Redefining the concept of protease-activated receptors: cathepsin S evokes itch via activation of Mrgprs

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Sensory neurons expressing Mas-related G-protein-coupled receptors (Mrgprs) mediate histamine-independent itch. We show that the cysteine protease cathepsin S activates MrgprC11 and evokes receptor-dependent scratching in mice. In contrast to its activation of conventional protease-activated receptors, cathepsin S-mediated activation of MrgprC11 did not involve the generation of a tethered ligand. We demonstrate further that different cysteine proteases selectively activate specific mouse and human Mrgrp family members. This expansion of our understanding by which proteases interact with G-protein-coupled receptors (GPCRs) redefines the concept of what constitutes a protease-activated receptor. The findings also implicate proteases as ligands to members of this orphan receptor family while providing new insights into how cysteine proteases contribute to itch.
The sensation of itch is relayed from the skin to the brain via a complex but orchestrated series of signals. Itch is initiated when exogenous or endogenous pruritogens activate receptors or channels on the peripheral projections of primary sensory neurons, the cell bodies of which reside in the dorsal root or trigeminal ganglia. Specific members of the family of Mas-related G-protein-coupled receptors (Mrgprs) are expressed by a subset of nociceptive fibres. These receptors have been shown to bind select pruritogens in the periphery and mediate non-histaminergic itch. For example, mouse MrgprA3 and human MRGPRX1 respond to chloroquine, an anti-malarial drug, and are responsible for relaying chloroquine-induced scratching in mice. Mouse MrgprC11 and human MRGPRX1 respond to a different subset of pruritogens including bovine adrenal medulla peptide (BAM8–22). MrgprC11 is activated by SLIGRL and SLIGKV, the tethered ligand peptides of respective mouse and human protease-activated receptor-2 (PAR2), while MRGPRX2 is activated by SLIGKV. Trypsin, a serine protease and conventional activator of PARs, does not activate MrgprD receptor expressed by nociceptive fibres. In transgenic mice in which a cluster of Mrgprs has been ablated, cutaneous exposure to these pruritogens evokes significantly less scratching compared with wild-type (WT) controls. These findings underscore the importance of this family of receptors to peripheral detection of non-histaminergic itch stimuli and the subsequent activation of itch-specific neural pathways. While several exogenous compounds trigger Mrgrp activation, endogenous ligands or modulators of MrgrpA3 and MrgprC11 receptors have yet to be identified.

Both serine and cysteine proteases have been implicated in triggering itch and inflammation in the skin. The plant cysteine protease mucunain, derived from the tropical bean plant commonly known as cowhage, and the human cysteine protease, cathepsin S (cat S), elicit itch in human volunteers. We demonstrated previously that cat S, mucunain and other plant cysteine proteases including papain, bromelain and ficin are capable of activating PAR2 and PAR4 (refs 10,11). It has been presumed that cysteine protease-evoked itch was induced via PAR2 activation. The possibility that activation of receptors other than PARs could be responsible for this sensation has not been investigated. MrgprC11 is activated by SLIGRL, a synthetic peptide generated based on the tethered sequence of the PAR2 N terminus following cleavage by serine proteases. This observation is surprising because Mrgprs are not members of the PAR family. MrgprC11 does not have either arginine or lysine residues in its N-terminal extracellular domain. This receptor would neither be cleaved nor in theory activated, by serine proteases including trypsins and kallikreins. In contrast, the MrgprC11 receptor N terminus contains glycine and leucine residues, both cleavage targets for aspartyl and cysteine proteases. Here, we formally investigated whether select cysteine proteases are capable of cleaving and activating MrgprC11 receptors in vitro, and determined the significance of MrgprC11 signalling in mediating cat S-induced scratching in vivo.

The results demonstrate that cathepsin S provokes itching by cleavage of MrgprC11 at a specific site, resulting in activation of this receptor without generating either a tethered or diffusible ligand. Although cathepsin S can activate PAR2 receptors, such an activation is not necessary for itching provoked by this protease. Hybrid receptors in which the N terminus of MrgprC11 was replaced with the N terminus from either the β2AR or MC1R were also activated by cathepsin S. These findings highlight the possibility that cysteine proteases may exert effects on other non-PAR GPCRs and further suggest that proteases are capable of modulating GPCRs by multiple mechanisms.

Results
Cysteine proteases cleave the N terminus of MrgprC11. MrgprC11 plays a critical role in mediating pruriception from the periphery. These receptors are known to bind several well-recognized pruritogens, including peptides resulting from the cleavage of PARs. Whether or not proteases have any direct effect on Mrgrp receptors was not known. To address this possibility, we asked whether cysteine proteases could cleave the extracellular N-terminal portion of MrgprC11. HeLa cells were transfected with a construct encoding MrgprC11 tagged with Gaussia luciferase at its N terminus and then treated with cat S or papain. Receptor cleavage was assessed by measuring levels of luminescence.

