Activation of mas-related G-protein–coupled receptors by the house dust mite cysteine protease Der p1 provides a new mechanism linking allergy and inflammation

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Cysteine and serine proteases function via protease-activated and mas-related G-protein–coupled receptors (Mrgprs) to contribute to allergy and inflammation. Der p1 is a cysteine protease and major allergen from the house dust mite and is associated with allergic rhinitis and allergic asthma. Der p1 activates protease-activated receptor 2 and induces the release of the pro-inflammatory cytokine IL-6 from cells. However, the possibility that Der p1 acts on Mrgprs has not been considered. We report here that ratiometric calcium imaging reveals that Der p1 activates the human receptor MRGPRX1 and the mouse homolog MrgprC11, implicated previously in itch. Der p1 cleavage of N-terminal receptor peptides followed by site-directed mutagenesis of the cleavage sites links receptor activation to specific amino acid residues. Der p1 also induced the release of IL-6 from heterologous cells expressing MRGPRX1. In summary, activation of Mrgprs by the allergen Der p1 may contribute to inflammation.

Asthma and rhinitis are chronic widespread heterogeneous conditions. Asthma is characterized by difficulty breathing, wheezing, and cough, whereas symptoms of rhinitis include a running and itchy nose and tearing eyes. Environmental allergens are associated and shared between these two conditions. An important source of allergen is the house dust mite *Dermatophagoides pteronyssinus*, and a prominent allergen from the mite is the cysteine protease, Der p1 (1).

Although it has been established that Der p1 activation of protease-activated receptor 2 (PAR2) on lung epithelial cells results in the release of pro-inflammatory cytokines (2), recent reports and the complexity of biology led us to consider the possibility that Der p1 had the capacity to activate Mrgprs. Thus, it has been reported that endogenous and exogenous cysteine proteases activate not only PARs but also members of the Mrgpr family (3) and that hexapeptides derived from serine protease cleavage of PAR2 unexpectedly activate Mrgprs to cause itch (4). In addition, Mrgprs are expressed primarily on mast cells and sensory neurons where they serve as innate sensors, contribute to allergy, and mediate the nociceptive sensations pain and itch (3, 5). The appreciation that epithelia, mast cells, and sensory nerves together contribute to allergy, including asthma (6), provided further impetus for these studies.

Here we determine that Der p1 can activate distinct members of the Mrgpr family and identify the N-terminal amino acids in Mrgprs required for cleavage, that N-terminal peptides can activate certain Mrgprs and PARs, and that Der p1 activation of a Mrgpr induces the release of the pro-inflammatory cytokine IL-6. Taken together, these data support the possibility that the house dust mite allergen Der p1 may contribute to inflammation through activation of Mrgprs.

**Results**

**Der p1 activates specific Mrgprs**

Because Der p1 is an allergen, we first asked whether it could activate any of the human or mouse Mrgprs associated with allergy or itch. To address this question, we used ratiometric calcium imaging to determine whether Der p1 could activate heterologous cells that had been transfected with cDNAs encoding the human Mrgprs X1–X4 or the relevant mouse Mrgprs. The only Mrgprs activated by Der p1 were the human receptor MRGPRX1 and the mouse receptors MrgprC11 and MrgprA3. These receptors have each been linked to itch but not previously to allergy. Der p1 was also found to activate human PAR2 (Fig. 1). Receptor activation was blocked in all cases by E-64, an established irreversible inhibitor of cysteine proteases. This result establishes that protease activity was necessary for receptor activation. The data for each receptor are shown in Table 1.

**Der p1 cleavage of N-terminal receptor sequences**

We next sought to determine the cleavage sites associated with receptor activation by Der p1. Attention was directed to MRGPRX1 because it was the only human receptor that was activated by Der p1. Attention was also directed to the mouse receptor MrgprC11 because it was activated by Der p1 and is also activated by SLIGRL, the tethered ligand and hexapeptide ligand for PAR2. PAR2 served as a positive control. The N-terminal peptides of MRGPRX1, MrgprC11, and PAR2 were syn-
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Figure 1. Der p1 activates MRGPRX1, MrgprC11, and PAR2 but not MRGPRX2 in transfected HeLa cells. Fluorescence traces of Der p1 (2 μM) action on MRGPRX1 (A), MrgprC11 (B), PAR2 (C), and MRGPRX2 (D) were determined by ratiometric calcium imaging with Fura2. E-64 (10 μM), a cysteine protease inhibitor, blocked this activity. The arrows indicate the time of Der p1 addition to the wells. Individual colored traces represent independent cells.

