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# Naturally Occurring Missense MRGPRX2 Variants Display Loss of Function Phenotype for Mast Cell Degranulation in Response to Substance P, Hemokinin-1, Human B-Defensin-3, and Icatibant

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## Abstract

Human mast cells (MCs) express a novel G protein–coupled receptor (GPCR) known as Mas-related GPCR X2 (MRGPRX2). Activation of this receptor by a diverse group of cationic ligands such as neuropeptides, host defense peptides, and Food and Drug Administration–approved drugs contributes to chronic inflammatory diseases and pseudoallergic drug reactions. For most GPCRs, the extracellular (ECL) domains and their associated transmembrane (TM) domains display the greatest structural diversity and are responsible for binding different ligands. The goal of the current study was to determine if naturally occurring missense variants within MRGPRX2's ECL/TM domains contribute to gain or loss of function phenotype for MC degranulation in response to neuropeptides (substance P and hemokinin-1), a host defense peptide (human b-defensin-3) and a Food and Drug Administration–approved cationic drug (bradykinin B2 receptor antagonist, icatibant). We have identified eight missense variants within MRGPRX2's ECL/TM domains from publicly available exome-sequencing databases. We investigated the ability of MRGPRX2 ligands to induce degranulation in rat basophilic leukemia–2H3 cells individually expressing these naturally occurring MRGPRX2 missense variants. Using stable and transient transfections, we found that all variants express in rat basophilic leukemia cells. However, four natural MRGPRX2 variants, G165E (rs141744602), D184H (rs372988289), W243R (rs150365137), and H259Y (rs140862085) failed to respond to any of the ligands tested. Thus, diverse MRGPRX2 ligands use common sites on the receptor to induce MC degranulation. These findings have important clinical implications for MRGPRX2 and MC-mediated pseudoallergy and chronic inflammatory diseases.




## Keywords

Animals; beta-Defensins; Bradykinin; Cell Degranulation; Cell Line, Tumor; Humans; Ligands; Loss of Function Mutation; Mast Cells; Mutation, Missense; Nerve Tissue Proteins; Neuropeptides; Phenotype; Rats; Receptors, G-Protein-Coupled; Receptors, Neuropeptide; Substance P; Tachykinins

## Disciplines

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**Ibrahim Alkanfari, Kshitij Gupta, Tahsin Jahan, and Hydar Ali**

**Human mast cells (MCs) express a novel G protein–coupled receptor (GPCR) known as Mas-related GPCR X2 (MRGPRX2). Activation of this receptor by a diverse group of cationic ligands such as neuropeptides, host defense peptides, and Food and Drug Administration–approved drugs contributes to chronic inflammatory diseases and pseudoallergic drug reactions. For most GPCRs, the extracellular (ECL) domains and their associated transmembrane (TM) domains display the greatest structural diversity and are responsible for binding different ligands. The goal of the current study was to determine if naturally occurring missense variants within MRGPRX2's ECL/TM domains contribute to gain or loss of function phenotype for MC degranulation in response to neuropeptides (substance P and hemokinin-1), a host defense peptide (human β-defensin-3) and a Food and Drug Administration–approved cationic drug (bradykinin B2 receptor antagonist, icatibant). We have identified eight missense variants within MRGPRX2's ECL/TM domains from publicly available exome-sequencing databases. We investigated the ability of MRGPRX2 ligands to induce degranulation in rat basophilic leukemia–2H3 cells individually expressing these naturally occurring MRGPRX2 missense variants. Using stable and transient transfections, we found that all variants express in rat basophilic leukemia cells. However, four natural MRGPRX2 variants, G165E (rs141744602), D184H (rs372988289), W243R (rs150365137), and H259Y (rs140862085) failed to respond to any of the ligands tested. Thus, diverse MRGPRX2 ligands use common sites on the receptor to induce MC degranulation. These findings have important clinical implications for MRGPRX2 and MC-mediated pseudoallergy and chronic inflammatory diseases.** ***The Journal of Immunology*****, 2018, 201: 343–349.**