Figure 1 | Cysteine proteases cleave near the N terminus of MrgprC11. (a) Luminescence was measured after treating LucMrgprC11-transfected HeLa cells with cat S (2 μM) and papain (2 μM) in the presence or absence of E-64 (5 μM). Baseline luminescence has been subtracted. The data are presented as mean ± s.e.m. **P < 0.001; ***P < 0.0005; two-tailed unpaired t-test from a representative experiment that was performed three times. (b) Western blot of LucMrgprC11 N-terminal cleavage products following treatment of transfected HeLa cells with control buffer without proteases (lane 1), papain, 0.02 μM (lane 2) and cat S, 2 μM (lane 3). The lesser amount of luminescence from papain as compared with cat S in a is likely a result of higher enzyme activity and a broader range of cleavage sites for papain. Similarly, for b, a lesser concentration of papain as compared with cat S was used for incubation times to be the same for detection of luciferase. See the Methods and Discussion sections for additional comments. The LucMrgprC11 construct was functionally active (Supplementary Fig. 1).
luminescence in the supernatants (Fig. 1). Both papain and cat S induced cleavage of the MrgrpC11 N terminus as determined by luminescence (Fig. 1a). Incubation with E-64, an irreversible cysteine protease inhibitor, blocked protease-mediated cleavage of the N termini. In separate studies, tagged MrgrpC11-transfected cells were treated with cyanine proteases. Western blots of supernatants probed with an anti-G. luciferase antibody revealed a dense band at ~20 kDa in cells treated with cat S or papain, consistent with the mass expected of an N-terminal peptide tagged with G. luciferase (Fig. 1b). As expected, no bands were identified in controls in which transfected cells were not treated with protease. These results reveal that cyanine proteases cleave the N-terminal portion of MrgrpC11.

**Cysteine proteases activate MrgrpC11 in vitro.** We next asked whether cysteine proteases were capable of activating MrgrpC11. This receptor signals via Gq, resulting in phospholipase C activation and, via second messenger recruitment, increased intracellular free calcium and protein kinase C activation. To assess receptor activation, we performed calcium imaging using the ratiometric calcium indicator Fura-2 in HeLa cells transfected with MrgrpC11 following treatment with cat S or papain. Each protease elicited calcium responses (Fig. 2a), activating MrgrpC11 in a concentration-dependent manner with an effector concentration for half-maximum response of ~140 nM (Fig. 2b). Calcium responses were ablated when cells were treated with the protease inhibitor E-64 (Fig. 2a). In contrast to the robust responses induced by cyanine proteases, we confirmed that trypsin, a serine protease, does not activate MrgrpC11 (ref. 3). Western blotting revealed PKC phosphorylation at serine 660 (Ser660) following treatment with cat S and papain (Fig. 2c). Quantification of the bands revealed an approximate 4.6-fold increase in p-PKC associated with protease activation relative to controls (Supplementary Fig. 3).

We next assessed whether cyanine proteases activate endogenously expressed Mrgrp receptors on dorsal route ganglion (DRG) neurons. Both cat S and papain treatment evoked calcium signals in DRGs from WT mice (Fig. 2d). Fewer DRG neurons harvested from mice in which a cluster of Mrgrp genes, including MrgrpC11, have been deleted (Mrgrp cluster Δ−/− mice), responded to cat S (Fig. 2d). The lack of response of these neurons to papain is likely due to receptor inactivation by papain under the conditions used. Because cat S is known to activate PAR2 when it is expressed in heterologous cell lines, we evaluated the effect of cat S on DRGs harvested from PAR2−/− mice. Of note, calcium responses to cat S remained intact in the absence of PAR2 receptors (Fig. 2d) These data support a role for protease-induced MrgrpC11 activation in DRGs and, importantly, identify cat S as a potential endogenous and relevant ‘ligand’ for this receptor. The average diameter of responsive neurons was 19.3 μm, comparable to that reported previously for Mrgrp-expressing itch neurons.

RNA interference (RNAi) knockdown of MrgrC11 and closely related MrgrpA3 was performed in DRG neurons (Fig. 3). Messenger RNA levels of both genes were significantly lower as confirmed by real-time PCR (Fig. 3d). Knockdown of MrgrpC11 only abolished the calcium response to the MrgrpC11 ligand cat S but not the MrgrpA3 ligand chloroquine. In contrast, knockdown of MrgrpA3 only affected the response to chloroquine but not the response to cat S, showing effectiveness and specificity of RNAi. Note that transient knockdown of MrgrpC11 totally abolished the cat S-induced response, while knockdown of MrgrpA3 only reduced the response to chloroquine. This result is consistent with the stronger knockdown effect of MrgrpC11. The observation that transient knockdown eliminates the calcium response to cat S suggests that the remaining calcium response in Mrgrp cluster knockout (KO) mice may be due to an otherwise unspecified compensation mechanism in the permanent KO. In summary, these data support a role for protease-induced MrgrpC11 activation in DRGs and, importantly, identify cat S as a potential endogenous and relevant ‘ligand’ for this receptor.

**Cathepsin S induces scratching via MrgrpC11.** Cat S has been shown previously to cleave and activate PAR2 in vitro13. It has been assumed, but never demonstrated clearly, that PAR2 activation is the mechanism by which cat S evokes scratching in mice and itch in humans. However, in light of our *in vitro* findings that cat S-induced calcium signalling in DRGs requires MrgrpC11 but not PAR2, we sought to determine the relative importance of these two receptors, if any, to cat S-induced scratching *in vivo*. We thus compared behavioural scratching responses with intradermal injection of cat S using the mouse cheek model in WT, Mrgrp cluster Δ−/− mice and PAR2−/− mice. Pain and itch can be readily distinguished using the cheek injection model13. We only observed itch associated hind-paw scratching, but not pain-related fore-paw wiping. Injection of cat S elicited robust scratching behaviour in WT mice, but was markedly reduced in Mrgrp cluster Δ−/− mice (Fig. 4a). Perhaps surprisingly, scratching in PAR2−/− mice was as robust as in WT littermates (Fig. 4b). These experiments underline the pivotal role of MrgrpC11 in mediating cyanine protease-induced itch.