| Table 1 | Activation of PAR2 and Mrgprs by Der p1 and Der f1 |
|---------|--------------------------------------------------|
| GPCR    | Der p1 2 | Der f1 2 |
| PAR2    | +        | +        |
| MRGPRX1 | +        | +        |
| MRGPRX2 | −        | −        |
| MRGPRX3 | −        | −        |
| MRGPRX4 | −        | −        |
| MrgprC11| −        | −        |
| MrgprA1 | −        | −        |
| MrgprA2 | −        | −        |
| MrgprA3 | −        | −        |
| MrgprA4 | −        | −        |
| MrgprA9 | −        | −        |
| MrgprB2 | −        | −        |
| MrgprB4 | −        | −        |
| MrgprB5 | −        | −        |


dthesized and incubated with Der p1, and the sequences of the resultant series of cleavage products were obtained via MS/MS arranged in descending order of ion abundance (Fig. 2). It is appreciated that strict quantitative conclusions cannot be drawn from this approach, but the findings establish sites of cleavage.

To account for the peptides identified by MS/MS in order of abundance, the major sites for Der p1 cleavage of the N terminus of MRGPRX1 were after Glu11, Leu22, and Asp2, respectively, as indicated below by the double arrows. These sites were followed by cleavage before Asp2, Leu8, and Leu12, as indicated by the regular arrows: 1M \( \downarrow \) D\( \uparrow \) PTIST \( \uparrow \ head LTTPINGTEETL \( \uparrow \) CYKQTLSL30. The preferred site for Der p1 cleavage of the N-terminal peptide of MRGPRX1 was deduced to be between Glu11 and Leu12. Both double and regular arrows are noted at this site to account for the two most abundant peptides and the less abundant peptides 6–8. In contrast, the peptides TPINGTET, TPINGTE, and TPINGTEE resulted from cleavage after Leu12, but because these peptides were not abundant, no arrow is included after Leu12.

The major sites for cleavage of the N terminus of MrgprC11 occurred after Asp2, Glu11, and Glu17 as indicated below by the bold arrows. These were followed by cleavage before Asp2 and before and after Ser12, as indicated by the regular arrows: 1M \( \downarrow \) D\( \uparrow \) PTISSHDTE \( \uparrow \) S \( \uparrow \) TPLNE \( \uparrow \) TGHPNCTPILTLS30.

Der p1 cleavage of the N terminus of PAR2 resulted in several peptides associated with pairs of internal cleavage sites. These are identified as Lys14/Gly23, Lys8/Gly23, and Ser6/Gly23 indicated below by the bold arrows. These were followed by Arg22/Gly23, Lys14, and Lys7/Gly23 as indicated by the regular arrows:

1GTNRSS \( \uparrow \) KG \( \uparrow \) R \( \uparrow \) SLIGK \( \uparrow \) VDGTSHVTG \( \uparrow \) KGVT27.

**Activity of Der p1-derived synthetic tethered peptides on Mrgprs and PAR2**

We next asked whether hexapeptides (and a nonapeptide in one case) derived from Der p1 cleavage of the N termini of MRGPRX1, MrgprC11, or PAR2 could activate receptors in either a cis or trans fashion (summarized in Table 2). The reason to consider the possibility of cis or trans activation is that SLIGRL, the hexapeptide derived from mouse PAR2, activates both human and mouse PAR2 (cis) and human MRGPRX2 and mouse MrgprC11 (trans) (3) but not other Mrgprs.

Der p1 cleavage of the PAR2 N terminus did not generate the conventional hexapeptide SLIGKV (the human ortholog of the mouse hexapeptide SLIGRL). However, from the data on relative ion abundance in Fig. 2, the hexapeptides VDGTSH, RSLIGK, and KGRSLI were synthesized. We asked whether these peptides could activate Mrgprs or PAR2. VDGTSH did not activate PAR2, MRGPRX1, MRGPRX2, or MrgprC11 (Fig. 3a). To address the possibility that the full-length and relatively most abundant cleavage product VDGTSHVTG might activate a receptor, this nonapeptide was synthesized. No activity was found (not shown). In contrast, RSLIGK activated both PAR2 (at 10 μM) and MRGPRX2 (at 50 μM) akin to previous findings with SLIGRL. However, RSLIGK failed to activate MrgprC11, which is in contrast to the overlapping hexapeptide SLIGRL, and RSLIGK did not activate MRGPRX1, even at 100 μM (Fig. 3a). KGRSLI had no activity on the receptors. SLIKGV and RSLIK activated PAR2 and MRGPRX2 in a dose-dependent manner (Fig. 3b).

