MrgX2 Is a High Potency Cortistatin Receptor Expressed in Dorsal Root Ganglion*

Nicola Robas, Emma Mead, and Mark Fidock†

From the Department of Target Genomics, Pfizer Global Research and Development, Sandwich, Kent, CT13 9NJ, United Kingdom

MrgX2 is a recently identified orphan G-protein-coupled receptor whose ligand and physiological function were unknown. Here we describe cortistatin, a neuropeptide for which no specific receptor has been identified previously, as a high potency ligand at MrgX2. Cortistatin has several biological functions including roles in sleep regulation, locomotor activity, and cortical function. Using a “reverse pharmacology” approach, we have identified a number of additional cyclic peptide agonists for MrgX2, determined their rank order of potency, and demonstrated that this receptor has a pharmacological profile distinct from the other characterized members of the Mrg (Mas-related genes) family. In MrgX2-expressing cells, cortistatin-stimulated increases in intracellular Ca2+ but had no effect on basal or forskolin-stimulated cAMP levels, suggesting that this receptor is Gi- coupled. Immunohistochemical and quantitative PCR studies show MrgX2 to have a limited expression profile, both peripheral and within the central nervous system, with highest levels in dorsal root ganglia.

G-protein-coupled receptors (GPCRs)† make up the largest and most diverse family of transmembrane proteins, which respond to a wide variety of stimuli including biogenic amines, peptides, bioactive lipids, hormones, and light. Agonist binding to these receptors activates intracellular signaling events, mediated by G-proteins, such as modulation of adenylate cyclase or Ca2+ mobilization (1, 2). Completion of the human genome sequencing project has identified ~140 “orphan” GPCRs for which the ligand and function are unknown (3–5). We have used bioinformatic and tissue distribution analysis to prioritize those orphans with potential therapeutic relevance followed by a “reverse pharmacology” approach to identify cognate and surrogate ligands (6, 7). The orphan receptor MrgX2 is a member of a family of ~5 human, 31 mouse, and 2 rat coding sequences that are related to the Mas1 oncogene (8). This group of genes has been termed Mrg (Mas-related genes) or SNSR (sensory neuron-specific G-protein-coupled receptors). Tissue distribution studies have shown these receptors to be expressed mainly in the small sensory neurons of the dorsal root ganglia (DRG), suggesting a role in nociception. To date, cognate ligands have been identified for five of these receptors: proenkephalin A gene products, especially BAM22 (EC50 ~13 nM), have been shown to potently activate human MrgX1 (SNSR3) (9). The neuropeptide RF amides are agonists at murine MrgA1 (NPFF, EC50 ~200 nM), MrgC11 (NPFF, EC50 ~54 nM), and MrgA4 (NPAF, EC50 ~60 nM) (10). Adenine has been identified as a high affinity ligand (Kᵅ ~18 nM) for a rat member of the Mrg family (11). However, none of these ligands are high potency agonists at MrgX2.

In an attempt to identify the cognate ligand for MrgX2, we screened a library of ~1200 endogenous GPCR ligands including small drug-like molecules, peptides, and bioactive lipids. Here we describe cortistatin-14 as a high affinity ligand for MrgX2 and examine the effects of other cyclic peptides at this receptor. In addition, immunohistochemistry and quantitative PCR were used to describe the tissue distribution of MrgX2 and determine co-expression with cortistatin.