**MrgrpC11 N-terminal cleavage sites.** We next sought to determine whether cleavage of the extracellular N-terminal domain of MrgrpC11 is necessary for protease-induced receptor activation, and if so, to identify the relevant cleavage sites. As per the MEROPS database, preferred amino acids for cleavage by cat S and papain include leucine at P2 and glycine, serine and aspartic acid at P1. Using site-specific mutagenesis, we generated a series of single-, double- and triple-substitution mutants near the MrgrpC11 N terminus (Table 1; Supplementary Information Note 1). Calcium imaging of HeLa cells transfected with these mutant receptors revealed that leucine13 was a critical residue for cat S-mediated cleavage and activation. Papain required both aspartic acid and leucine13 for receptor cleavage and activation (Table 1; Supplementary Table 1). None of the other candidate residues including glycine14, serine6,15 and leucine16 were required for receptor activation by either protease.

To determine the specific protease cleavage sites, a peptide corresponding to the 28N-terminal residues of MrgrpC11 was synthesized, MDPTISSHDGDSTPL13 NETGHPNCTPILT. The aspartic and leucine residues required for papain and cat S activity are indicated. This peptide was incubated with cat S or papain and then subjected to mass spectrometry (MS/MS) peptide sequence analysis, as described in Supplementary Table 1 and Note 1, as we have done previously with cat S and PAR2 (ref. 17). Cat S cleavage occurred between N16 and E17 with L13 at the P2 position, MDPTISSHDGDSTPL13N ETGHPNCTPILT, consistent with the site-directed mutagenesis approach above. Substituting arginine for leucine13 completely eliminated the response to cat S (Table 1; Fig. 5b). Papain preferred L13 at the P2 position and cleavage between N16 and E17 but no additional specificity was apparent, and cleavage otherwise occurred at multiple sites.

**MrgrpC11 is not activated by tethered or diffusible ligands.** As conventional PARs are activated by tethered ligands generated following protease-mediated cleavage of their N-terminal domains, we sought to determine whether such peptides could activate MrgrpC11. We generated a series of eight peptides (Fig. 5a; Supplementary Table 2) ranging from 6 to 15 amino
The percentage of DRG neurons activated by papain (20 μM) was significantly reduced in MRG cluster knockout mice as compared with WT mice (n = 3, P < 0.001; two-tailed unpaired t-test). The percentage of DRG neurons activated by cat S (5 μM) is not significantly different between WT and PAR2+/− mice (n = 3, P = 0.74). (e) Representative calcium traces of cat S (5 μM) in DRG neurons from WT, MRG cluster Δ−/− and PAR2−/− mice. Each trace represents responses from a single neuron. Arrows indicate application of cat S.

Figure 2 | Cysteine proteases activate MrgrprC11 in heterologous cells and DRG neurons and induce PKC phosphorylation. (a) Single-cell calcium imaging in HeLa cells transfected with MrgrprC11 cDNA following treatment with cat S (2 μM, solid line), papain (2 μM, dashed line), cat S + E-64 (10 μM; dotted line), papain + E-64 (10 μM; dash-dotted line) and trypsin (10 nM) dash-double-dotted line. The concentration of trypsin is typical of that used in studies of PARs. Higher concentrations of trypsin, including those in the micromolar range, did not activate MrgrprC11. Additional calcium responses are detailed in Supplementary Fig. 2. (b) Concentration–effect curves for protease agonists cat S (squares) and papain (circles) on MrgrprC11. Error bars represent ± s.e.m. from three sets of experiments. (c) Phosphorylation of serine 660 as a measure of second messenger is revealed by western blot. HeLa cells were transfected with MrgrprC11 (lanes 1–3), or non-transfected HeLa cells (lanes 4–6) were treated with buffer alone (lanes 1 and 4), cat S (lanes 2 and 5) or papain (lanes 3 and 6). Band quantification and full scans of the blots are provided in Supplementary Fig. 3. (d) The percentage of DRG neurons activated by cat S (5 μM) was significantly reduced in Mrgr cluster knockout mice as compared with WT DRG neurons (n = 3, P < 0.001). The percentage of DRG neurons activated by papain (20 μM) was reduced to zero in Mrgr cluster knockout mice as compared with WT mice (n = 3, ***P < 0.001; two-tailed unpaired t-test). The percentage of DRG neurons activated by cat S (5 μM) is not significantly different between WT and PAR2−/− mice (n = 3, P = 0.74). (e) Representative calcium traces of cat S (5 μM) in DRG neurons from WT, Mrgr cluster Δ−/− and PAR2−/− mice. Each trace represents responses from a single neuron. Arrows indicate application of cat S.