In addition to FcεRI, mast cells (MCs) express a novel seven transmembrane (TM) domain receptor known as Mas-related G protein–coupled receptor (GPCR) X2 (MRGPRX2) (1, 2). Emerging evidence suggests that MRGPRX2 contributes to the pathogenesis of a number of chronic inflammatory diseases and is responsible for injection site reactions to opioids and other Food and Drug Administration–approved cationic drugs (3–9). For example, expression of MRGPRX2 is upregulated in skin MCs of patients with chronic urticaria when compared with MCs of normal subjects (7). Furthermore, MRGPRX2-mediated MC activation by the neuropeptide (NP) substance P (SP) contributes to the pathogenesis of chronic urticaria. Hemokinin-1 (HK-1) is a novel NP, which is released from Ag/IgE–activated MCs and human bronchial cells and causes contraction of human bronchi ex vivo (10, 11). We recently reported that MRGPRX2 is expressed at low levels in nonasthmatic lung MCs, but its expression is significantly upregulated in asthmatic lung MCs and demonstrated that HK-1 causes MC degranulation via this receptor (12).

Human β-defensin-3 (hBD3) in epithelium and MRGPRX2-expressing MCs are present in healthy gingiva, and their levels are elevated in patients with chronic periodontitis (6, 13). Furthermore, hBD3 causes MC degranulation via MRPGRX2 (14). Icatibant, a bradykinin $B_2$ receptor antagonist used for the treatment of hereditary angioedema, promotes MC degranulation via MRPGRX2 and causes injection site erythema and swelling in nearly every patient (15, 16). These findings suggest that activation of MRGPRX2 by a diverse group of ligands such as SP, HK-1, hBD3, and icatibant contribute to the pathogenesis of chronic urticaria, asthma, periodontitis, and pseudoallergic reactions. Modeling and mutagenesis studies with SP and various opioid ligands led to the suggestion that different agonists interact with different amino acid residues in MRGPRX2's predicted ligand-binding pocket to induce MC degranulation (17, 18). However, the sites of interaction of HK-1, hBD3, and icatibant on MRGPRX2 for MC degranulation have not been determined.

Recent crystallography data obtained for a number of GPCRs and comparison of sequence homology have provided prediction regarding the regions of GPCRs that are involved in ligand binding and G protein coupling. The seven TM bundles are connected by three extracellular (ECL) loops (ECL1, ECL2, and ECL3) and three intracellular (ICL) loops (ICL1, ICL2, and ICL3). The ECL part also includes the N-terminus, and the ICL part includes the helix VIII and a C-terminal sequence. GPCRs can be divided into modules. The ECL and their closest TM regions (see Fig. 1A)

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Abbreviations used in this article: CADD, combined annotation–dependent depletion; ECL, extracellular; FAM-SP, fluorescence-labeled SP; GPCR, G protein–coupled receptor; hBD3, human β-defensin-3; HDP, host defense peptide; HK-1, hemokinin-1; ICL, intracellular; MAF, minor allele frequency; MC, mast cell; MRGPRX2, Mas-related GPCR X2; NHLBI-GO-ESP, National Heart, Lung, and Blood Institute Grant Opportunity Exome Sequencing Project; NP, neuropeptide; PolyPhen-2, Polymorphism Phenotyping version 2; RBL, rat basophilic leukemia; RBL-MRGPRX2, RBL cell stably overexpressing MRGPRX2; SP, substance P; TM, transmembrane; WT, wild-type.

have the greatest structural diversity and are responsible for the binding of diverse ligands. By contrast, the ICL and its closest TM regions are responsible for G protein coupling and downstream signaling (19).