EXPERIMENTAL PROCEDURES

Fluorometric Imaging Plate Reader Assay (FLIPR®—HEK293-Ga15 cells, obtained from Aurora Biosciences Inc. (San Diego, CA), and native HEK293 cells were grown in Dulbecco’s minimum essential medium supplemented with 10% fetal calf serum and l-glutamine (2 mM) at 37 °C in 5% CO2, 95% air. These cells were transiently transfected with the expression plasmid encoding MrgX2 (coding sequence as GenBankTM AY042214) using LipofectAMINE PlusTM reagent (Invitrogen), according to the manufacturer's instructions. Twenty-four h after transfection, the HEK293 and HEK293-Ga15 cells were separately seeded into black clear-bottomed, 96-well, poly-D-lysine-coated plates at a density of 5 × 104 cells/well. The cells were cultured for a further 24 h. The medium was then removed from the cells and replaced with 100 µl of warm (37 °C) dye loading solution (50 µg of Fluo4 (Molecular Probes) in 20 µl of Me2SO + 20 µl of 20% pluronic acid, added to 11 ml of serum-free Dulbecco’s modified Eagle’s medium containing 2.5 mM probenecid). The plates were then incubated for 1 h at 37 °C. The cells were subsequently washed three times with 150 µl of wash buffer/well (Hank’s balanced salt solution, 2.5 mM probenecid, 0.1% bovine serum albumin, pH 7.4). The plates were incubated at room temperature for 30 min prior to processing within the FLIPR®. Small molecule ligands and bioactive lipids were screened at a concentration of 10 µM, and peptides were screened at a concentration of 1 µM. Peptides were diluted to the appropriate concentration in Hank’s balanced salt solution, 0.1% bovine serum albumin. For dose-response studies, all data points were carried out in triplicate. Mock-transfected cells (transfected with empty plasmid) were screened in parallel to MrgX2-transfected cells.

Cyclic AMP-responsive Element (CRE)-Luciferase Assay—HEK293 cells were cultured to ~80% confluence, at which time they were cotransfected with plasmids pCRE-Luc (CRE-luciferase, Stratagene) and a construct containing MrgX2 using the lipid transfection reagent LipofectAMINE PlusTM (Invitrogen) as recommended by the manufacturer. Cells were cultured for 24 h after transfection before being washed with phosphate-buffered saline (pH 7.4), recovered in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum, and seeded as 100 µl aliquots (5 × 104 cells) into white 96-well tissue culture plates. Following a further 24 h incubation, 3-isobutyl-
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RESULTS

Functional Activation of MrgX2—We initially screened HEK293-Ga15 cells transiently transfected with MrgX2 against a library of 1200 known GPCR ligands using the measurement of intracellular calcium concentration to detect activation of the receptor. Dose-response studies on the activating ligands showed cortistatin-14 (CST-14) to be the highest potency agonist with an EC50 of 25 nM (Fig. 1). A number of other cyclic peptides showed high nanomolar potency at MrgX2 (Table I). No significant change in efficacy was seen in response to these agonists. No response to these peptides was seen in the mock-transfected cells.

To investigate the downstream signaling of MrgX2, we mon-
itored the increase or decrease of intracellular cAMP in response to MrgX2 activation, using a CRE luciferase reporter system. No changes in cAMP levels were observed in MrgX2-transfected cells in response to cortistatin, whereas the same transfected population of cells elicited a potent response to CST-14 in the FLIPR® Ca²⁺/H11001 assay. To further define the native signaling mechanism of MrgX2, the receptor was transfected into native HEK293 cells, and the response to CST-14 was determined in the FLIPR® Ca²⁺/H11001 assay. An EC₅₀ of 25 nM was obtained, which was equivalent to the value generated using the HEK293-Gα15 cells. These results taken together indicate that this receptor is not Gₛ- or Gᵢ-coupled and that it signals via a Gᵦ-coupled mechanism.

Human Tissue Distribution Studies—Quantitative PCR was used to determine the distribution of MrgX2 and cortistatin in human tissues. Highest levels of MrgX2 were observed in lumbar DRG and MrgX2 (black bars) and CST (gray bars) expression levels were determined. Each column represents the mean of triplicate values. Each tissue is normalized to β-actin.