Proteases activate β2AR and MC1R–MrgrprC11 hybrid receptors. We have shown thus far that protease-mediated cleavage of MrgrprC11 causes receptor activation without the formation of either a tethered or diffusible ligand. Another possible explanation of the ability of cysteine proteases to activate Mrgrprs is that proteolysis of the extracellular domain results in a direct conformational change of the intracellular portion of the receptor that allows G-coupled signalling to occur. If this is the case, a mutant MrgrprC11 in which the native N terminus has been substituted with an unrelated GPCR that can also be cleaved by cysteine proteases might be activated by proteolysis. To evaluate this possibility, we substituted the N terminus of MrgrprC11 with either that of the β2-adrenergic receptor (β2AR) or the melanocortin-1 receptor (MC1R). These receptors are class A GPCRs, such as Mrgrprs, but they couple primarily to Gs. We selected β2AR and MC1R because of the deep knowledge surrounding their structure and function. Each of these receptors contains residues that can be cleaved by cysteine proteases. However, biogenic amines and melanocortins, respectively, not proteases, are their conventional ligands. As predicted, both cat S and papain treatment of HeLa cells transfected with hybrid receptors β2AR–MrgrprC11 or MC1R–MrgrprC11 resulted in robust calcium signalling. As controls, protease treatment did not result in calcium mobilization in HeLa
cells expressing native β2AR or MC1R (Fig. 6). Taken together with the site-specific mutagenesis results, these findings argue that cat S- or papain-mediated cleavage of specific N-terminal residues directly triggers conformational changes in the transmembrane and/or intracellular domains of the MrgprC11 receptor that lead to downstream signalling.

Effect of cysteine proteases on other Mrgprs. To determine whether cysteine protease-mediated receptor activation was specific to MrgprC11, we examined whether cat S and papain were capable of triggering calcium mobilization in HeLa cells transfected with other members of the Mrgpr family, including human MRGPRX1–4 as well as mouse MrgprA3, which mediates chloroquine-induced itch. We found that in addition to activating human PAR2 and mouse MrgprC11, cat S activated human MRGPRX2 (Table 2; Supplementary Fig. 5). Cat S failed to activate other human MRGPRs and mouse MrgprA3 (Table 2). In contrast, papain activated human MRGPRX1, but had no effect on the other human MRGPRs (Table 2; Supplementary Fig. 5). As expected, trypsin failed to activate these Mrgprs (Fig. 2a; Table 2). These results demonstrate that cysteine proteases activate only

Figure 3 | Acute knockdown of MrgprC11 abolishes DRG response to cathepsin S. (a) MrgprA3 knockdown DRG neurons responded normally to cat S (5 μM), while MrgprC11 knockdown DRG neurons showed no response (n = 4, P < 0.05). In contrast, chloroquine (CQ) (2 mM) induces calcium responses in ~4% of MrgprC11 knockdown DRGs, while knockdown of MrgprA3 markedly reduced the response to CQ (n = 4, P < 0.05). (b) Representative calcium traces from MrgprA3 knockdown DRG neurons in response to cat S and chloroquine. (c) Representative calcium traces from MrgprC11 knockdown DRG neurons in response to cat S and chloroquine. Each trace represents responses from a single neuron. Arrows indicate application of cat S. (d) Relative messenger RNA (mRNA) level of MrgprA3 and MrgprC11 in the respective knockdown DRGs as determined by real-time PCR. The data are presented as mean ± s.e.m. *P < 0.05, **P < 0.01; ***P < 0.001; two-tailed unpaired t-test.
select Mrgrps, and that cat S, in particular, activates mouse and human Mrgrps that have been implicated in itch transmission.

Discussion
Endogenous and exogenous proteases have long been implicated in the pathogenesis of many inflammatory diseases, including skin diseases. Our previous work showed that endogenous cat S and other proteases trigger histamine-independent itch. Transgenic mice in which cat S is overexpressed spontaneously develop an intensely pruritic, eczematous disorder resembling atopic dermatitis. In addition, cat S levels are selectively upregulated in psoriatic keratinocytes, a condition also marked by itch. In vivo correlates of these observations were demonstrated as the capacity of cat S to activate DRG neurons from Mrgr cluster mice was markedly diminished while not significantly diminished in PAR2 mice. RNAi knockdown of Mrgr specifically abolished the response of DRG neurons to cat S. In contrast, knockdown of MrgrA did not diminish the response of DRG neurons to cat S. It is not clear why the decrease in scratching responses in Mrgr cluster mice, while significant, was not as substantial as the decrease in Ca^2+ signals in DRG neurons from these mice. An explanation is that cat S activates PAR2 on keratinocytes, resulting in the release of thymic stromal lymphopoietin, a skin-derived cytokine that has been shown to induce itch via neuronal receptors for this molecule. In addition, while PAR2 does not appear to have a direct role in cat S-evoked itch in WT mice, a non-native role may be unmasked in the absence of Mrgr. Our data also identify the cysteine protease cat S as a functional endogenous ligand for Mrgrs, currently considered as orphan receptors. Whether other endogenous cysteine proteases activate Mrgrs awaits future investigations.

Conventional PARs are GPCRs that are uniquely activated by proteolysis. In this scenario, proteases bind and specifically cleave residues in the N-terminal domain of the receptor to unmask a ‘tethered’ ligand that subsequently interacts with another portion of the receptor to trigger intracellular signalling. Similarly, PARs can be activated by synthetic hexapeptides that mimic the first six amino acids of the unmasked N terminus. The data presented here are the first to demonstrate that GPCRs other than PARs can be activated by proteolysis, as cysteine proteases activate MrgrC11. Site-specific mutagenesis revealed that cat S requires L15 and papain requires D9 and L15 for cleavage, while data from other ‘non-PAR’ GPCRs and further suggest that proteases are capable of modulating GPCRs by multiple mechanisms.

