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Salusin β is a surrogate ligand of the mas-like G protein-coupled receptor MrgA1 ☆

Zhiwei Wang [a], Toshio Takahashi [b], Yumiko Saito [c], Hiroshi Nagasaki [d], Nga Kim Ly [a], Hans-Peter Nothacker [a], Rainer K. Reinscheid [a], Jun Yang [e], Jaw Kang Chang [e], Masayoshi Shichiri [f], Olivier Civelli [a,*]

[a] Department of Pharmacology, University of California, Irvine, CA 92697, United States
[b] Suntory Institute for Bioorganic Research, Wakayamadai, Shimamotocho, Mishimagun, Osaka, 618-8503, Japan
[c] Department of Pharmacology, Saitama Medical School, Morohongo, Saitama, 350-0495, Japan
[d] Nagoya University, School of Medicine, Clinical Research Bldg., Nagoya City, Aichi, 466-8550, Japan
[e] Phoenix Pharmaceuticals, Inc., Belmont, CA 94002, United States
[f] Tokyo Medical and Dental University Medical Hospital, Yushima, Bunkyo-ku, Tokyo, 113-8519, Japan

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Abstract

The mas-like G protein-coupled receptors form a subfamily of G protein-coupled receptors that includes variable member numbers across different species and that have been shown to bind a wide variety of ligands from peptides to amino acid derivatives. While screening a library of peptides against different orphan G protein-coupled receptors, we found that human salusin β activates the mouse mas-like G protein-coupled receptor, mMrgA1 with an EC_{50} of about 300 nM. Salusin β is a bioactive peptide recently discovered through bioinformatics analysis which stimulates arginine–vasopressin release from rat pituitary and causes rapid and profound hypotension and bradycardia. However, when we further analyzed the generality of the mMrgA1 activation, we found that human salusin β does not activate corresponding human mas-like G protein-coupled receptors. Our results show that human salusin β is a surrogate ligand of the mouse MrgA1 and raises a cautionary flag for experiments that analyze the pharmacological profiles of mas-like G protein-coupled receptors from different species.

Keywords: mas-like G protein-couple receptor; Salusin; Pain

1. Introduction

The mas-related G protein-coupled receptors (Mrgs) (Dong et al., 2001; Zylka et al., 2003), also called Sensory Neuron-Specific G protein-coupled Receptors (SNSRs) (Lembo et al., 2002), are highly expressed in small diameter sensory neurons of the trigeminal nerve, dorsal root ganglia and skin. They form a particular group of G protein-coupled receptors in that they do not have obvious orthologs in different species. Excluding the pseudogenes, there exist 19 mouse Mrgs (8 mMrgAs, 5 mMrgBs, 1 each in mMrgC, mMrgD, mMrgE, mMrgF, mMrgG, mMrgH), 10 human (MrgX1, 2, 3, 4, D, E, F and G, MAS1 and MAS1L) and 12 rat genes (1 rMrgA, 5 rMrgBs, 1 each in rMrgC, rMrgD, rMrgE, rMrgF/RTA, rMrgG, rMrgH). Their unique localization suggested that they may be important in modulating nociception (Dong et al., 2001). Indeed, Grazzini et al. (2004) demonstrated that SNSR1 (MrgX3) played a role in pain perception. However the precise natures of the endogenous ligands for these receptors are far from clear. Dong et al. (2001) first reported that RFamide peptides activate some of the mas-like G protein-coupled receptors. However a more careful study by Han et al. (2002) demonstrated that RFamide peptides more specifically activated mMrgC11 and worked very poorly on mMrgA1. Subsequent studies have also identified other potential ligands: bovine adrenal medulla peptide 22 (BAM22) on SNSR3/4 (MrgX1) (Lembo et

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* Corresponding author. Room 369, Med Surge II, Department of Pharmacology, University of California, Irvine, CA 92697, United States. Tel.: +1 949 824 2522; fax: +1 949 824 4855.
E-mail address: ocivelli@uci.edu (O. Civelli).

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al., 2002); adenine on rat Mrg10 (Bender et al., 2002); cortistatin on MrgX2 (Robas et al., 2003); β-alanine on MrgD (Shinohara et al., 2004) and more recently proadrenomedullin N-terminal peptides (PAMP) on MrgX2 (Kamohara et al., 2005).

