Sensory Neuron-Specific GPCR Mrgprs Are Itch Receptors Mediating Chloroquine-Induced Pruritus

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SUMMARY

The cellular and molecular mechanisms mediating histamine-independent itch in primary sensory neurons are largely unknown. Itch induced by chloroquine (CQ) is a common side effect of this widely used antimalarial drug. Here, we show that Mrgprs, a family of G protein-coupled receptors expressed exclusively in peripheral sensory neurons, function as itch receptors. Mice lacking a cluster of Mrgpr genes display significant deficits in itch induced by CQ but not histamine. CQ directly excites sensory neurons in an Mrgpr-dependent manner. CQ specifically activates mouse MrgprA3 and human MrgprX1. Loss- and gain-of-function studies demonstrate that MrgprA3 is required for CQ responsiveness in mice. Furthermore, MrgprA3-expressing neurons respond to histamine and coexpress gastrin-releasing peptide, a peptide involved in itch sensation, and MrgprC11. Activation of these neurons with the MrgprC11-specific agonist BAM8-22 induces itch in wild-type but not mutant mice. Therefore, Mrgprs may provide molecular access to itch-selective neurons and constitute novel targets for itch therapeutics.

INTRODUCTION

Itch, formally known as pruritus, has been defined as an “unpleasant skin sensation that elicits the desire or reflex to scratch” (Ikoma et al., 2006). Primary sensory neurons in dorsal root ganglia (DRG) play an essential role in generating itch by detecting pruritogenic stimuli through their peripheral axons in the skin and mucosal surfaces and sending the signals to the spinal cord via their central axons (Paus et al., 2006). The best-characterized itch mediator is histamine, which is mainly secreted by skin mast cells and excites nearby sensory fibers by acting on histamine receptors (Alving et al., 1991). Histamine-induced itch in humans can be almost completely blocked by histamine receptor H1 antagonists. However, the blockers are ineffective in many other itch conditions, such as those arising from atopic dermatitis, renal and liver diseases, the side effects of drugs, plant toxins, and mechanical stimuli (Paus et al., 2006). These observations, together with other electrophysiological and molecular studies, strongly imply the existence of histamine-independent types of itch (Davidson et al., 2007; Johanek et al., 2007; Johanek et al., 2008). A major hurdle to investigating histamine-independent itch is the lack of information about the receptors directly activated by nonhistaminergic pruritogens, as well as molecular markers for itch-sensing neurons in the DRG.

Chloroquine (CQ) is a drug that has long been used in the treatment and prevention of malaria. One major side effect of this drug is itch, which is very common among black Africans (up to 70%) but less common in other races. Pruritus is a major cause of noncompliance in the treatment of malaria as ~30% of African patients refused further CQ treatment because of unbearable itch (Mnyika and Kihamia, 1991; Sowunmi et al., 2000). This noncompliance may lead to the development and spread of CQ-resistant Plasmodium falciparum. CQ-induced itch is not considered an allergic reaction since pruritus is seen after first exposure (Ademowo et al., 1998; Olatunde, 1977). More importantly, it cannot be treated effectively by antihistamine drugs, suggesting that a histamine-independent pathway is involved (Abila et al., 1994; Ezeamuzie et al., 1990). CQ-induced itch is also well documented in mice. Subcutaneous CQ injection in wild-type (WT) mice acutely evokes a pronounced
scratching behavior (Green et al., 2006). Interestingly, mice lacking gastrin-releasing peptide receptor (GRPR), which is specifically expressed in dorsal horn neurons of the spinal cord, exhibit severely reduced itch responses even by various pruritogens, including CQ (Sun and Chen, 2007). Furthermore, mice with GRPR-expressing dorsal horn neurons selectively ablated showed profound scratching deficits, whereas pain behaviors were unaffected in these animals (Sun et al., 2009). These findings suggest that both GRPR and the second-order neurons in the spinal cord marked by GRPR are important for transmitting itch signals from primary sensory afferents. However, it is unknown whether CQ directly activates primary sensory fibers in the skin and whether cell surface receptors are involved in the process.

Several G protein-coupled receptors (GPCRs) have been shown to be essential in generating itch, including histamine receptors and protease-activated receptors (PARs) (Shim and Oh, 2008; Steinhoff et al., 2003). Mrgprs (also named Mrg/SNSR) are a family of orphan GPCRs consisting of more than 50 members in the mouse genome that can be grouped into several subfamilies: MrgprA1–A22, MrgprB1–B13, MrgprC1–C14, and MrgprD–G (Dong et al., 2001; Zylka et al., 2003). Strikingly, the expression of Mrgprs, including MrgprA1, MrgprA4, MrgprB5, MrgprC11, and MrgprD, is restricted to subsets of small-diameter sensory neurons in DRG and trigeminal ganglia and has not been detected in the central nervous system or in the rest of the body (Dong et al., 2001; Zylka et al., 2003). Similarly, human MrgprXs are also selectively expressed in DRG neurons (Lembo et al., 2002).