Classically, GPCRs are thought to become activated when ligands bind in a pocket formed by the transmembrane helices thereby triggering exposure of cytoplasmic domains required for coupling to heterotrimeric G-protein subunits. PARs, on the other hand, become activated when proteolysis allows for tethered ligands to interact with residues in the second extracellular loop, subsequently resulting in conformational changes in the transmembrane helices. While the exact mechanism by which
proteolysis triggers activation of MrgrpC11 remains unknown, we suggest that the N-terminal domain interacts with the extracellular loops of the receptor to lock it in an inactive state. Cleavage of the N terminus by cysteine proteases may release this lock, shifting transmembrane and cytoplasmic domains as suggested with traditional PARs. Our observations that calcium signalling was evoked by cathepsin-induced proteolysis of the β2AR and MC1R N-terminal exchange mutants are consistent with this hypothesis. The mechanism underlying this observation deserves further study.

It is interesting that although N-terminal MrgrpC11 peptides did not activate Mrgrp signalling, SLIGRL, a PAR2 N-terminal peptide, is capable of inducing itch via activation of MrgrpC11 (ref. 3), at least at micromolar concentrations. Whether this effect occurs naturally is not clear. The concept that N-terminal peptides released into the extracellular milieu following PAR cleavage may exert a biologic effect independent of PARs has been reported. PAR1 cleavage by thrombin generates an N-terminal peptide known as parstatin, which is capable of inhibiting vascular endothelial cell growth factor- and fibroblast growth factor-induced angiogenesis in vitro and in vivo. It also plays a role in modulating platelet function 27,28. Whether protease-mediated cross-signalling between PAR2 and MrgrpC11 serves a relevant role in coupling itch and inflammation in vivo is not known.

Cysteine proteases, in particular cysteine cathepsins, are widely expressed in mammalian tissues and have been implicated in the pathophysiology of a diverse array of inflammatory, autoimmune

Figure 5 | N-terminal peptides do not activate MrgrpC11 and cat S requires L15 for activity. (a) A series of eight MrgrpC11 N-terminal peptides was generated based on possible protease cleavage sites. (b) Calcium imaging of HeLa cells transfected with MrgrpC11 following treatment with a representative MrgrpC11 N-terminal peptide, C11-1 100 μM, the hexapeptide tethered ligand ETGHPN at the cat S cleavage site. (c) Cat S does not activate the MrgrpC11 in which L15 has been changed to R. Note that SLIGRL activates the native and mutant receptors. Arrows represent addition of test reagent.

Figure 6 | Cathepsin S and papain activate MrgrpC11 downstream signalling in N-terminal exchange receptors. (a) Cat S and papain activate β2AR-MrgrpC11 but not the WT AR as determined by calcium imaging. (b) Cat S and papain activate MC1R-MrgrpC11 but not WT MC1R as determined by calcium imaging. See Supplementary Fig. 4 for additional details.
Table 2 | Responses of PAR2 and Mrgrps following treatment with cat S, papain and trypsin as determined by calcium imaging.

| GPCR          | Cat S, 2 μM | Papain, 2 μM | Trypsin, 10 nM |
|---------------|-------------|--------------|---------------|
| hPAR2         | +           | +            | +             |
| hMRGPRX1      | +           | +            | +             |
| hMRGPRX2      | +           | +            | +             |
| hMRGPRX3      | +           | +            | +             |
| hMRGPRX4      | –           | –            | –             |
| mMRgrpC1      | +           | +            | +             |
| mMRgrpA3      | +           | +            | +             |

<cat S, cathepsin 5; Mrgrps, Mas-related G-protein-coupled receptors; PAR2, protease-activated receptor-2. * indicates that the indicated receptor was activated. Representative calcium imaging data are presented in Supplementary Fig. S5.>

and allergic diseases, as well as in neuropathic pain15,29–32. Recent work in murine colitis models demonstrates that macrophage- and microglial cell-derived cat S induces colonic inflammation and pain by activating PAR2 on different cell types, linking increased paracellular permeability in colonocytes with noceceptive hyper-excitability and neurogenic inflammation in primary spinal afferent neurons15,33–35. We suggest that cat S may similarly act as a key regulator linking itch and inflammation in the skin. Our work reveals a novel mechanism by which cat S accomplishes its diverse effects: activation of MrgrpC11.

Increased cat S expression has been reported in several common inflammatory skin conditions, including atopic dermatitis, psoriasis and seborrhoeic dermatitis14,19,36. Under these conditions, cat S may similarly act as a key regulator linking itch and inflammation in the skin. Our work reveals a novel mechanism by which cat S accomplishes its diverse effects: activation of MrgrpC11.