Salusin β is the predicted product of a cDNA discovered by Shichiri et al. (2004). It might be cleaved from a precursor protein that contains a signal peptide, indicative of a secretory protein. The precise in vivo structure of salusin is currently unknown. In analogy to other prohormone precursors it can be assumed that primary processing might generate a 50-mer peptide that might be further cleaved at a pair of Arginine residues, yielding the hypothetical peptides salusin α and salusin β. It has been found that intravenous (i.v.) injection of salusin β to rats causes rapid, profound hypotension and bradycardia. The peptide also stimulates arginine–vasopressin release from rat pituitary in vitro. The identity of the receptor is currently unknown. More recent reports demonstrate that reduced cardiac contractility is unlikely to be a major mechanism for rapid and profound hypotension induced by salusin β (Yu et al., 2004; Izumiyama et al., 2005). Yu et al. (2004) further showed that both peptides improved calcium uptake and protein synthesis in neonatal rat cardiomyocytes.

In an effort to identify ligands of orphan G protein-coupled receptors (Civelli et al., 2001), we screened a collection of orphan receptors against a library of peptides. We found that mouse MrgA1 was activated by human salusin β. This interaction is selective since salusin β did not activate human MrgX1 X2, X3, X4, MrgD, MrgE, MrgF, MrgG, MAS1, MAS1L, rat RTA, rMrgA10, mouse MrgA4, and MrgC11. However, the fact that a human peptide could not activate any of the known human mas-like G protein coupled receptors and the fact that mouse salusin β is only a partial agonist at mMrgA1 leads us to conclude that salusin β represents only a surrogate ligand of mMrgA1.

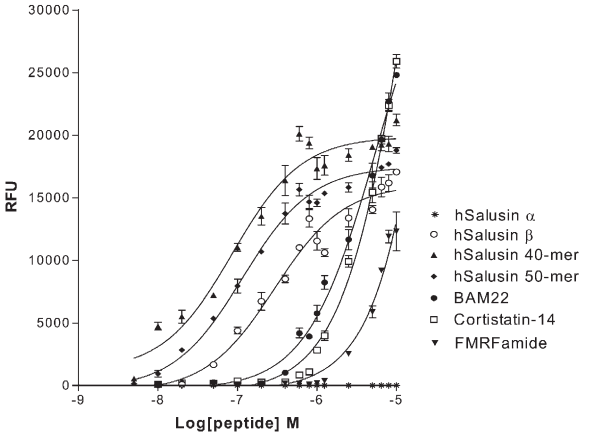
2. Materials and methods

2.1. Materials

HEK293T cells were cultured in DMEM/10% fetal bovine serum (FBS). All G protein-coupled receptors used in the study were amplified from either human, mouse or rat cDNA library (Clontech, Palo Alto, CA) and cloned into pcDNA3.1(−) (Invitrogen, Carlsbad, CA). The sequences were confirmed by sequencing from both ends and with internal primers by Laragen (Los Angeles, CA). Fluo-4 AM was purchased from Molecular Probes, OR. Salusin α and β were synthesized by Peptide Institute Inc. of Japan. Mouse salusin β and 40-mer were synthesized at Suntory Institute for Bioorganic Research, Japan. Human salusin 40-mer and 50-mer were provided by Phoenix Pharmaceuticals, Inc., CA. Lipofectamine was purchased from Invitrogen, Carlsbad, CA. All other reagents were purchased from Sigma, St. Louis, MO.