Mrgprs can be activated by peptides terminating in RF/Y-G or RF/Y-amide, such as molluscan FMRFamide and mammalian neuropeptide FF (NPFF), neuropeptide AF (NPAF), RF/Y-amide, such as molluscan FMRFamide and mammalian cyto-stimulating hormone (C24). These peptides can activate heterologously expressed mouse MrgprA1, MrgprA4, and MrgprC11 and human MrgprX1 with different sensitivities (Dong et al., 2001; Han et al., 2002). Therefore, the deleted cluster represents ~50% of the potentially functional Mrgpr repertoire and contains most MrgprA and MrgprC genes, as well as some members of the MrgprB subfamily. The deleted Mrgpr genes are specifically expressed in DRG (Dong et al., 2001; Han et al., 2002; Zylka et al., 2003). MrgprA6, A9, A11, B1, B2, B6, B8, B10, and D–G are not included in this deletion based on the Mouse Genome Project and RT-PCR experiments (data not shown). Notably, MrgprB1 and MrgprB2, which were not deleted, are expressed in the skin but not in DRG (Zylka et al., 2003).

Mating between mice heterozygous for the cluster deletion (Mrgpr-clusterΔ−/−) produced offspring with the expected Mendelian distribution of gender and genotype. Homozygous Mrgpr-clusterΔ−/− mice are viable, fertile, and generally indistinguishable from WT littermates in appearance, body weight, overt behavior, and gross anatomy. The motor function of Mrgpr-clusterΔ−/− mice is also normal as determined by the rotorod test (data not shown). Furthermore, Mrgprs are not required for neuronal survival, fate determination or differentiation of small-diameter sensory neurons (Figure 1C; Supplemental Data available online).

**Mrgpr-clusterΔ−/− Mice Exhibit Severe Reduction in CQ-Induced Scratching**

Activation of small-diameter sensory neurons in DRG can generate different types of somatosensation, including pain and itch with specific and distinct behavioral responses. For instance, pain and itch cause withdrawal and scratching responses, respectively (Ikoma et al., 2006). We next investigated whether the deletion of Mrgpr genes affects behavioral responses to pain- and itch-inducing stimuli. Mrgpr-clusterΔ−/− mice responded normally to acute noxious heat, cold, mechanical, and chemical stimulation as compared with WT littermates (Figures 2A–2F). Thus, acute pain sensation appears to be unaffected in Mrgpr-clusterΔ−/− mice. In addition, Mrgpr mutant mice exhibited modest but statistically significant increases only at certain testing time points in inflammatory hyperalgesia induced by complete Freund’s adjuvant (CFA) or carrageenan injection. No significant difference was found in neuropathic pain caused by L5 spinal nerve ligation between mutant and wild-type mice (Figure S1).

In addition to pain, we evaluated chemically induced itch responses in Mrgpr-clusterΔ−/− mice. No significant difference was found between Mrgpr-clusterΔ−/− and WT mice in the total number of scratching bouts induced by histamine over a period of 30 min (Figure 2G). Consistent with this result, WT and mutant mice also showed similar responses to compound 48/80, a drug that elicits mast cell degranulation and induces histamine-dependent itch (Kuraishi et al., 1995; Nakayama et al., 2002) (Figure 2H). These results suggest that Mrgprs are not involved in histamine-dependent itch.

Strikingly, itch induced by CQ was strongly reduced in Mrgpr-clusterΔ−/− mice. Figure 2I shows the time course of scratching bouts at 5 min intervals after CQ injection. Typically, the first bout

**RESULTS**

**Targeted Deletion of a Cluster of Mrgpr Genes**

Many Mrgpr genes are clustered together on mouse chromosome 7 (Dong et al., 2001; Zylka et al., 2003). To determine the function of Mrgprs in vivo while overcoming the potential problem of gene redundancy, we generated a mouse line in which a cluster of Mrgpr genes was deleted (Figures 1A and 1B). The deleted 845 kilobase region comprises ~30 Mrgpr genes, 12 of which (MrgprA1–A4, A10, A12, A14, A16, A19, B4, B5, and C11) have intact open reading frames (ORFs; Figure 1A). No other ORF is present in this region, according to the Mouse Genome Project. Although the mouse Mrgpr superfamily consists of over 50 members, more than half are pseudogenes, and only ~24 genes have intact ORFs (Dong et al., 2001; Han et al., 2002). Therefore, the deleted cluster represents ~50% of the potentially functional Mrgpr repertoire and contains most MrgprA and MrgprC genes, as well as some members of the MrgprB subfamily. The deleted Mrgpr genes are specifically expressed in DRG (Dong et al., 2001; Han et al., 2002; Zylka et al., 2003). MrgprA6, A9, A11, B1, B2, B6, B8, B10, and D–G are not included in this deletion based on the Mouse Genome Project and RT-PCR experiments (data not shown). Notably, MrgprB1 and MrgprB2, which were not deleted, are expressed in the skin but not in DRG (Zylka et al., 2003).