Hexapeptides associated with N-terminal cleavage products of MRGPRX1 and MrgprC11 by Der p1 were also synthesized and evaluated for their capacity to activate Mrgprs and PAR2. These peptides were PTISTL, LTPING, and CYKQT from MRGPRX1 and PTISSH, TPLNE, and TGHPN from MrgprC11. Only CYKQT had activity, and this activity was in a trans fashion on MRGPRX2. CYKQT activated its parent MRGPRX1 and either MrgprC11 or PAR2 (Fig. 4). The results are summarized in Table 2.

**Der p1 activity on N-terminal mutants**

In an effort to complement the cleavage studies and to determine the importance of the relevant residues with respect to cleavage by Der p1, site-directed mutagenesis was used to convert selected residues to histidine. Histidine was selected...
because it is one of the least preferred amino acids associated with Der p1 activity. The amino acids Asp², Glu¹¹, and Leu²² in the N terminus of MRGPRX1 associated with cleavage by Der p1 are at the P1 position and were converted individually to His. The N-terminal residues Asp², Glu¹¹, and Glu¹⁷ of MrgprC11 at the P1 position of Der p1 cleavage were also altered to His. Similarly, the PAR2 N-terminal amino acids Gly⁸ and Gly²³ in the P1 position and Gly¹³ in the P2 position were changed to His. Lys¹⁴ was not considered for mutagenesis because Der p1 does not prefer Lys in the P1 position. When tested for receptor activation, Der p1 failed to activate the mutant receptors MRGPRX1E11H, MrgprC11E11H, and MrgprC11E17H, PAR2G8H, and PAR2G13H. However, Der p1 activated the remaining mutants MRGPRX1D2H, MrgprC11D2H, and PAR2G23H (Fig. 4).

The results reveal that Asp in the second position of the N termini of MRGPRX1 and MrgprC11 are not crucial for Der p1 cleavage because receptor activity was maintained following site-directed mutagenesis. In contrast, residues Glu¹¹ and Leu²² in MRGPRX1 and Glu¹¹ and Glu¹⁷ in mMrgprC11 were important for receptor function as the mutant receptors lost activity. Similarly, site-directed mutagenesis of glycines 8 and 13 resulted in loss of activity to Der p1. Finally, Gly²³ in the P1 position was common for Der p1 cleavage; this residue was not found to be important for activation of PAR2 by Der p1.

Der p1 induction of the pro-inflammatory cytokine IL-6 is associated with activation of Mrgprs and PAR2

We employed ELISA to establish that Der p1 induced the release of IL-6 from heterologous cells expressing MRGPRX1, MrgprC11, or PAR2 (Fig. 5A). The Mrgpr/PAR2-activating peptides RSLIGK and SLIGKV induced IL-6 from cells expressing these receptors and also cells expressing MRGPRX2 (Fig. 5, B and C). Control cells transfected with vector alone did not release IL-6 into the medium.

We next turned to A549 cells, a line derived from human lung epithelia, considered a model with biological relevance to asthma. Der p1 is known to induce the production of IL-6 from A549 cells. RT-PCR revealed abundant transcripts corresponding to PAR2, and transcripts corresponding to MRGPRX1 and MRGPRX2 were present (Fig. 6A). To determine that Der p1 activation of MRGPRX1 and PAR2 could each contribute to the induction of IL-6, we used siRNA to knock down expression of these receptors individually and together and then stimulated the cells with Der p1. Knockdown of MRGPRX1 and PAR2 modulated IL-6 induction by Der p1 (Fig. 6B). The decrease in Der p1 induction of IL-6 following knockdown of MRGPRX1 is ~5%. In comparison, the decrease in Der p1 induction of IL-6 following knockdown of PAR2 is more substantial. These data are consistent with the lower level of transcription of MRGPRX1 as compared with PAR2.

Discussion

There are more than 10 species of house dust mites releasing protease allergens consisting of cysteine and serine proteases.
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(a)

![Graphs showing fluorescence over time for different proteases and hexa-peptide concentrations.]