2.2. Fluorometric Imaging Plate Reader Assay (FLIPR)

The assay was performed as reported earlier (Saito et al., 1999; Nothacker et al., 1999). Briefly, HEK293T cells were grown in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% fetal calf serum at 37 °C in 5% CO_2. The cells were transiently transfected with the plasmids encoding the G protein-coupled receptors used in this report using Lipofectamine reagent according to the manufacturer's instructions. Cells were seeded into poly-D-lysine-coated black wall, clear-bottom 96-well plates 24 h later at a density of 80,000 cells per well. Twenty-four hours later the medium was removed and replaced with 100 μl of dye loading solution (2 μM of Fluo-4AM dissolved in pluronic acid in 1× Hank's buffer supplemented with 20 mM HEPES, pH 7.4) for 1 h at 37 °C. The cells were then washed 3 times with 1× Hank's buffer with 20 mM HEPES before the FLIPR assay. The ligands



hSalusin (50-mer)	AIFIFIRWLLKLGHHGRAPPRRSGALPPAPAAPRPALRAQRAGPAGPGAK-NH_2
hSalusin (40-mer)	AIFIFIRWLLKLGHHGRAPPRRSGALPPAPAAPRPALRAQ
hSalusin β	AIFIFIRWLLKLGHHGRAPP
hSalusin α	SGALPPAPAAPRPALRAQRAGPAGPGAKG
BAM22	YGGFMRRVGRPEWWMDYQKRYG
Cortistatin-14	PCKNFFWKTFSSCK
FMRFamide	FMRF-NH_2

Fig. 1. Dose response curves of intracellular Ca^{++} mobilization induced by different concentrations of human salusin β, salusin α, salusin 40-mer, salusin 50-mer, BAM22, cortistatin-14, and FMRFamide in an HEK/293T cell line stably expressing mMrgA1. Data represent the mean ± S.E.M. of triplicate measurements.

Table 1
EC_{50} values for agonist compounds at mMrgA1

Peptide	EC_{50} (nM)±S.E.M
Cortistatin-14	>1000
Bovine adrenal medulla peptide 22	>1000
FMRF–NH_2	>1000
FMRF–OH	NR
Kisspeptin	NR
LRLRF–NH_2	NR
Met-enkephlin-RF–OH	NR
Met-enkephlin-RF–NH_2	NR
Neuromedin B	NR
Neuropeptide AF	NR
Neuropeptide FF	NR
Neuropeptide B	NR
Neuropeptide W	NR
Orexin A	NR
Prolactin releasing peptide-20	NR
QRFP	NR
γ1 MSH	>1000
Human salusin α	NR
Human salusin β	295.8±4.0
Human salusin (40-mer)	87.9±5.1
Human salusin (50-mer)	116.8±2.9
Mouse salusin β (20-mer)	130.2±5.7
Mouse salusin α	NR
Mouse salusin β (20-mer)–NH_2	90.8±12.6
Mouse salusin β (21-mer)	826.5±30.6
Mouse salusin β (40-mer)	931.7±25.6

Data represent concentration–response curve in triplicate. The functional Ca^+ response was measured using the FLIPR assay system (see Materials and methods).
NR: no response at 1 μM.
>1000: has response at 1 μM but not saturated at 10 μM, thus EC_{50} cannot be determined.

were added into the cells within 4 s and the intracellular Ca^+ concentration was monitored at 488 nm over a period of 4 min.

2.3. Cyclic AMP assay

Stable mMrgA1-expressing HEK293T cells were seeded into poly-D-lysine-coated 6-well plates at a density of 350,000 cells per well and grown for 24 h before the assay. Cells were incubated with assay buffer (1× Hank's buffer, 20 mM HEPES, pH 7.4, and 0.3 mM IBMX) for 10 min and with 1 μM forskolin with or without different concentrations of salusin β for 15 min. Ice-cold ethanol was added to a final concentration of 65% (v/v) and the cells were stored at −80 °C until used for cAMP measurement. The content of cAMP in the lysate was quantified with the cAMP [^{125}I] Biotrak scintillation proximity assay (SPA) system (Amersham Biosciences, NJ) measured with Trilux MicroBeta 1450 (Wallac).

2.4. Assay of MAPK activity

Cells were incubated in 12-well poly-D-lysine-coated plates at 900,000 cells per well overnight. The cells were then changed to serum-free medium for 2 h before incubation with peptide in Hank's/HEPES buffer for various times. The reactions were stopped by adding 200 μl cold RIPA buffer (10 mM Tris, pH 7.5, 15 mM NaCl, 5 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 1.5% NP-40). Cell lysates were centrifuged at 20,000 *g* for 30 min and part of the supernatant was used for loading onto 7.5% SDS-polyacrylamide gel electrophoresis (PAGE). Phosphorylated extracellular signal regulated kinase 1/2 (ERK1/2) was detected by anti-phospho-p44/42 MAPK (Thr202/Tyr204) antibody (Cell Signaling) and peroxidase-conjugated Affini-Pure Goat anti-rabbit IgG (H+L) (Jackson Immunoresearch). The bands were then visualized by ECL™ Western Blotting Detection System (Amersham Biosciences, NJ).