We have identified eight naturally occurring missense variants within MRGPRX2's ECL and TM domains from three publicly available databases: the National Heart, Lung, and Blood Institute Grant Opportunity Exome Sequencing Project (NHLBI-GO-ESP), 1000 Genomes Project, and Exome Aggregation Consortium (ExAC). The goal of the current study was to determine if any of these variants display gain or loss of function phenotype for MC degranulation in response to NPs (SP and HK-1), a host defense peptide (HDP; hBD3), and a cationic drug (bradykinin B2 receptor antagonist, icatibant). The data presented in this article provide novel insights on the impact of single naturally occurring mutation on MRGPRX2 activation by a diverse group of cationic ligands and have important implications for MC-mediated health and disease.

## Materials and Methods

### *Materials*

All cell culture reagents and Indo-1 were purchased from Invitrogen (Gaithersburg, MD). SP, fluorescence-labeled SP (FAM-SP), hBD3, and icatibant were purchased from AnaSpec (Fremont, CA). Human HK-1 was purchased from α Diagnostic (San Antonio, TX). MRGPRX2 plasmid–encoding hemagglutinin-tagged human MRGPRX2 in pReceiver-MO6 vector was obtained from GeneCopoeia (Rockville, MD). Amaxa transfection kit (Kit V) was purchased from Lonza (Gaithersburg, MD). PE anti-human MRGPRX2 was obtained from BioLegend (San Diego, CA). QuikChange II Site-Directed Mutagenesis Kit was purchased from Agilent Genomics (Santa Clara, CA).

### *Cell culture*

Rat basophilic leukemia (RBL)–2H3 cells were maintained as monolayer cultures in DMEM supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (100 μg/ml) (20).

### *Site-directed mutagenesis*

QuikChange II Site-Directed Mutagenesis Kit (Agilent) was used to generate MRGPRX2 variants. DNA sequencing was performed to confirm the nucleotide sequences for each construct. The following forward and reverse primers were used for each variant: N16H: Forward: 5′-GGGCTTGGTCATTTCCATGCACTGTTGTACTTTCTGT-3′ Reverse: 5′-ACAGAAAGTACAACAGTGCATGGAAATGACCAAGCCC-3′; L31V: Forward: 5′-GAAGACCGGGATCACGGTCTCCTTGCCAC-3′ Reverse: 5′-GTGGCAAGGAGACCGTGATCCCGGTCTTC-3′; V43I: Forward: 5′-TTTCCTACCAGCCCGATCAGGGCAATGAAAAGG-3′ Reverse: 5′-CCTTTTCATTGCCCTGATCGGGCTGGTAGGAAA-3′; F78L: Forward: 5′-GGAAGCAGAGTAAGAGGAAGTCGGCCCCG-3′ Reverse: 5′-CGGGGCCGACTTCCTCTTACTCTGCTTCC-3′; G165E: Forward: 5′-GCTGAGCATCTTGGAAGAGAAGTTCTGTGGCTTCT-3′ Reverse: 5′-AGAAGCCACAGAACTTCTCTTCCAAGATGCTCAGC-3′; D184H: Forward: 5′-CTCTGGTTGGTGTCAGACATTTCATTTCATCACTGC-3′ Reverse: 5′-GCAGTGATGAAATGAAATGTCTGACACCAACCAGAG-3′; W243R: Forward: 5′-CTGCCCTTTGGCATTCAGCGGTTCCTAATATTATGGAT-3′ Reverse: 5′-ATCCATAATATTAGGAACCGCTGAATGCCAAAGGGCAG-3′; H259Y: Forward: 5′-AAGGATTCTGATGTCTTATTTTGTTATATTCATCCAGTTTCAGTTGTC-3′ Reverse: 5′-GACAACTGAAACTGGATGAATATAACAAAATAAGACATCAGAATCCTT-3′.