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was observed within 1 to 2 min after injection, and scratching peaked within 15 min in WT mice. In contrast, Mrgpr-cluster \( ^{-/-} \) mice showed a delayed occurrence of the first scratching behavior (WT 57.8 \( \pm \) 25.4 s versus KO 280.5 \( \pm \) 35.0 s; \( p = 0.0004 \)). The total number of scratching bouts induced by CQ was 278 \( \pm \) 21 in WT mice and 104 \( \pm \) 13 in Mrgpr-cluster \( ^{-/-} \) mice (Figure 2I). Interestingly, injection of a CQ precursor, quinoline, did not evoke any scratching behavior (see below for quinoline’s structure and inability of activating Mrgprs) in both WT and mutant mice (Figure 2J). However, immediately after quinoline treatment, CQ injection at the same location induced robust scratching behavior in WT mice, and the number of scratches induced by this treatment was again severely reduced in Mrgpr-cluster \( ^{-/-} \) mice (Figure 2J). These results suggest that CQ-induced itch, but not histaminergic itch, is affected in the cluster deletion mice. Similarly, intradermal injection of CQ in rats evoked profound scratching responses, whereas quinoline did not (Figure S2A). These data provide further evidence that CQ-evoked itch is well conserved.

Previous studies have indicated that CQ can cause mast cell degranulation (Green and Lim, 1989; Nosal et al., 1991). To determine whether this effect on mast cells contributes to CQ-evoked scratching behavior, we repeated the experiment on SASH mice, which lack mast cells due to a chromosomal inversion in the regulatory element of the Kit gene (Yamazaki et al., 1994). As compared to WT controls, SASH mice exhibited a modest but significant reduction in CQ-induced scratching behavior (Figure 2K). The mast cell deficiency in these mice was confirmed by a dramatic decrease in the level of histamine released upon skin mast cell degranulation (Figure 2L). These results confirm that degranulation of mast cells induced by CQ contributes to scratching behavior, which may account for the residual response to CQ in Mrgpr-cluster \( ^{-/-} \) mice.
CQ Directly Excites DRG Neurons in an Mrgpr-Dependent Manner

Since CQ-induced itch is not considered an allergic reaction, we hypothesized, given our results, that this type of itch results from a direct activation of DRG neurons by the drug. If so, then the behavioral deficit seen in mutant mice would be attributed to a direct activation of DRG neurons by the drug. If so, then the behavioral deficit seen in mutant mice would be attributed to a direct activation of DRG neurons by the drug. If so, then the behavioral deficit seen in mutant mice would be attributed to a direct activation of DRG neurons by the drug. If so, then the behavioral deficit seen in mutant mice would be attributed to a direct activation of DRG neurons by the drug. If so, then the behavioral deficit seen in mutant mice would be attributed to a direct activation of DRG neurons by the drug. If so, then the behavioral deficit seen in mutant mice would be attributed to a direct activation of DRG neurons by the drug. 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neurons in DRG and that Mrgprs are required for this effect. Similar to the mouse results, CQ induces robust activation in a subset of rat DRG neurons as determined by both an increase in \([\text{Ca}^{2+}]_i\) and generation of APs (Figure S2).

**CQ Specifically Activates Mouse MrgprA3 and Human MrgprX1**

The *Mrgpr-cluster D/C0/C0* behavioral and cellular loss-of-function phenotypes strongly suggest that Mrgprs function as cell-surface receptors for CQ. To test this possibility directly, we examined whether Mrgprs have the ability to confer sensitivity to CQ on heterologous cells. We cloned each of the 12 Mrgprs that were deleted in *Mrgpr-cluster D/C0/C0* mice (Figure 1A) into a mammalian expression vector and transfected them individually into human embryonic kidney (HEK) 293 cells. By fusing green fluorescent protein (GFP) to the C termini of the Mrgpr coding sequences, we were able to visualize both transfected cells and the proper membrane localization of the receptors. Our previous studies demonstrated that GFP does not disturb the normal function of Mrgprs (Dong et al., 2001; Han et al., 2002). Increased \([\text{Ca}^{2+}]_i\) resulting from activation of the receptors was monitored by calcium imaging. Among the 12 mouse Mrgprs, only MrgprA3 conferred a strong response to CQ on HEK cells, whereas the other receptors conferred either weak or no responses to the drug (Figures 4A–4D and 5B, and data not shown). MrgprA1, MrgprA4, and MrgprC11 were activated by their peptide agonists FMRF, NPFF, and BAM8-22, respectively, confirming that they are functional receptors but insensitive to CQ (Figures 4A, 4C, and 4D). Furthermore, MrgprA3-expressing HEK cells did not respond to BAM8-22 or histamine, indicating that MrgprA3 is a specific receptor for CQ (Figure 4B). Conversely, histamine receptor H1-expressing HEK293 cells failed to show any response to CQ (Figure 4F).

