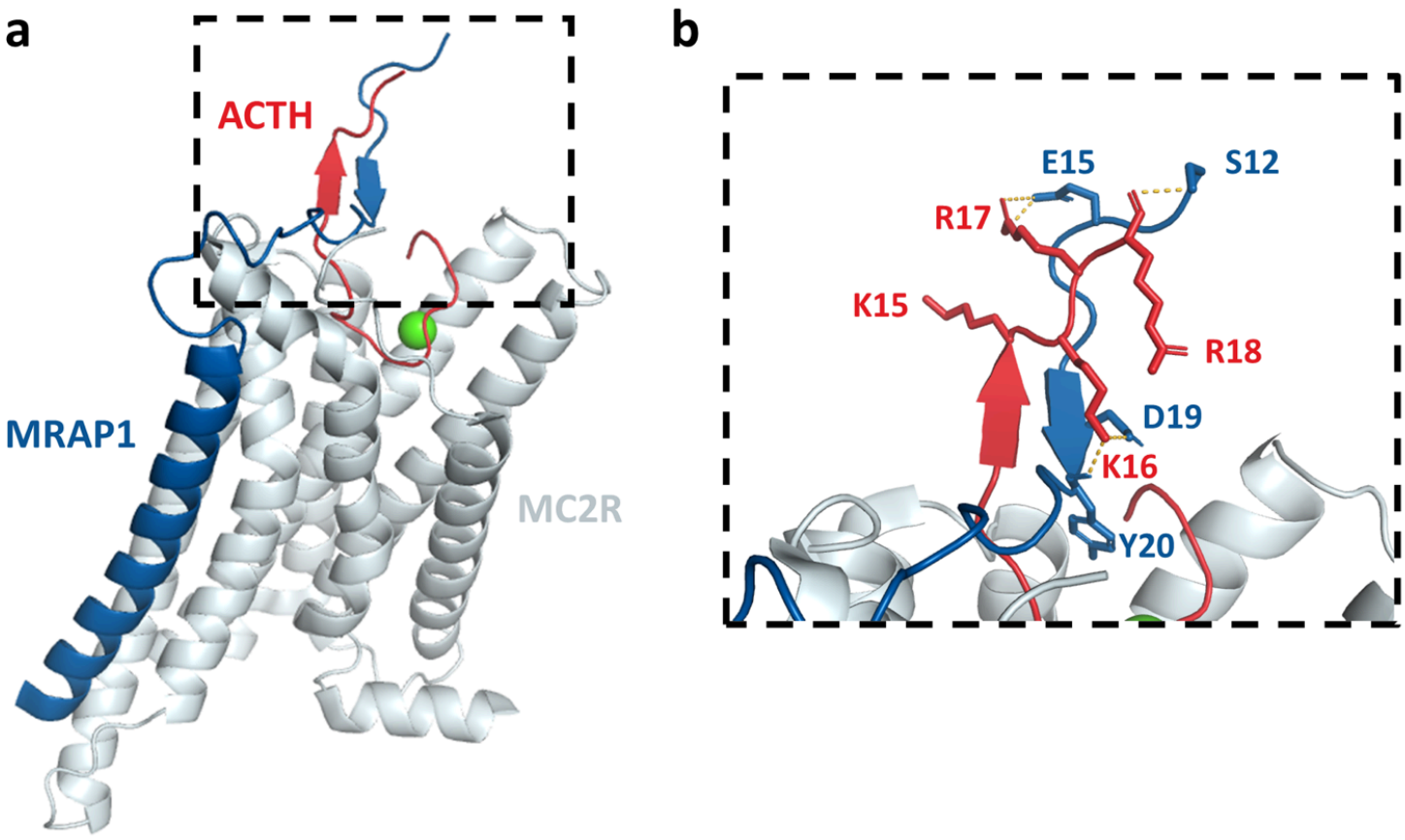

Figure 1

(a) Cryo-EM structure (PDB code 8GY7)(Luo et al. 2023)of MRAP1, MC2R, and ACTH. (b) Detailed hypothesized interactions between MRAP1 and the K15-K16-R17-R18 basic tetrapeptide sequence of ACTH.