First, as we demonstrated here, cat S induces itch directly by activating MrgrpC11. Second, cat S-mediated activation of PAR2 may induce itch if PAR2 N-terminal peptides bind to MrgrpC11 on primary sensory nerves. In addition, PAR2 activation in keratinocytes may trigger the release of thymic stromal lymphopoietin, which in turn activates cutaneous sensory neurons that express transient receptor potential ankyrin channel A1 (TRPA1), leading to itch stimulation and neurogenic inflammation25. Recognizing that proteases can recruit multiple distinct pathways to induce itch and inflammation may help drive the development of more effective therapeutic strategies to treat inflammatory diseases, shifting the focus from blocking individual downstream receptors to inhibiting protease–receptor complexes.

Methods

Reagents, peptides and antibodies. Recombinant cat S was generated as previously described37,38 and diluted in 1 × PBS, pH 7.4, 5 mM EDTA, 5 mM dithiorthreitol and 5 mM cystine chloride. Papain (Sigma–Aldrich) was diluted in 0.1 × PBS, 50 mM sodium acetate, pH 6.5, 5 mM EDTA, 5 mM dithiorthreitol and 5 mM cystine chloride. Crystalline papain is more active and has a broader sequence of MrgprC11 were obtained from GenScript and diluted in PBS. The 28 amino-acid N-terminal peptide MDPTISSHDTESTPLNETGHPNCTPILT was made by Peptide 2.0 (Chantilly, VA). The cysteine protease inhibitor E-64 was made by Bioreagents EZ-run prestained rec protein ladder, cat # 3603500 from Thermo Fisher. All other reagents were purchased from Invitrogen, unless otherwise noted.

Mice. WT and PAR2 KO mice were obtained from Jackson Laboratories. Mrgrp cluster A/C2 mice were generated by X.D. as previously described37. The mouse studies were performed at Johns Hopkins and approved by the IACUC at that institution.

Cell culture. HeLa cells were obtained from the ATCC and maintained in MEM supplemented with fetal bovine serum, 1-glutamine, penicillin and streptomycin (Fisher Biochemicals).

Dissociated DRG neurons were prepared from WT, Mrgrp cluster A/C2−/−, PAR2−/− and Pirt-GCaMP3 mice as reported previously37. DRGs were collected from all spinal levels of 4-week-old mice, placed in cold medium and treated with enzyme solution at 37 °C. Following titration and centrifugation, cells were suspended, plated on glass coverslips coated with poly-nlysine and laminin, incubated at 37 °C and used within 48 h.

Cloning of WT MrgrpC11 and generation of mutants. The coding region of MrgrpC11 was cloned by PCR from mouse genomic DNA using the forward and reversed primers 5'-GCGCTGAGACGAGCATCCTCATCAG-3' and 5'-GCGAAAGTTCAATATCTGTTTGAATCTC-3'. C11 N-terminal mutants were made by site-specific mutagenesis procedures using the primer pairs listed or昇 现 in Supplementary Table S3 to change the listed residues to isoleucine or arginine at the P2 site. Leucine at P2 is the preferred site for cleavage by cathepsin S as in native MrgrpC11, with isoleucine less so and arginine not at all.

Preparation of G. luciferase–MrgrpC11 fusion cDNAs. G. luciferase complementary DNA (cDNA) was cloned, without its termination codon, into pcDNA3.1 (+) as an Xhol–Xhol fragment. MrgrpC11 cDNA was fused as an Xhol–Xhol–XhoI unit at the 5' terminus of the luciferase cDNA to obtain the vector pLucMrgrpC11N.

Preparation of MrgrpC11 with [βAR2 and MC1R amino termini. The MrgrpC11-coding region without its N terminus from amino-acid 34 to its C terminus was cloned as an EcoRI–HindIII DNA into pcDNA3.1 (+) to obtain the vector pcMrgprA3/C11. The N termini of [βAR2 and MC1R from amino acids 1–34 and 1–37, respectively, were isolated as Xhol–EcoRI fragments by PCR, and cloned into pLucMrgrpC11N to generate the vectors pARCh11 and pMC1RC11 for expression of the chimeric receptors [βAR2–MrgrpC11 and MC1R–MrgrpC11.

Calcium imaging. HeLa cells were grown to confluence, trypsinized and then transfected with the pcDNA vector carrying the G. luciferase–MrgrpC11 fusion construct, MrgrpC11 cdna or salmon sperm DNA as a control using Lipofectamine 2000. Transfected cells were plated into 96-well glass-bottom plates at 50,000 cells/well and maintained at 37 °C in 5% humidified CO2 for 3 h after which the medium was replaced. Twenty-four to 48 h after transfection, cells were incubated for 60 min at room temperature with 2 μM of Fura-2-acetoxyethyl ester (Fura-2) diluted in complete DMEM. Following Fura-2 loading, the medium was replaced with 90 μL of HEPE5-buffered saline (20 mM HEPE5, 115 mM NaCl, 1.1 mM Na2PO4, 0.8 mM MgCl2, 1.3 mM glucose, pH 7.4) and cells were used immediately for calcium imaging. A luciferase–receptor construct was used in all transfections to establish consistency of transfection efficiency.

Ratiometric calcium imaging was performed using a Zeiss Axiovert 200 M microscope equipped with a spinning filter wheel and Axiovision software (version 4.6). Peptide and protease agonists, cat S or papain (10 μM) were added 15 s after initiating image acquisition and typically represented by down arrows in the figures. Images were acquired at time 0 and every 5 s thereafter for at least 90 s. All images obtained during each acquisition period were analysed. Each experiment was performed at least three times, and ratiometric changes were measured in at least three independent experiments. Representative cells depicted in the figures in the Results or Supplementary Information.