The human Mrgpr family (i.e., MrgprXs) is much smaller than the murine family. Although the human and mouse genes share strong sequence homology, they do not form clear orthologous pairs. We found that MrgprX1-expressing HEK293 cells
responded to CQ, whereas MrgprX2- and X3-expressing cells were completely insensitive to the drug (Figure 4E and data not shown). Together, these data suggest that CQ directly activates mouse MrgprA3 and human MrgprX1 in heterologous cells with high specificity.

In order to determine the lowest concentrations of CQ capable of activating MrgprA3 and X1, we performed dose-response experiments in HEK293 cells. These experiments indicated that the receptors could be activated by the drug at micromolar concentrations with the mouse receptor showing 10-fold higher sensitivity than the human receptor (Figure 5B and 5D). EC50s for MrgprA3 and MrgprX1 are 27.55 ± 2.03 and 297.68 ± 2.10 μM, respectively (see the Discussion regarding the low potency of CQ on MrgprX1). Besides CQ, we also determined the sensitivities of these receptors to other structurally related compounds (i.e., quinoline, quinine, and serotonin) (Figure 5A). Quinoline is used as an intermediate in the production of various compounds including CQ. Despite the presence of a bicyclic structure, quinoline completely failed to activate MrgprA3 and MrgprX1, suggesting that the side chain in CQ is also necessary for activation (Figures 5B and 5D). Consistently, quinoline does not induce any scratching behavior in mice (Figure 2J). Serotonin failed to activate MrgprA3 at a concentration of 100 μM (Figure 5B). Quinine is another drug used to treat malaria, and its side effects also include itch. However, it is unclear whether quinine-induced itch is an allergic response (Gonzalez et al., 2002; Kanny et al., 2003). Unlike CQ, quinine weakly activates MrgprA3 (Figure 5B).

**MrgprA3 Is the Major Receptor Mediating CQ Responsiveness in DRG Neurons**

We and other groups have shown that MrgprA3 is expressed in a small subset (i.e., 4%-5%) of WT DRG neurons (Dong et al., 2001; Liu et al., 2008; Zylka et al., 2003). The population of MrgprA3+ neurons is small in comparison to that expressing another Mrgpr member, MrgprD (Figure 6A). According to our previous studies, MrgprA3 has the highest expression level among all MrgprAs in adult mouse DRG, whereas MrgprA1 is dramatically downregulated to expression in few neurons, all of which are also MrgprA3+ (Zylka et al., 2003). To confirm the expression profile of MrgprA3, we performed RT-PCR on various adult mouse tissues. Among the tissues tested, MrgprA3 is found exclusively in DRG and nodose ganglia (Figure 6B). The low intensity of the MrgprA3 band as compared to that of GAPDH is consistent with the fact that only a small percentage of neurons express MrgprA3 in these ganglia. Human MrgprX1 exhibits a similar expression pattern (Lembo et al., 2002). This
result also suggests that mast cells are unlikely to express MrgprA3. We did not see MrgprA3 in the skin, which contains many mast cells, nor did we see MrgprA3 expression in primary mast cells enriched from skin (Figure 6B) or bone marrow-derived mast cells (data not shown). Therefore, the transmission of CQ-induced itch signal by MrgprA3 likely occurs in primary sensory neurons in DRG and not other cell types in the skin.

To determine whether the expression of MrgprA3 in DRG neurons correlates with CQ sensitivity (also 4%–5%), we performed single-cell RT-PCR for the gene on individual DRG neurons responsive to CQ as determined by calcium imaging. Eight out of nine CQ-responding neurons expressed MrgprA3 mRNA, whereas none of the 11 CQ-insensitive neurons showed detectable levels of the receptor transcript (Figure 6C). Since MrgprA3 expression in mouse DRG neurons correlates very well with CQ sensitivity, we examined whether specific knockdown of MrgprA3 would abolish CQ responsiveness. Strikingly, WT DRG neurons failed to respond to CQ after electroporation with siRNA specifically targeted against MrgprA3, whereas a control siRNA had no effect on CQ sensitivity (Figures 6D–6F). These data strongly suggest that MrgprA3 is the main receptor mediating CQ-evoked responses in mice. Unlike mouse MrgprA subfamily which consists of 22 members, rats have only one MrgprA. Consistently, using a single-neuron RT-PCR technique, we found that all of CQ-sensitive rat DRG neurons express rat MrgprA (n = 10).