### *Transfection of RBL cells and flow cytometry*

Cells ($1.5 \times 10^6$) were transfected with plasmids (1.5 μg) encoding MRGPRX2 or its missense variants using the Amaxa Nucleofector Device and Amaxa Kit V according to the manufacturer's protocol. For stable transfection, cells were cultured in the presence of G-418 (1 mg/ml) and used within 1 mo of transfection (21). For transient transfection, cells were used within 16–20 h after transfection. To detect MRGPRX2 expression, cells ($0.5 \times 10^6$) were incubated with PE-conjugated anti-MRGPRX2 Ab, washed in FACS buffer, fixed, and analyzed on a BD LSR II flow cytometer. For FAM-SP binding, RBL cells stably overexpressing MRGPRX2 (RBL-MRGPRX2) were used (12). Untransfected RBL cells and RBL-MRGPRX2 ($0.5 \times 10^6$) were incubated with FAM-SP (1 μM, 60 min, 4°C). The cells were washed twice with FACS buffer and analyzed by flow cytometry.

### *Calcium mobilization*

$Ca^{2+}$ mobilization was determined as described previously (21). Briefly, cells ($2 \times 10^6$) were loaded with 1 μM Indo-1 acetoxymethyl ester for 30 min at room temperature. Cells were then washed and resuspended in 1.5 ml of HEPES-buffered saline. The cells were then stimulated with SP, and $Ca^{2+}$ mobilization was determined using a Hitachi F-2700 Fluorescence Spectrophotometer with an excitation wavelength of 355 nm and an emission wavelength of 410 nm.

### *Degranulation*

Cells ($5 \times 10^4$) were seeded into 96-well plates in a total volume of 50 μl HEPES buffer containing 0.1% BSA and exposed to ligands for 30 min. Cells without treatment were designated as controls. For total β-hexosaminidase release, unstimulated cells were lysed in 50 μl of 0.1% Triton X-100. Aliquots (20 μl) of supernatants or cell lysates were incubated with 20 μl of 1 mM p-nitrophenyl-N-acetyl-β-D-glucosamine for 1 h at 37°C. The reaction was stopped by adding 250 μl of a 0.1 M $Na_2CO_3$/0.1 M $NaHCO_3$ buffer, and absorbance was measured at 405 nm (21).

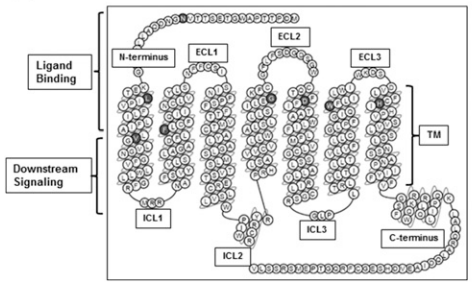
## Results

### *Identification of D184H and G165E as loss of function MRGPRX2 variants for SP, HK-1, hBD3, and icatibant in stably transfected RBL-2H3 cells*

To identify naturally occurring missense variants in the ECL and TM domains of MRGPRX2, we searched three publicly available databases: NHLBI-GO-ESP, 1000 Genomes, and ExAC. We found eight targets in the *MRGPRX2* gene with missense mutations (Fig. 1A). Amino acid change for each mutation is shown in Fig. 1B. Data from a computational protein prediction program, Polymorphism Phenotyping version 2 (PolyPhen-2), under the HumDiv model on a scale of 0 (benign) to 1 (damaging) are shown in Fig. 1B (22). The G165E and D184H variants have the lowest minor allele frequency (MAF; average <0.01%), whereas the N16H variant has the highest MAF (8.95%). Combined annotation–dependent depletion (CADD) is an in silico tool used for scoring the deleteriousness of single nucleotide variants as well as insertion/deletion variants in the human genome (23). A CADD score of >10 indicates that a variant may be deleterious. As shown in Fig. 1B, all eight MRGPRX2 variants targeted for the current study have CADD scores of <10. Thus, the combined in silico analyses suggest that although some of the MRGPRX2 variants may be damaging (PolyPhen-2 score >0.5), these mutations are rare (MAF <1%) and may not be associated with pathogenicity (CADD <10) (Fig. 1).