![Localization of MrgX2 and the ligand cortistatin in human tissues.](image)

**Fig. 2.** Localization of MrgX2 and the ligand cortistatin in human tissues. Human cDNA (multiple tissue panel, Clontech) was used as a template and MrgX2 (A), and cortistatin (B) transcript levels were determined by quantitative PCR. As shown in C, total RNA was isolated from thoracic, lumbar, and cervical DRG and MrgX2 (black bars) and CST (gray bars) expression levels were determined. Each column represents the mean of triplicate values. Each tissue is normalized to β-actin.
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Fig. 3. Localization of MrgX2 in DRG using immunohistochemistry. Spinal cord DRG magnified ×60 is shown. Arrow indicates small diameter neuron stained with MrgX2-specific anti-peptide antibody.

Structure-activity relationship studies on the cortistatin peptide show that the extracyclic N-terminal proline and the C-terminal lysine amide are essential for its distinct biological properties (19). This suggests that both of these residues are required for binding of cortistatin to its specific receptor. Interestingly, MrgX2 exhibits high affinity for CST-14 and CST-17, which contain both the N-terminal proline and the C-terminal lysine amide. However, the ligand affinity drops dramatically for somatostatin-14, which lacks these two critical amino acids but shares 11 of its remaining 12 residues with cortistatin. The somatostatin analogue octreotide is unable to functionally activate MrgX2 further, indicating that the structural determinants required for MrgX2 activation are located within the extreme N- and C-terminal residues of the peptide (Fig. 4).

Within the brain, the highest levels of cortistatin expression are found in the cerebral cortex. MrgX2 is not detected in the cortex by quantitative PCR or by immunohistochemistry and thus is unlikely to play a role in the modulation of cortical activity. Quantitative PCR studies show cortistatin to have a broad tissue distribution as compared with the limited expression of MrgX2. This is not unexpected as a number of the diverse actions of cortistatin have been shown to be mediated via interactions with the widely distributed somatostatin family of receptors (17). However, as discussed previously, the existence of a cortistatin-specific receptor is suggested by pharmacological evidence that cortistatin exerts some of its effects via somatostatin receptor-independent pathways. Quantitative PCR and immunohistochemistry data show that MrgX2 is expressed at highest levels in the small diameter neurons of the DRG (Fig. 3), which include primary sensory fibers associated with acute and neuropathic pain. We have also detected the presence of cortistatin mRNA in human DRG by quantitative PCR, and it is interesting to speculate that this ligand-receptor pair may have a role in nociception, but this has yet to be established. However, it may prove challenging to use an animal model to define the physiological effects of MrgX2 activation by cortistatin as bioinformatic similarity searches have shown no mouse or rat orthologues of MrgX2. This divergent cross-species receptor evolution is not unique to the Mrg family. The recently ligand-paired human receptor GPR8 has been shown to be absent in rodents (20), although its high affinity ligand, neuropeptide W, is present in these species (21). Despite these challenges, the identification of MrgX2 as the first human cortistatin-prefering receptor, together with the co-localization of receptor and ligand in the DRG, should promote investigation into the role of cortistatin and its receptor in nociception.

MrgX2 is the sixth member of the Mrg family to be ligand-paired. The neuropeptides NPFF, NPAF, and BAM22 and the purine adenine have been shown to be agonists of MrgA1, MrgA4, MrgX1, and a rat Mrg receptor, respectively. Recently, peptides containing the C-terminal consensus sequence −RF(Y)G or −RF(Y) amide have been identified as agonists at the MrgA1 and MrgC11 receptors. BAM22 and NPFF activate MrgX2 with an EC₅₀ of ~400 nM, whereas NPAF and adenine are inactive at this receptor. All of these receptors share 45–65% amino acid identity with MrgX2 and exhibit overlapping expression within small diameter fibers of the DRG. However,
the structure activity relationships and rank order of potency of their respective ligands are distinct. Thus nociceptive sensory neurons show not only molecular diversity but also a surprising degree pharmacological diversity, suggesting the involvement of the Mrg family in subtle regulation and fine-tuning of nociceptive responses.

Our data have contributed additional facets to the already complex pharmacology of the Mrg family. The future development of MrgX2-selective agonists and antagonists will further elucidate the physiological role of this receptor and its involvement in the biological functions of cortistatin.

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