**MrgprA3 and MrgprX1 Rescue the Phenotypes of Mrgpr-Deficient Neurons**

We next asked whether MrgprA3 or MrgprX1 can rescue the phenotypes of DRG neurons from Mrgpr-clusterΔ−/− mice. To answer this question, we electroporated the Mrgpr expression constructs used in the heterologous studies into dissociated adult DRG neurons from these mice. After 24 hr in culture, expression and membrane localization of the transfected Mrgprs in the mutant neurons could be readily visualized by GFP (Figure 6G). Strikingly, all MrgprA3-expressing mutant neurons generated numerous APs in response to CQ treatment (Figure 6H), whereas neighboring GFP-negative neurons remained silent (n = 6, not shown). The number of APs generated in the GFP-positive neurons was comparable to that produced by CQ treatment of WT DRG neurons, indicating a nearly complete rescue by MrgprA3. Similar results were obtained for Mrgpr-deficient neurons electroporated with MrgprX1 (Figure 6J). In contrast, fewer than half of the MrgprA1-electroporated neurons elicited a few APs in response to CQ (Figure 6I). Rescue by MrgprA3 and MrgprX1 was also seen using calcium imaging, with an increase in [Ca2+]i induced by CQ (Figures 6K–6M). Together, these results strongly suggest that mouse MrgprA3 and human MrgprX1 are the major CQ receptors in DRG neurons. Expression of rat MrgprA in Mrgpr-clusterΔ−/− DRG neurons conferred CQ sensitivity upon them, whereas rat MrgprC did not (Figures S2D and S2E).

**CQ-Sensitive Neurons Also Respond to Histamine and Capsaicin**

To further define the population of CQ-sensitive neurons in DRG, we examined the responses of these cells to other well-characterized chemicals. Many studies utilizing multiple approaches have shown that histamine- and capsaicin-responding cells largely overlap (Schmelz et al., 2003; Shim et al., 2007). Consistent with previous reports, we found that
**B**

- **MrgrprA3**
- **GAPDH**

**C**

- **MrgrprA3**
- **β-actin**

**D**

- **siRNA MrgrprA3**
  - BAM
  - CQ

**E**

- **siRNA MrgrprC11**
  - CQ
  - BAM
  - CQ

**F**

- **siRNA MrgrprA3**
- **siRNA MrgrprC11**

**G**

- **KO+MrgrprA3**
- **CQ**

**H**

- **KO+MrgrprA1**
- **CQ**

**I**

- **KO+MrgrprX1**
- **CQ**

**J**

- **KO+MrgrprX1**

**K**

- **KO+MrgrprA3**

**L**

- **KO+MrgrprA1**
  - FMRF

**M**

- **KO+MrgrprX1**
  - BAM 8-22
87% of histamine-sensitive DRG neurons also responded to capsaicin as monitored by an increase in [Ca^{2+}], using calcium imaging. Interestingly, all CQ-responding neurons in DRG cultures were also activated by both histamine and capsaicin (Figure 7A). Furthermore, we noticed that CQ-sensitive cells have a narrow range of cell diameters, whereas histamine-sensitive neurons have a wide range (Figure S3). Therefore, the small population of CQ-sensitive neurons in WT DRG defines a unique and specific subset of histamine- and capsaicin-sensitive neurons.

**MrgprA3-Expressing Neurons Are Likely to Be Itch-Selective Neurons**

The finding that MrgprA3-positive neurons are sensitive to both histamine and CQ raises the interesting possibility that these neurons are itch-selective neurons. Gastrin-releasing peptide (GRP), a ligand for GRPR, is expressed in a subset of DRG neurons (Sun and Chen, 2007). To look for overlap between GRP and MrgprA3 expression in DRG neurons, we carried out double-staining experiments for these two genes. Strikingly, 93% of MrgprA3-positive neurons also expressed GRP, providing strong evidence that MrgprA3-expressing neurons may play important roles in itch sensation (Figures 7F and S4).

Our previous data have shown that expression of MrgprC11 largely overlaps with that of MrgprA3 (Zylka et al., 2003). Consistently, all BAM8-22-responsive neurons (i.e., 3.6% of total WT DRG neurons) also responded to CQ (Figures 7C–7E). Importantly, no DRG neurons from Mrgpr-clusterΔ−/− mice responded to BAM8-22 (Figure 7C). As we expected, intradermal injection of BAM8-22 induced strong scratching behavior in WT mice, whereas mutant mice exhibited a dramatic reduction in the response evoked by the peptide (Figure 7B). Together, these data suggest that activation of MrgprA3- or MrgprC11-expressing neurons by their specific agonist (i.e., CQ and BAM8-22, respectively) can evoke scratching behavior and further support that these neurons are involved in itch sensation.

**DISCUSSION**

It has been known for a long time that both pain and itch are initiated and modulated by small-diameter sensory neurons in DRG (Caterina and Julius, 1999; Ikoma et al., 2006; Shim and Oh, 2008). Compared to pain, our knowledge of itch especially histamine-independent itch at cellular and molecular levels is poor. Here, we present evidence showing that sensory neuron-specific Mrgps are receptors mediating CQ-induced itch.

**Mrgpr-clusterΔ−/− mice exhibit a severe reduction in CQ-induced scratching behavior, whereas histamine-mediated itch and acute pain are completely normal. The residual CQ-induced scratching behavior seen in mutant animals is likely due to an indirect effect on skin sensory nerves. Both our behavioral data from SASH mice and complete elimination of CQ-sensitive neurons in **Mrgpr-clusterΔ−/−** DRG support this notion and suggest that the residual CQ-induced response in **Mrgpr-clusterΔ−/−** mice results from degranulation of skin mast cells caused by the drug, a phenomenon observed in previous studies (Green and Lim, 1989; Nosal et al., 1991).