Studies on the effects of missense and other mutations on GPCR functions are routinely conducted with transiently transfected HEK293 cells (17, 18, 24–26). However, our previous studies on the regulation of MRGPRX2 have been performed with stably transfected rodent MC line (RBL-2H3 cell) that does not endogenously express the receptor (6, 12). To determine if any of the MRGPRX2 variants shown in Fig. 1 display gain or loss of function for MC activation, we first generated stable transfectants in RBL cells. The stable transfection procedure involves cDNA nucleofection followed by culturing cells in the presence of selection marker G418. We were quite surprised to find cells expressing three mutants (N16H, W243R, or H259Y) did not survive the G418 selection procedure. We found that 5 d after the start of G418 selection, cells expressing the wild-type (WT) receptor were almost fully covering the surface of the tissue culture dish, but only few cells could be detected in N16H, W243R, or H259Y transfectants. Triton X-100–lysed cells were used to measure total β-hexosaminidase content as an assay to quantitate cell number. As shown in Fig. 2A, unlike the situation with cells

A




B

| rs ID | Protein change | PolyPhen-2 | MAF (%) NHLBI-GO-ESP | 1000 Genome | ExAC | Average | CADD |
|---|---|---|---|---|---|---|---|
| rs11024970 | N16H | 0.937 | 8.70 | 11.30 | 6.85 | 8.95 | 1.1 |
| rs145992601 | L31V | 0.001 | 0.20 | 0.20 | 0.20 | 0.20 | 0.001 |
| rs11823569 | V43I | 0.502 | 0.91 | 0.66 | 0.24 | 0.60 | 6.35 |
| rs79763999 | F78L | 0.129 | 0.35 | 0.32 | 0.10 | 0.25 | 6.01 |
| rs141744602 | G165E | 0.567 | < 0.01 | not available | < 0.01 | <0.01 | 4.32 |
| rs372988289 | D184H | 0.736 | < 0.01 | not available | < 0.01 | <0.01 | 4.03 |
| rs150365137 | W243R | 0.760 | 0.43 | 0.14 | 0.33 | 0.30 | 2.15 |
| rs140862085 | H259Y | 0.009 | 0.03 | 0.04 | 0.01 | 0.02 | 0.001 |

**FIGURE 1.** Naturally occurring MRGPRX2 variants identified from NHLBI-GO-ESP, 1000 Genomes Project, and ExAC databases. (**A**) Serpentine diagram of the secondary structure of human MRGPRX2. Each circle represents amino acid residue with one letter code. Solid background denotes the eight naturally occurring missense variants used in the current study. ECL, ICL, and TM domains are shown. (**B**) Amino acid change for each MRGPRX2 variant, PolyPhen-2 score, MAF, and CADD score are shown.

expressing the WT receptor, we could not detect any β-hexosaminidase in transfectants with cDNA-encoding N16H, W243R, or H259Y (Fig. 2A). We therefore focused our initial studies on variants L31V, V43I, F78L, G165E, and D184H, which stably express on the cell surface similar to the WT receptor (Fig. 2B). Although MRGPRX2 is activated by multiple ligands, SP is probably the most well characterized (1, 2, 5, 7, 16, 18). We therefore tested the effects of SP on $Ca^{2+}$ mobilization and degranulation in cells expressing these variants. We found that the variants L31V, V43I, and F78L responded normally to SP for $Ca^{2+}$ mobilization and degranulation (Fig. 2C, 2D). By contrast, when compared with the WT receptor, $Ca^{2+}$ mobilization and degranulation to SP were substantially inhibited in cells expressing G165E and D184H variants (Fig. 2C, 2D),