Primary DRGs cultured from WT, Mrgrp cluster A/C2−/− and PAR2−/− mice were loaded with Fura-2 for 30 min at 37 °C for ratiometric calcium imaging as described above following application of cat S and papain (5 and 20 μM, respectively). For experiments incorporating RNA interference, MrgrpA3 and MrgrpC11 on-target short interfering RNAs (siRNAs) were purchased from Thermo Scientific. MrgrpA3 or MrgrpC11 siRNA (0.175 nmol) were electroporated, respectively, into DRG neurons from Pirt-GCaMP3 mice that had been generated previously27. Electroporation of dissociated DRG neurons with Mrgrp-expression constructs was carried out using Mouse Nucleofector Kit (Amaxa Biosystems) following the manufacturer’s instructions. Electroporated neurons were plated and cultured as described above and used within 72 h.

Green fluorescence from genetically encoded calcium GCaMP3 was detected using a single-photon Ca2+ imaging with a 700 Zeiss confocal microscope, using the 488-nm line of a solid-state laser for excitation of Pirt-GCaMP3 and a 488-nm laser main dichroic beam splitter and a 505–555 nm variable secondary dichroic mirror to detect the emission of green fluorescence.
Real-time quantitative PCR. Total RNA was extracted from cultured DRGs 3 days after siRNA electroporation using an Rneasy micro kit (Qiagen). Purified RNA was quantified on a Nanodrop 2000 UV spectrophotometer (Thermal Scientific) at 260 nm and assayed for purity using the 260/280-nm ratio. DNA was reverse transcribed with the Superscript First Strand Synthesis System (Invitrogen). PCR amplifications were carried out with a Step One Plus real time PCR system (Applied Biophysics). PCR conditions were 95 °C for 3 min and 50 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. Primers as listed below: MrgrpC11-F 5′-AGATGCAA CACCCCGAGAAG-3′; MrgrpC11-R 5′-TGGATGCACTGGTATGAC-3′; MrgrpA3-F 5′-ACAACAGCCGCAAGCTACA-3′; MrgrpA3-R 5′-ACTCTCCAG GGAAGGTTTGTG-3′; Actin-F 5′-CGTGGACACGCTCAGCAGCT-3′; Actin-R 5′-CCACTTGCGTCTTACAC-3′. All primers were intron spanning to avoid genomic DNA contamination. Melting curve analysis was applied to all final PCR products after the cycling protocol. Samples were run in triplicate, and threshold cycle (Ct) values from each reaction were averaged. Relative messenger RNA levels of MrgprA3 and MrgprC11 were calculated from threshold cycles and normalized to the actin level.

Western blot analysis for luciferase. HeLa cells transfected with MrgrpC11 cDNA were analysed using ratimetric imaging as described above with cat S and papain at concentrations from 1 nM to 10 μM. Each of the concentration-dependent readings was performed in triplicate. Maximum intensities at all of the concentrations were plotted against doses of papain, using GraphPad Prism software. Error bars represent s.e.m. within an experiment, although these experiments were performed multiple times with similar results.

Luciferase assay. MrgrpC11 plasmid DNA (10 μg) or salmon sperm as control was transfected into 2 × 106 HeLa cells as described above and plated into 10-cm dishes. Forty-eight hours after transfection, the cells were washed with PBS, scraped and pelleted. Cells were suspended in a volume of 200 μl PBS and 100 μl of a luciferase assay mixture was added to each well in triplicate in 96-well plates. After incubation for 10 min at room temperature, control cells were not treated with protease. Luciferase activity was quenched by adding 300 μl of complete DMEM, after which the cells were centrifuged and supernatants (150 μl) were assayed in triplicate for luminescence according to the instructions of the manufacturer (New England Biolabs). These experiments were performed multiple times with protease activity being quenched at many different time points so as to catch luminescence associated with papain, as this protease has the capacity to digest luciferase.

Luciferase expression was quantified using ImageJ. The distinct roles of two GPCRs, MrgrpC11 and PAR2, in itch and hyperalgesia. Sci. Signal. 4, ra45 (2011).

Wilson, S. R. et al. TRPA1 is required for histamine-independent, Mas-related G protein-coupled receptor-mediated itch. Nat. Neurosci. 14, 595–602 (2011).

Sikand, P., Dong, X. & LaMotte, R. H. BAM8-22 peptide produces itch and nociceptive sensations in humans independent of histamine release. J. Neurosci. 31, 7563–7567 (2011).

Leombo, P. M. et al. Proenkephalin A gene products activate a new family of sensory neuron–specific GPCRs. Nat. Genet. 5, 201–209 (2002).

Ramachandran, R. & Hollenberg, M. D. Proteinases and signalling: pathophysiological and therapeutic implications via PARs and more. Br. J. Pharmacol. 153(Suppl 1): S263–S282 (2008).

Liu, Q. et al. Mechanisms of itch evoked by beta-ala-lanine. J. Neurosci. 32, 14532–14537 (2012).

Reddy, V. B., Iuga, A. O., Shimada, S. G., LaMotte, R. H. & Lerner, E. A. Cowhage-evoked itch is mediated by a novel cysteine protease: a ligand of protease-activated receptors. J. Neurosci. 28, 4331–4335 (2008).