Since Mrgrp members are highly homologous to each other, especially the MrgrpA subfamily (70%–80% identity), it is surprising to find that only MrgprA3 shows strong activation by CQ. The most divergent regions of Mrgprs are localized to the extracellular loops, consistent with the differences in their ligand preferences (Dong et al., 2001; Yang et al., 2005). Bioinformatic analysis of Mrgrp sequences suggest that positive selection likely accounts for the amino acid substitutions in the extracellular domains (Choi and Lahn, 2003; Yang et al., 2005). Interestingly, human MrgrpX1 can respond to both CQ and BAM8-22, whereas mouse MrgpA3 and MrgprC11 are specific receptors for these two agonists, respectively. The agonist selectivity of the mouse receptors supports the conclusion made from statistical analysis that adaptive evolution of Mrgrp family contributes to its expansion in the mouse genome.

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**Figure 6. MrgprA3 Is Required for CQ Responsiveness in Mouse DRG Neurons**

(A) Fluorescent in situ hybridization of DRG sections with MrgprA3 (green, arrowheads) and MrgprD (red). The white dashed line outlines the DRG.

(B) RT-PCR analysis of 14 mouse tissues or cell types for expression of MrgprA3. The only tissues containing MrgprA3 are WT DRG and nodose ganglia. Notably, no band was found in Mrgpr-clusterΔ−/− DRG, confirming that MrgprA3 was deleted in Mrgpr-clusterΔ−/− mice.

(C) Single-cell RT-PCR was performed on individual DRG neurons with the responsiveness to CQ (1 mM) established by calcium imaging (shown here are 12 representative neurons). MrgprA3 mRNA was detected in eight out of nine CQ-responsive neurons (+) but was not detected in any of the 11 CQ-unresponsive neurons (−). For a negative control, sample of bath solution was used (Bath); diluted total DRG cDNA was used as positive control (DRG). Arrows indicate predicted product size for MrgprA3 (150 bp) and β-actin (302 bp). No product was detected in RT controls from MrgprA3-expressing cells (n = 8).

(D and E) Representative traces from three different WT DRG neurons electroporated with siRNAs in calcium-imaging assays. CQ-induced increase in [Ca^{2+}]i was completely lost in WT neurons electroporated with MrgprA3 siRNA (D). However, these neurons (normally express both MrgprA3 and MrgprC11) are still sensitive to BAM8-22 (BAM). Twenty-four BAM8-22-sensitive neurons were analyzed. As a control, CQ responsiveness in WT neurons electroporated with MrgprC11 siRNA remained intact (ten CQ-sensitive neurons were analyzed) (E). But MrgprC11 siRNA completely abolished BAM8-22 sensitivity.

(F) The efficiency and specificity of MrgprA3 siRNA were tested by cotransfection of HEK293 cells with MrgprA3 siRNA and expression constructs of MrgprA3 or MrgprC11. Western blot shows that MrgprA3 siRNA specifically knocked down the expression of MrgprA3 but not MrgprC11.

(G–M) MrgprA3 and MrgprX1 selectively rescued CQ responsiveness in Mrgpr-clusterΔ−/− DRG neurons. Visualization of Mrgpr-clusterΔ−/− DRG neurons that express MrgrpA3-GFP protein is shown (G). Note the membrane and axon localization (arrowheads) of MrgprA3-GFP in DRG neurons. All Mrgpr-clusterΔ−/− neurons electroporated with MrgprA3 fired a train of APs upon CQ treatment (n = 6) (H). Fewer than half of MrgprA17-electroporated neurons (three out of seven) elicited a few APs upon CQ treatment (I). Most Mrgpr-clusterΔ−/− neurons electroporated with MrgprX1 (five out of seven GFP-positive neurons recorded) also generated a train of APs in response to CQ (J).

(K–M) Each figure shows typical calcium traces from three different neurons. All MrgprA3-expressing Mrgpr-clusterΔ−/− neurons showed increased [Ca^{2+}]i in response to CQ (1 mM) but not BAM8-22 (2 μM) (K). All MrgprA17-electroporated mutant neurons showed a strong response to FMRF (2 μM), whereas only a small portion responded to CQ (L). Electroporation of MrgprX1 rendered Mrgpr-clusterΔ−/− DRG neurons sensitive to both CQ and BAM8-22 (M).
Figure 7. CQ Responsiveness Defines a Specific Subpopulation of DRG Neurons

(A) CQ-responsive neurons represented a small population of DRG neurons that also responded to histamine (50 μM) and capsaicin (1 μM) with increased [Ca^{2+}]_{i} monitored by calcium imaging.

(B) The total scratching bouts during the first 30 min after BAM8-22 intradermal injection (50 μl of 1 mM). WT mice exhibited significantly stronger scratching responses after injection than did Mrgr-cluster.D^{-/-} littermates (n = 8 per genotype; *p < 0.05).