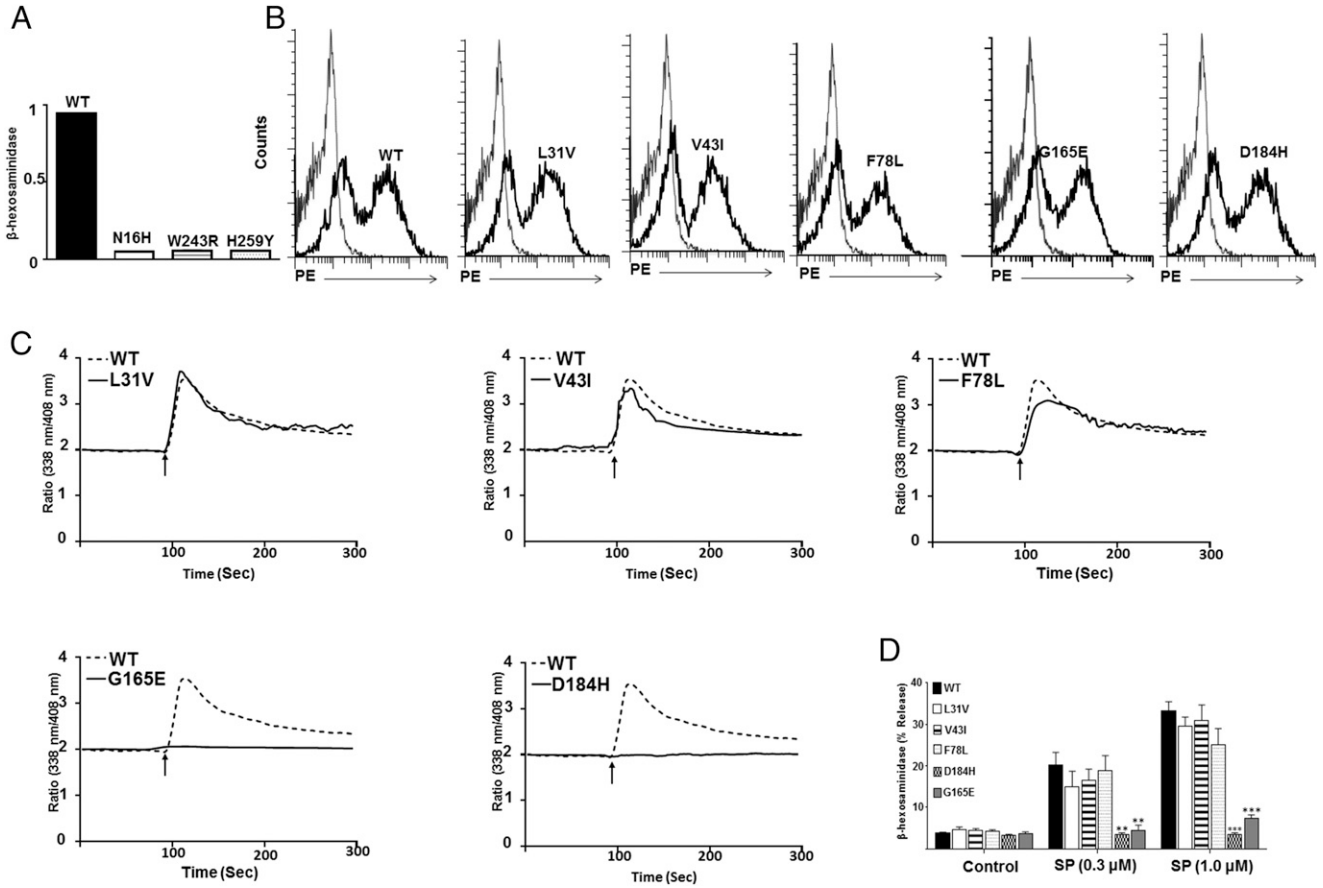



**FIGURE 2.** Effects of naturally occurring MRGPRX2 mutations (L31I, V43I, F78L, G165E, and D184H) on cell surface expression, SP-induced $Ca^{2+}$ mobilization and degranulation in stably transfected RBL cells. (**A**) Cells were transfected with cDNA-encoding WT, N16H, W243R, or H253Y variant, transferred to 24-well plate, and G418 was added to the culture medium 16 h after transfection. After 5 d, nonadherent cells were removed, adherent cells were lysed, and total β-hexosaminidase content was determined. (**B**) Flow cytometry was performed with PE–anti-MRGPRX2 Ab to determine cell surface expression of WT and variants in stably transfected RBL cells. Representative histograms for WT/variant (thick line) and control untransfected cells (thin line) are shown. (**C**) Cells expressing WT and MRGPRX2 variants were loaded with Indo-1 and ICL $Ca^{2+}$ mobilization in response to SP (1 μM) was determined. Data shown are representative of three independent experiments. (**D**) Cells were exposed to buffer (control) or SP (0.3 and 1 μM) for 30 min, and β-hexosaminidase release was determined. All data points are expressed as mean ± SEM of three experiments performed in triplicate. Statistical significance was determined by two-tailed unpaired *t* test. $^{**}p < 0.01$, $^{***}p < 0.001$.