(b)

![Graphs showing fluorescence vs. log of hexa-peptide concentration for PAR2 and MRGPRX2.]

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Figure 3. Receptor activation by hexapeptides derived from Der p1 cleavage of PAR2. a, fluorescence traces of MRGPRX1, MrgprC11, PAR2, and MRGPRX2 produced by RSLIGK, SLIGKV, and VDGTSH. RSLIGK activated PAR2 and MRGPRX2 but not other Mrgprs (left column). SLIGKV activates PAR2 and MrgprC11 as expected, in addition to MRGPRX2 (middle column). VDGTSH was inactive (right column). The concentration of the peptides is indicated in the figures. The arrows indicate the time of Der p1 addition to the wells. Individual colored traces represent independent cells.

Figure 4. The effect of Der p1 (2 μM) on histidine-substituted receptor mutants reveals residues necessary for activity. Transfected cells were loaded with Fura2, and ratiometric calcium imaging was performed. MRGPRX1, MrgprC11, and PAR2 N-terminal histidine mutants treated with Der p1 at 2 μM final concentration. The arrows indicate the time of Der p1 addition to the wells. Individual colored traces represent independent cells.

Figure 5. Induction of IL-6 by Der p1 (A), RSLIGK (B), and SLIGRL (C) in HeLa cells expressing Mrgprs and PAR2. Der p1 (100 nM), RSLIGK (100 μM), and SLIGRL (100 μM). The data indicate values ± S.E. *, p ≤ 0.01; **, p ≤ 0.001. p values greater than 0.05 are considered non-significant (ns).

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Figure 6. Induction of IL-6 in A549 pulmonary epithelial cells by Der p1 is dependent in part on MRGPRX1. a, MRGPRX1, MRGPRX2, and PAR2 are transcribed in A549 cells. * indicates the band that corresponds to MRGPRX1. b, knockdown of MRGPRX1 and PAR2 modulates IL-6 induction by Der p1 in A549 cells. *, p < 0.05; ***, p < 0.005. p values greater than 0.05 are considered non-significant (ns).

There are more than 20 Der p1-like cysteine proteases that are thought to contribute to chronic allergic reactions by releasing inflammatory cytokines and mediators, including IL-4, IL-5, IL-6, IL-13, IL-25, TSLP, TNFα, histamine, and prostaglandins (2, 7). Asokananthan et al. (7) showed that activation of PAR2 by Der p1 induced the production of IL-6 from transfected cells and A549 lung epithelial cells. We confirm and broaden their findings with the demonstration that Der p1 not only activates PAR2 but also activates the human receptor MRGPRX1 and the mouse homolog MrgprC11. The data presented here also reveal that IL-6 is induced when cells transfected with these Mrgprs were exposed to Der p1. These findings broaden the potential range by which Der p1 may lead to inflammation that links interactions between Der p1 and Mrgprs, not only PAR2, on endothelial cells, mast cells, and sensory neurons. Human MRGPRX1 and MRGPRX2 are expressed primarily on sensory nerves and mast cells, respectively, and appear to be expressed on A549 cells, indicating that functional

Table 3
Der p1 cleavage preferences of amino acids Gly, Leu, Asp, Glu, and His
The preferences are taken from the MEROPS database (www.merops.sanger.ac.uk) (11). (Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party hosted site.)

| Amino acid | P4 | P3 | P2 | P1 | P1’ | P2’ | P3’ | P4’ |
|------------|----|----|----|----|----|----|----|----|
| Gly        | ***| ***| *****| ****| ****| *****| ****| ***|
| Leu        | ***| ***| ****| ***| ***| ***| ***| ***|
| Asp        | **| **| **| **| **| **| **| **|
| Glu        | * | * | **| ***| ***| **| ***| **|
| His        | * | * | ***| ***| ***| ***| ***| * |