3. Results

We have screened a variety of synthetic peptides against a host of orphan G protein-coupled receptors heterologously expressed in human HEK293 cells. Their putative activities were monitored by intracellular Ca^{++} release using the FLIPR assay configuration. We have found that human salusin β activates the mouse MrgA1 (mMrgA1) with an EC_{50} of 295.8±4.0 nM (Fig. 1). We then tested several peptides and transmitters that have been shown to be ligands of mas-like G protein-coupled receptors (Table 1). We found that none were able to activate mMrgA1 at significant concentrations. (BAM22, cortistatin-14 and FMRFamide were able to activate mMrgA1, but they did so at concentrations close to 1 μM and failed to reach saturation). These data show that the activation by salusin β is specific.

Salusin α failed to activate mMrgA1 (Fig. 1). This is of particular interest since salusin α has been shown to elicit physiological effects similar to that of salusin β. Based on the published sequence of human prepro-salusin, we hypothesized that the salusin α and β sequence may act cooperatively to activate the receptor. We therefore synthesized two extended peptides, human salusin 50-mer and 40-mer (Fig. 1). Human salusin 50-mer includes the salusin β and the amidated salusin α sequences, human salusin 40-mer extends also toward the C

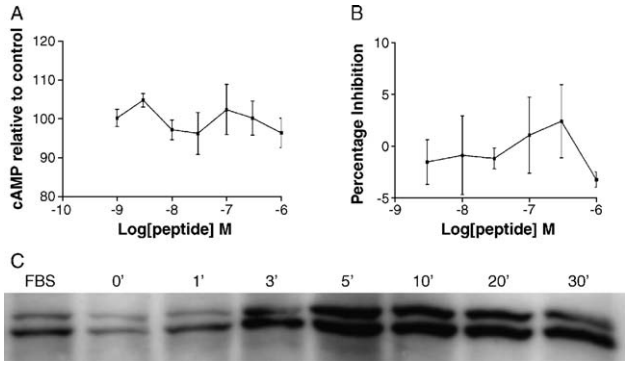


Fig. 2. Effect of human salusin β on cAMP production (2A), on forskolin-induced cAMP production (2B) and on ERK 1/2 phosphorylation (2C). Inhibition of cAMP accumulation were determined on cells stimulated with 1 μM forskolin and increasing concentrations of human salusin β. Results are expressed as percentage inhibition o forskolin-stimulated cAMP level. Curves are plotted as mean+S.E.M. from three determinants. 2C. Human salusin β stimulates MAPK signaling pathway in mMrgA1 stable cell line. Cells were treated with 1 μM of human salusin β for the time as indicated. FBS indicates the cells were treated for 20 min with 10% fetal bovine serum. The two bands shown are the phosphorylated forms of ERK 1/2 (p42 and p44).

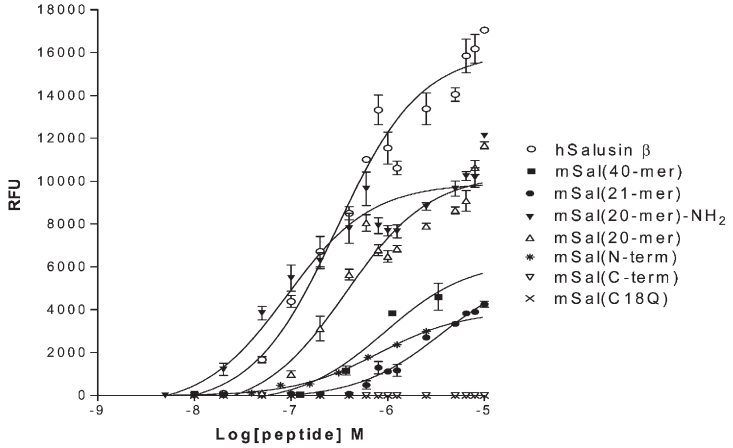
terminus but ends at a potential Arg cleavage site inside the salusin α sequence. Both peptides strongly activated mMrgA1 with EC_{50}s of 87.9 and 116.8 nM for the 40-mer and 50-mer respectively. This shows that the full sequence of salusin α is not required for mMrgA1 activation. It should be noted, however, that salusin α and β structures are significantly different in their primary structure making it unlikely that they act on the same receptor.