(C and D) As determined by calcium imaging, 3.6% of WT DRG neurons responded to BAM8-22 (2 μM) with increased [Ca^{2+}]_{i} and all of them are also CQ-sensitive (D), whereas Mrgr-cluster.D^{-/-} DRG neurons failed to respond to the drug (n = 3 per genotype) (C).

(E) The Venn diagram illustrates the relationships of histamine- (His), capsaicin- (Cap), chloroquine- (CQ), and BAM8-22- (BAM) responsive neurons in adult DRG. The sizes of the circles are proportional to the sizes of the cell populations.
According to our dose-response curves, EC\textsubscript{50}s of CQ for MrgrpA3 and human MrgrpX1 are 27.55 ± 2.03 and 297.68 ± 2.10 μM, respectively. Although the concentration of CQ in patient plasma is in the micromolar range, excretion of the drug is quite slow and it is deposited in tissues in considerable amounts (Adam et al., 2004; Evans et al., 2005; Onyeji and Ogunbona, 2001). Since CQ binds strongly to melanin that is synthesized by melanocytes, it accumulates at very high levels in the skin and other pigmented tissues to reach high micromolar to millimolar concentrations (Dencker et al., 1975; Olatunde, 1971; Sams and Epstein, 1965; Tanaka et al., 2004). The high level of CQ (i.e., high micromolar to millimolar concentrations) is also required to induce scratching behavior in mice based on our and other group’s dose-response studies (data not shown) (Green et al., 2006). In addition, patients prone to CQ-induced itch accumulate higher concentrations of CQ in their skin than those not prone to the side effect (Olatunde, 1971). The different levels of CQ in the skin of the two groups are likely due to different rates of metabolism of the drug (Onyeji and Ogunbona, 2001). Besides the level of CQ in the skin, mouse strain comparison and human familial clustering of itch studies suggest that genetic variability also contributes to phenotypic differences in CQ-induced itch (Ajayi et al., 1989; Green et al., 2006). The high polymorphism seen in both mouse and human Mrgrp genes may provide a molecular explanation for the variability in itch levels among different individuals (Dong et al., 2001; Yang et al., 2005).

Our heterologous studies indicate that MrgrpA3 is the major receptor for CQ among the 12 deleted Mrgrps. Other Mrgrps excluded in the cluster deletion are unlikely to be involved in CQ signaling in DRG neurons because of the total loss of CQ response in mutant DRG. Consistently, the percentage of CQ-sensitive DRG neurons (i.e., 4%-5% of total DRG neurons) matches that of MrgrpA3-expressing cells as determined by in situ hybridization on adult DRG sections (Liu et al., 2008). More importantly, our single-neuron RT-PCR results indicate that MrgrpA3 expression correlates almost perfectly to CQ responsiveness. Furthermore, both gain- and loss-of-function studies firmly establish that MrgrpA3 is required for CQ responsiveness in mice.

This small population of CQ-sensitive neurons marks a subset of histamine- and capsaicin-responsive cells in DRG, and it has a uniform cell size as compared to the total histamine-sensitive population. According to different reports, the percentage of histamine-sensitive cells in the DRG ranges from 15% to 40% (Han et al., 2006; Kim et al., 2004; Nicolson et al., 2002). It is unlikely that all of these cells are pruriceptive neurons. In fact, human microneurography studies suggest the sensory fibers that respond to histamine with sustained discharges are responsible for itch, whereas those weakly activated by histamine are involved in pain processing (Schmelz et al., 1997). The strong histamine-responsive fibers comprise only a small portion of all unmyelinated sensory fibers, and the majority of them are heat responsive. Recent studies have shown that TRPV1, a molecular sensor for capsaicin and heat, functions downstream of histamine receptors and is required for histamine-induced DRG neuron activation and itch behavior (Shim et al., 2007). These studies also raise the interesting possibility that a subset of capsaicin- and heat-sensitive neurons mediates itch. Therefore, it would be important to know whether the 4%-5% of total DRG neurons activated by CQ is selective for itch. Activation of CQ-sensitive neurons by BAM8-22 through MrgrpC11 also induces scratching response, providing further evidence that CQ-sensitive neurons may be itch-selective neurons. Finally, overlap between MrgrpA3- and GRP-expressing neurons in DRG leads us to propose a model for CQ signal transduction: CQ directly activates a subset of primary sensory fibers in the skin through MrgrpA3. This leads to the release of GRP into the dorsal horn of the spinal cord, where it activates a subset of dorsal horn neurons through GRPR. Therefore, the identification of Mrgrps as receptors for CQ may open new avenues for the exploration of itch-selective neuron development and function, as well as the development of novel anti-itch drugs.