The cleavage preferences for Gly, Asp, Glu, Leu, and His as obtained from the MEROPS website. Gly at P1–P4 and P1’–P3’; Leu at P2–P4, P1’, P3’, and P4’; Asp at P1, P3, and P4; and Glu at P1, P2, and P2’–P4’ are preferred. His is rarely associated with cleavage by Der p1 and was thus selected for site-directed substitution reported here. Analysis of the MS/MS data reveals that Der p1 cleaved the synthetic MRGPRX1 N terminus preferentially after Asp, Glu, and Leu; MrgprC11 after Asp and Glu; and PAR2 after Gly. The residues Asp⁷, Glu¹¹, and Leu²² in MRGPRX1 and the amino acids Asp⁷, Glu¹¹, and Glu¹⁷ in MrgprC11 are all in P1 positions. For PAR2, the residues Gly⁸, Gly²³, and Gly¹³ are in P1 and P2 positions, respectively. The preferred cleavages were at Glu¹¹ in MRGPRX1, Glu¹⁷ in MrgprC11, and Gly¹³ and Gly²³ in PAR2. The cleavage sites following Der p1 treatment of N-terminal peptides correlated with receptor activation following site-directed mutagenesis. Asp-to-His mutants of both MRGPRX1 and MrgprC11 were activated by Der p1, whereas Glu-to-His and Leu-to-His mutants were not. Similarly, the Gly⁸-to-His mutant of PAR2 was activated by Der p1, whereas Gly¹³-to-His and Gly²³-to-His mutants were inactive. Thus, Glu¹¹ and Leu²² in MRGPRX1 and Glu¹¹ and Glu¹⁷ in MrgprC11 are necessary for Der p1 activation. Gly⁸ in P1 and P2’, Gly¹³ in P2, and Gly²³ in P1 positions of PAR2 are needed for activation by Der p1.

With respect to PAR2, the most abundant N-terminal peptide associated with Der p1 cleavage was VDGTSHTVTG. Neither VDGTSHTVTG nor its related hexapeptide VDGTSHT could activate Mrgprs or PAR2. In contrast, RSLIGKV, the hexapeptide associated with the next most abundant cleavage product had the same degree of activity on PAR2 as SLIGKV, the mouse homolog of SLIGRL. KGRSLI, the hexapeptide associated with the third most abundant product, was also inactive. SLIGKV, the hexapeptide associated with the fourth most abundant product, was active and is a known serine protease–tethered ligand (8). RSLIGKV also activated MRGPRX2, although at a higher concentration than SLIGKV.

The data reported here reveal that Der p1 can activate MRGPRX1 and that a peptide derived from MRGPRX1 can activate MRGPRX2. The results confirm activation of PAR2 by Der p1 and that receptor activation results in the release of IL-6. These findings may have clinical relevance with respect to allergic asthma and allergic rhinitis via a mechanism of neurogenic inflammation that links interactions between Der p1 and Mrgprs, not only PAR2, on endothelial cells, mast cells, and sensory neurons. Human MRGPRX1 and MRGPRX2 are expressed primarily on sensory nerves and mast cells, respectively, and appear to be expressed on A549 cells, indicating that functional
expression on bronchial epithelium could occur. PAR2- and MRGPRX2-expressing mast cells are present in the bronchial epithelium and exposed to Der p1 (1, 5). In conclusion, MRGPRX1 and MRGPRX2 may contribute to house dust mite allergy. Antagonists that prevent the activation of these receptors may have therapeutic value in asthma and allergy.

Experimental procedures

Cell lines, chemicals, and reagents

HeLa cells were obtained from the ATCC and maintained in DMEM, 10% FBS, L-glutamine, penicillin, and streptomycin (Thermo Fisher). Der f1 from Dermatophagoides farinae and Der p1 from D. pteronyssinus were obtained from mite culture and isolated by affinity chromatography, and purity was determined to be >95% based on silver-stained SDS-PAGE. (Indoor Biotechnologies). A formal measure of protease activity was not available. N-terminal peptides from the human receptors MRGPRX1, 30 residues, and PAR2, 27 residues, and the mouse receptor MrgrpC11, 30 residues, were synthesized by Peptide 2.0 Inc. SLIGRL, RSLIGK, and other peptides based on Der p1 cleavage were synthesized by Genscript. The irreversible cysteine protease inhibitor E-64 and IL-6 ELISA kits were from Sigma.

cDNAs

cDNAs encoding Mrgprs were isolated by PCR using forward and reverse primers and human or mouse genomic DNAs as templates. Human receptors are in capital letters, whereas mouse Mrgprs begin with “M” followed by lowercase letters. Primers for MRGPRX1, MRGPRX2, and MrgrpC11 are shown. Equivalent primers were used to isolate cDNAs encoding additional Mrgrps in Table 1: MRGPRX1 forward, GCCTCGGCTGAGCATGGATCAAACC; MRGPRX1 reverse, GCCTCGGCTGAGCATGGATCAAACC; MRGPRX2 forward, GCCTCGAGCATGATGGTCAACC; MRGPRX2 reverse, GCAGAGCTTCTTCTACACCAAGACTGCTTCTCG; MrgrpC11 forward, GCCTCGAGACATGAGCTTCTATATTCAACCATCTC; and MrgrpC11 reverse, GAGAGCAGTTTTGCTTCTGAAATC. PAR2 cDNA was obtained from Life Technologies. Each of the cDNAs was cloned into the expression vector pcDNA3.1(−).