Since G protein-coupled receptors are known to utilize various signaling pathways dependent on the cellular environments, we tested whether mMrgA1 activation would also affect other secondary messenger responses. We found that mMrgA1 activation did not positively or negatively affect adenylyl cyclase activity (Fig. 2A and B) but potently stimulated ERK 1/2 phosphorylation (Fig. 2C). We also transfected mMgrA1 in a different cellular environment, Chinese Hamster Ovary (CHO) cells, and found that in this cell system it also activates intracellular Ca^{++} release. Together these data show that mMrgA1 acts predominantly via the Gαq pathway.

To search for the selectivity of the response, we tested human salusin β against a variety of mouse and rat mas-like G protein-coupled receptors, e.g. mMrgA4, mMrgC11 and rMrgA10, and four receptors that are not mas-related, e.g. GPR10, GPR14, GPR24, GPR54. We found that human salusin β was unable to activate any of these receptors (data not shown).

We then investigated whether mouse salusin β could activate mMrgA1. Since the mouse salusin β has not been isolated, we resolved to find it through genomic sequence analysis. We compared the human genomic DNA draft sequence NT_008470.16 to the mouse genomic DNA draft sequence NT_039206.2 which led to the identification of the mouse preprosalusin containing the salusin β sequence. The exact delineation of the active peptide sequence could not however be determined with certainty (Fig. 3). The human salusin β peptide is 20 residues long which comparatively would terminate the mouse peptide at an Ala residue. C-terminally to this residue is a Gly that could serve as an amidating donor site. We thus synthesized both amidated and non-amidated 20-mer mouse salusin β as well as a non-amidated 21-mer. As shown in Fig. 3, while the 21-mer was very poor at activating mMrgA1 (EC_{50} of 826.5 nM), the two 20-mers (either amidated or non-amidated) activated it with EC_{50}s of 90.8 and 130.2 nM respectively. However, when compared to human salusin β, the mouse peptides behaved as partial agonists of the mMrgA1 (Fig. 3). We therefore reasoned that in the human salusin β sequence there might be motif(s) that may permit a higher degree of activation. We attempted to find these motifs by carrying out a structure–activity relationship study. Mouse salusin β is identical to its human ortholog at its N-terminus but diverges in its C-terminus. We synthesized a peptide (mSal(N-term)) containing the first 10 residues of mouse salusin β and one containing the remaining 10 residues (mSal(C-term)) and tested them for activity on mMrgA1 expressing cells. mSal(N-term) retained partial activity while mSal(C-term) lost all activity. This indicates that the N-terminus carries structural features that are important for salusin β activity and helps explain its evolutionary conservation.

If salusin is the endogenous ligand of the mouse MrgA1, it should also activate a human mas-like G protein-coupled receptor. We therefore tested the MAS1 and 9 human mas-like G protein-coupled receptors known to exist, namely MrgX1, X2, X3, X4, D, E, F, G, and MAS1L. All nine receptors were



hSalusin β	AIFIFIRWLLKLGHHGRAPP
mSal(40-mer)	AIFIFIRWLLAVWHHGGAPAGRCGALPPAPAASRAPLRTQ
mSal(21-mer)	AIFIFIRWLLAVWHHGGAPAG
mSal(20-mer)-NH_2	AIFIFIRWLLAVWHHGGAPA-NH_2
mSal(20-mer)	AIFIFIRWLLAVWHHGGAPA
mSal(N-term)	AIFIFIRWLL
mSal(C-term)	AVWHHGGAPA
mSal(C18Q)	CGALPPAPAASRAPLRTQ

Fig. 3. Dose response curves of intracellular Ca^{++} mobilization induced by different concentrations of human salusin β, mouse salusin β (21-mer), mouse salusin β (20-mer), mouse salusin β (20-mer) amidated form, mouse 40-mer, mouse salusin β N-terminal fragment and C-terminal fragment. Data represent the mean ± S.E.M. of triplicate measurements.