Reddy, V. B. & Lerner, E. A. Plant cysteine proteases that evoke itch activate protease-activated receptors. Br. J. Dermatol. 163, 532–535 (2010).

Reddy, V. B., Shihamda, S. G., Sikand, P., Lamorte, R. H. & Lerner, E. A. Cathepsin S elicits itch and signals via protease-activated receptors. J. Invest. Dermatol. 130, 1468–1470 (2010).

Rawlings, N. D., Barrett, A. J. & Bateman, A. MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Res. 40, D343–D350 (2012).

Shimada, S. G. & LaMotte, R. H. Behavioral differentiation between itch and pain in mice. Pain 139, 681–687 (2008).

Kim, N. et al. Overexpression of cathepsin S induces chronic atopic dermatitis in mice. J. Invest. Dermatol. 132, 1169–1176 (2012).

Cattaruzza, F. et al. Cathepsin S is activated during colitis and causes visceral hyperalgesia by a PAR2-dependent mechanism in mice. Gut 64, 1057–1068 (2011).

Elmariah, S. B., Reddy, V. B. & Lerner, E. A. Cathepsin S signals via PAR2 and generates a novel tethered ligand receptor agonist. PLoS ONE 9, e97902 (2014).

Deschamps, K. et al. Genetic and pharmacological evaluation of cathepsin s in a mouse model of asthma. Am. J. Respir. Cell Mol. Biol. 45, 81–87 (2011).

Schonefuss, A. et al. Upregulation of cathepsin S in psoriatic keratinocytes. Exp. Dermatol. 19, 880–888 (2010).

Pignanato, F., Picci, F., Pesciulli, L. & Lotti, T. Itch in psoriasis: epidemiology, clinical aspects and treatment options. Clin. Dermatol. Investig. Dermatol. 2, 9–13 (2009).

Globes, D., Bayliss, M. S. & Harrison, D. J. The impact of itch symptoms in dermatologic correlates. Arch. Dermatol. 124, 1052–1057 (1988).

Vilardo, J. P., Jean-Alphonse, F. G. & Gardella, T. J. Endosomal generation of different GPCRs. Nat. Chem. Biol. 10, 707–708 (2014).

Tsvetanova, N. G., Iraniadej, R. & von Zastrow, M. G protein-coupled receptor (GPCR) signaling via heterotrimeric G proteins from endosomes. J. Biol. Chem. 290, 6689–6696 (2015).

Wilson, S. R. et al. The epithelial cell-derived atopic dermatitis cytokine TSLP activates neurons to induce itch. Cell 155, 285–293 (2013).

Li, T. H., Grossmann, M. B. & Lerner, E. A. G protein-coupled receptors. I. Diversity of receptor-ligand interactions. J. Biol. Chem. 273, 17299–17302 (1998).

Zania, P. et al. Parmesan, the cleaved peptide on proteinase-activated receptor 1 activation, is a potent inhibitor of angiogenesis. J. Pharmacol. Exp. Ther. 328, 378–389 (2009).
28. Furman, M. I. et al. The cleaved peptide of PAR1 results in a redistribution of the platelet surface GPIb-IX-V complex to the surface-connected canalicular system. Thromb. Haemost. 84, 897–903 (2000).
29. Inaoka, T. et al. Molecular cloning of human cDNA for cathepsin K: novel cysteine proteinase predominantly expressed in bone. Biochem. Biophys. Res. Commun. 206, 89–96 (1995).
30. Mohamed, M. M. & Sloane, B. F. Cysteine cathepsins: multifunctional enzymes in cancer. Nat. Rev. Cancer 6, 764–775 (2006).
31. Clark, A. K. et al. Inhibition of spinal microglial cathepsin S for the reversal of neuropathic pain. Proc. Natl Acad. Sci. USA 104, 10655–10660 (2007).
32. Gupta, S., Singh, R. K., Dastidar, S. & Ray, A. Cysteine cathepsin S as an immunomodulatory target: present and future trends. Expert Opin. Ther. Targets 12, 291–299 (2008).
33. Steinhoff, M. et al. Agonists of proteinase-activated receptor 2 induce inflammation by a neurogenic mechanism. Nat. Med. 6, 151–158 (2000).
34. Jacob, C. et al. Mast cell tryptase controls paracellular permeability of the intestine. Role of protease-activated receptor 2 and beta-arrestins. J. Biol. Chem. 280, 31936–31948 (2005).
35. Zhao, P. et al. Cathepsin S causes inflammatory pain via biased agonism of PAR2 and TRPV4. J. Biol. Chem. 289, 27215–27234 (2014).
36. Viode, C. et al. Cathepsin S, a new pruritus biomarker in clinical dandruff/seborrhoeic dermatitis evaluation. Exp. Dermatol. 23, 274–275 (2014).
37. Kim, Y. S. et al. Central terminal sensitization of TRPV1 by descending serotonergic facilitation modulates chronic pain. Neuron 81, 873–887 (2014).

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Author contributions
V.R. and E.A.L. conceived the overall design of the study. V.R. performed in vitro studies that were designed and analysed by E.A.L. E.A. and S.B.E. contributed to data interpretation. S.S. performed behavioural and DRG neuron studies that were designed, analysed and interpreted by X.D. All authors contributed to the writing and review of the manuscript.

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