Transfection and ratiometric calcium imaging

HeLa cells were transiently transfected with pcDNA3.1 (Invitrogen) vectors carrying cDNAs for the specific receptors using Lipofectamine 2000 (Thermo Fisher) and analyzed via ratiometric calcium imaging as described (9). Imaging was performed immediately after loading cells with Fura-2, using a Zeiss Axiovert 200M microscope platform equipped with a flipping filter wheel. Axivision software (version 4.6) was used for image analysis of cells excited at 340 and 380 nm. Proteases were added at 15 s after the start of the excitation procedure. Images were taken every 5 s, including at zero time. Ratiometric changes were measured in 10–20 cells in each individual experiment. Maximum fluorescence of the cells in each image was calculated and plotted against time in seconds. Traces of three representative cells from each image are shown in the figures.

Proteases were used at a concentration of 2 μM. Concentration–effect curves were not generated, because proteases were not active at concentrations below 100 nM. Because the specific activity of the preparation of Der p1 has not been established, it is possible that a different method or preparation may be more active.

Concentration–effect measurements for SLIGKV and RSLIGK

HeLa cells transfected with human PAR2 and MRGPRX2 cDNAs were subjected to ratiometric imaging as described above with SLIGKV and RSLIGK at concentrations from 1 μM to 1 mM. Each of the concentration-dependent readings was performed in triplicate. Maximum intensities at each of the dilutions were calculated and plotted against concentration using GraphPad Prism software. The error bars represent S.E.

Der p1 cleavage of N-terminal peptides

The N-terminal peptides of the receptors were as follows: MRGPRX1, MDPTISTLDTELTPINGTEETLCYKQT; MrgrpC11, MDPTISSHDTESPLNETGPNCT; PAR2, GTNRSKGRSLIGKDTSHTGKVT. Conditions for cleavage were as reported previously (9). Briefly, 100 μmol of each peptide was dissolved in 50 mM Tris, pH 7.0, 0.1 M NaCl, 0.1 mM EDTA, and 0.01% Tween 20. 100 μl of each mixture were incubated with Der p1 at 1 μM final concentration at 25 °C for 20 min. Acetonitrile and formic acid were added to final concentrations of 2 and 0.1%, respectively, and samples were immediately frozen at −80 °C. Cleavage peptides were subjected to MS/MS analysis at the Harvard Microchemistry & Proteomics Analysis Facility (Cambridge, MA) (10).

N-terminal mutagenesis

The preferred sites for proteolysis by Der p1, as identified by MS/MS, were aspartic acid, glutamic acid, leucine, and glycine. These residues were changed to histidine by site-specific mutagenesis using the primer sets below. His was selected, because it was one of the few amino acids not preferred by Der p1. The codons for H, CAC, or CAT, is highlighted in bold italics in the forward primer sequences. Following PCR using Phusion DNA polymerase (New England BioLabs), the reaction mixture was treated with DpnI and transformed into Mach1 Escherichia coli cells. DNAs from three colonies were made for each mutant, verified by sequencing, and transfected into HeLa cells.

The amino acids Asp5, Glu11, and Leu22 were individually changed to H in the N-terminal region of human MRGPRX1: MRGPRX1D2H forward, CTCGAGCTGAGCATGATGGATCAAACC; MRGPRX1 reverse, GCCTCGAGCATGATGGATCAAACC; MRGPRX2 forward, GCCTCGAGCATGATGGTCAACC; MRGPRX2 reverse, GCAGAGCTTCTTCTACACCAAGACTGCTTCTCG; MrgrpC11 forward, GCCTCGAGACATGAGCTTCTATATTCAACCATCTC; and MrgrpC11 reverse, GAGAGCAGTTTTGCTTCTGAAATC.