**EXPERIMENTAL PROCEDURES**

**Generation of Mrgrp-cluster;\textsuperscript{−/−} Mice**

to delete a cluster of Mrgrp genes in the mouse germline, we constructed two replacement vectors for MrgrpA1 and MrgrpB4, which reside on either end of the Mrgrp cluster. The entire ORFs of both MrgrpA1 and MrgrpB4 are encoded by a single exon. The arms of the MrgrpA1 and MrgrpB4 targeting constructs were obtained by PCR amplification from 129/SvJ genomic DNA with the Expand High Fidelity PCR System (Roche). Details of the generation of Mrgrp-cluster;\textsuperscript{−/−} mice are available in the Supplemental Data.

**Behavioral Studies**

All behavioral tests were performed with an experimenter blind to genotype. The mice were 2- to 3-month-old males (20–30 g) that had been backcrossed to C57Bl/6 mice for at least ten generations. Rat studies were done with 4-week-old CD animals (Charles River Laboratories). All experiments were performed under the protocol approved by the Animal Care and Use Committee of Johns Hopkins University School of Medicine. Pruritic compounds (i.e., histamine, compound 48/80, and CQ) were subcutaneously injected into the nape of the neck after acclimatization, and scratching behavior was observed for 30 min. A bout of scratching was defined as continuous scratch movements with hind paws directed at the area around the injection site. Scratching behavior was quantified by recording the number of scratching bouts at 5 min intervals over the 30 min observation period. Details for other behavior assays are available in the Supplemental Data.

**Cultures of Dissociated DRG Neurons**

DRG from all spinal levels of 4-week-old mice or rats were collected in cold DH10 medium and treated with enzyme solution at 37°C for 30 min. A bout of scratching was defined as continuous scratch movements with hind paws directed at the area around the injection site. Scratching behavior was quantified by recording the number of scratching bouts at 5 min intervals over the 30 min observation period. Details for other behavior assays are available in the Supplemental Data.

(F) WT adult DRG sections were doubly stained by in situ hybridization for MrgrpA3 (blue) and immunostaining with anti-GRP antibody (brown). Most MrgrpA3\textsuperscript{+} cells (51 out of 55) express GRP. Arrowheads indicate MrgrpA3/GRP-coexpressing neurons. Arrows indicate MrgrpA3\textsuperscript{+/}GRP\textsuperscript{−} cells. All error bars represent the SEM.
CaCium Imaging
Neurons or HEK293 cells were loaded with fura 2-acetomethoxy ester (Molecular Probes) for 30 min in the dark at room temperature or 45 min at 37° C, respectively. After washing, cells were imaged at 340 and 380 nm excitation to detect intracellular free calcium. Calcium-imaging assays were performed with an experimenter blind to genotype. Each experiment was done at least three times, and at least 100 cells (neurons or HEK293 cells) were analyzed each time.

RNA Interference
MrgprA3 and MrgprC11 on-target siRNAs were purchased from Thermo Scientific. 0.175 nmol MrgprA3 or MrgprC11 siRNA was electroporated into WT DRG neurons, respectively. After 3 days of culture, neurons were replated on glass coverslips for calcium imaging. HEK293 cells in 12-well plates were cotransfected with 2 μg Mrgpr-expression constructs and 2 μg siRNA with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. After 1 day of culture, cells were lysed for western blot.

Whole-Cell Current-Clamp Recordings of Cultured DRG Neurons
Neurons plated on coverslips were transferred into a chamber with the extra-cellular solution. Patch pipettes had resistances of 2–4 MΩ. In current-clamp recordings, action potential measurements were performed with an Axon 700B amplifier and the pCLAMP 9.2 software package (Axon Instruments). Neurons were perfused with 1 mM CO2 for 20 s. All experiments were performed at room temperature (−25° C). Details are available in the Supplemental Data.

In Situ Hybridization and Immunostaining
In situ hybridization was performed as previously described (Dong et al., 2001). For MrgprA3 and GRP double staining, after in situ hybridization for MrgprA3, DRG sections were incubated overnight at 4° C with Rabbit anti-GRP antibody (Innoston, 1:1000) in PBS/1% donkey serum/0.3% Triton X-100. After washing twice in PBS, sections were incubated with biotinylated secondary antibody (Jackson, 1:200) for 2 hr at room temperature and then washed twice in PBS and incubated with ABC mix (Elite ABC kit, Vectastain) in PBS at RT for 1 hr. Sections were washed twice in PBS and incubated with 3,3′-Diaminobenzidine/H2O2 in PBS for color development.

Single-Cell RT-PCR
Plated neurons with CQ responsiveness established by calcium imaging were individually harvested into a glass pipette and transferred into PCR tube. Reverse transcription was done by Super-Script III CellsDirect (Invitrogen) as previously described (Kwong et al., 2008). Details for PCR conditions are available in the Supplemental Data.

Data Analysis
Data are presented as mean ± standard error of mean (SEM). Statistical comparisons were made with an unpaired Student’s t test, and differences were considered significant at p < 0.05.

SUPPLEMENTAL DATA
Supplemental Data include Supplemental Experimental Procedures and four figures and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)01492-5.

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