Table 2
EC_{50} (± S.E.M.) of different compounds at human Mrgs

	Salusin β	BMA22	Cortistatin	Adenine	β-alanine
MrgX1	NR	313.2+15.9	NR	NR[a]	NR[a]
MrgX2	NR	NR	208.7+2.5	NR[a]	NR[a]
MrgX3	NR	NR	NR	NR[a]	NR[a]
MrgX4	NR	NR	NR	NR[a]	NR[a]
MrgD	NR	NR	NR	NR[a]	57.8+3.5 (μM)
MrgE	NR	NR	NR	NR[a]	NR[a]
MrgF	NR	NR	NR	NR[a]	NR[a]
MrgG	NR	NR	NR	NR[a]	NR[a]
MAS1	NR	NR	NR	NR[a]	NR[a]
MAS1L	NR	NR	NR	NR[a]	NR[a]

Values are in nM except as indicated. NR indicates that there was no response at 1 μM. NR[a] indicates there was no response at 100 μM.

cloned and expressed, but, to our surprise, none were activated by human salusin β (Table 2). MAS1 was not activated by salusin β either. This shows that the interaction between the mouse MrgA1 and human salusin β is specific to and only to that receptor.

4. Discussion

In this paper, we have demonstrated that an orphan G protein-coupled receptor, mMrgA1, is activated specifically by human salusin β. This activation is independent of the cellular environment since it occurs in both CHO and HEK293 cells. Mouse salusin β, which shares the same N-terminus as its human counterpart, activates also mMrgA1 but does it as a partial agonist. While it is perplexing that a human peptide would activate a mouse but not a human mas-like G protein-coupled receptor, it is not without precedence. Bender et al. (2002) reported that rat MrgA10 is activated by adenine yet it has been reported that adenine could not activate any of the human mas-like G protein-coupled receptors, namely MrgX1-MrgX4 (2002). We confirmed this result by testing adenine at concentrations up to 100 μM (Table 2). It has also been shown that L-carnosine can activate rat MrgD/TGR7 but not human and mouse MrgD (Shinohara et al., 2004). The only agonist found thus far able to activate a human, mouse and rat mas-like G protein-coupled receptor is β-alanine that specifically activates TGR7/MrgD (Shinohara et al., 2004). But MrgD is the only mas-like G protein-coupled receptor known to have orthologs in these three species. The majority of mas-like G protein-coupled receptors lack clear orthologs. This might be due to unequal crossover such as nonhomologous meiotic recombination or to de novo retrotransposition (Zylka et al., 2003).

In summary, the salient fact of this study is the discovery that human salusin β while able to activate a mouse mas-like G protein-coupled receptor does not activate any of the known human mas-like G protein-coupled receptors. Our results lead us to propose salusin β as a surrogate ligand of mMrgA1. While it is possible that new G protein-coupled receptors may still be discovered, a true human MrgA1 ortholog would have to share close homology with the mouse receptor to be able to be activated by salusin β since our studies show that the receptor activation depends on a tight structure–activity relationship. One would expect that such a closely-related human receptor would have been discovered by sequence database searches. Since this is presently not the case, one has to also conclude that the carrier for salusin β biological activity, if any, is still unknown.

The mas-like G protein-coupled receptor family stands out as a G protein-coupled receptor family that breaches pharmacological selectivity in natural ligand–receptor interaction. It should be emphasized however that most of the ligands that were paired to mas-like G protein-coupled receptors were found through the application of the reverse pharmacology approach that we also applied in this study. Inherent to this approach is the possibility of finding surrogate ligands, i.e. molecules which activate a given receptor but might not necessarily represent natural ligands. Our findings therefore call for caution in discovering endogenous ligands of mas-like G protein-coupled receptors with a ligand matching approach and indicate that the transmitter that naturally activates mMrgA1 has still to be found.

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