The amino acids Asp5, Glu11, and Glu17 were individually changed to H in the N-terminal region of mouse MrgrpC11: MrgrpC11D2H forward, AGACTCGAGACATGATGGATCAAACC; MrgrpC11 reverse, GCCTCGAGACATGAGCTTCTATATTCAACCATCTC; and MrgrpC11 reverse, GAGAGCAGTTTTGCTTCTGAAATC.

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The amino acids Asp5, Glu11, and Glu17 were individually changed to H in the N-terminal region of mouse MrgrpC11: MrgrpC11D2H forward, AGACTCGAGACATGATGGATCAAACC; MrgrpC11 reverse, GCCTCGAGACATGAGCTTCTATATTCAACCATCTC; and MrgrpC11 reverse, GAGAGCAGTTTTGCTTCTGAAATC.

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ATGGTTGATGATCAGCTTCTCAGTCT; MrgprC11E11H forward, TCACTCCACAGACACACACTTCACACTCCACTGAAAT; MrgprC11E1H reverse, ATCCAGTGGTGTAGTGTTGTGTGCGGATGTA; MrgprC11E17 forward, TCTACCAGTACATCGTGTTCACCCAAC; and MrgprC11E17 reverse, GTGGGGATGACCGTGTCTTCC; PAR2G13H reverse, ACCAATAAGGCTTCTGTGTTTCT; PAR2G8H reverse, AATAGATCCTCTAAAACAC

The amino acids Gly8, Gly13, and Gly23 were individually changed to His in the N terminus of human PAR2: PAR2G8H forward, AACTGTAACTGC

RT PCR of A549 cell line RNA

Total RNA was extracted from A549 cells using TRIzol (Invitrogen), and the resulting RNA was treated with RNase-free DNase. 20 μg of total RNA was converted into ssDNA by SuperScript III (Invitrogen). PCR was carried out using the following forward and reverse primers: MRGPRX1 forward, GTGTCTGATCTGGTGTGGGATGT; MRGPRX1 reverse, TCTACACCACTGATCCCAAC; and MRGPRX2 reverse, GAAGAGATGATGAGGCTTGACAC; MRGPRX2 forward, TCTACACCACTGATCCCAAC; and PAR2G23H reverse, AACCTGAAT

siRNA transfection of A549 cells

A549 cells were cultured in 24-well plates at 300,000 cells/well/1 ml. Three sets of siRNA duplexes for hPAR2, MRGPRX1, and control were dissolved in RNase-free water to a concentration of 20 μM. 1.5 μl for each of the duplexes for each gene and control were placed into separate tubes (final concentration, 4.5 μl) containing 51 μl of Opti-MEM. 4.5 μl of Lipofectamine 2000 was placed in a separate tube with 51 μl of Opti-MEM and at 25 °C for 5 min. The duplexes and Lipofectamine 2000 were combined and incubated at 25 °C for 20 min, and the transfection complexes added to the cells. This procedure was done in three wells for each of the PAR2 siRNA, MRGPRX1 siRNA, PAR2 siRNA + MRGPEX1 siRNA, and control siRNA, and the plate was incubated at 37 °C in a CO2 incubator for 24 h. The medium was aspirated, and the cells were washed once in PBS. 300 μl of serum-free DMEM were placed in each of the wells, and Der P1 was added to each of the transfected wells, as well as three non-transfected wells to final concentration of 100 nM. IL-6 was measured as described below. The data presented are representative of three distinct experiments.

IL-6 measurement

IL-6 was assayed by ELISA per instructions provided by the vendor. In brief, 300,000 HeLa cells/well were plated into a 24-well plates and transfected with 5 μg of MRGPRX1, MRGPRX2, MrgprC11, or PAR2 or, as a control, vector pcDNA3.1(−) alone, in separate wells. 24 h after transfection, the medium was replaced with 300 μl of DMEM without serum. Der p1 (100 nM), SLIGRL (100 μM), and RSLIGK (100 μM) were added, and the plate was placed in an incubator for 24 h. The medium from each well was transferred into 1.5-ml microfuge tubes, spun for 2 min to pellet dead cells, and assayed (50 μl) in triplicate in 96-well plates. IL-6 was determined by interpolation from the standard curve using Prism 6 software (GraphPad) and plotted as pg/300,000 cells. This experiment was performed three times, with similar results obtained each time.

Author contributions—V. B. R. and E. A. L. were responsible for the conception and design of the studies and together interpreted the data and wrote the manuscript. V. B. R. performed the experiments.

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