despite normal cell surface expression (Fig. 2B). These findings are consistent with higher PolyPhen-2 scores for G165E and D184H when compared with L31A, V43I, and F78L variants (Fig. 1B).

Reddy et al. (17) recently showed that a point mutant of MRGPRX2 (E164R) was resistant to SP for $Ca^{2+}$ mobilization but responded normally to an HDP, LL-37. Based on this finding, it was proposed that different amino acid residues on MRGPRX2 are responsible for binding to different ligands. We therefore tested the effects of naturally occurring missense mutations (L31A, V43I, F78L, G165E, and D184H) on degranulation in response to HK-1, hBD3, and icatibant. As shown in Fig. 3, cells expressing L31V, V43I, or F78L variant responded to all ligands tested for degranulation, similar in extent to the WT receptor. By contrast, cells expressing G165E or D184H variant were resistant to HK-1, hBD3, and icatibant for degranulation. These findings suggest that SP, HK-1, hBD3, and icatibant all interact with the same amino acids on MRGPRX2's fourth and fifth TM domains (Fig. 1) to induce MC degranulation.

Our next goal was to perform binding studies to determine if reduced MC activation in missense variants correlates with correspondingly reduced ligand binding to MRGPRX2. Of the agonists used in this study, only SP is available as a fluorescent-labeled conjugate (FAM-SP). Our initial goal was to perform flow cytometry analysis to determine if FAM-SP binds to RBL cells stably expressing MRGPRX2 and to test if this binding could be blocked by unlabeled HK-1, hBD3, or icatibant. For these studies, we used RBL-MRGPRX2. We confirmed cell surface MRGPRX2 expression by flow cytometry using PE-conjugated anti-MRGPRX2 Ab (Fig. 4A). To validate the functional activity of FAM-SP for MRGPRX2, we compared its ability to induce degranulation in untransfected RBL cells and RBL-MRGPRX2. As shown in Fig. 4B, both unlabeled SP and FAM-SP induced degranulation in RBL-MRGPRX2, but not in untransfected cells, validating the specificity of FAM-SP for the receptor. However, flow cytometry experiment demonstrated that FAM-SP interact equally well with untransfected RBL cells and RBL-MRGPRX2 (Fig. 4C). These findings suggest that the amphipathic nature of SP facilitates a strong interaction with the plasma membrane even in the absence of MRGPRX2, thus making it difficult to perform the proposed binding studies.

### *Identification of W243R and H259Y as loss of function MRGPRX2 variants for SP, HK-1, hBD3, and icatibant in transiently transfected RBL cells*

The low PolyPhen-2 score for the MRGPRX2 variant H259Y (Fig. 1B) indicates that this mutation is benign. Thus, our inability to generate a stable transfectant expressing this variant in RBL cells was surprising. We therefore generated transient transfectant in RBL cells expressing H259Y. We also performed similar studies with two other variants with high PolyPhen-2 scores (N16H, 0.937 and W243R, 0.760) that did not survive the stable transfection procedure (Fig. 1A). We found that all three variants expressed on the surface of RBL cells (Fig. 5A). The N16H variant responded to SP for $Ca^{2+}$ mobilization and degranulation similar to the WT receptor (Fig. 5B, 5C). However, W243R and H259Y variants were resistant to SP-induced $Ca^{2+}$ mobilization and degranulation (Fig. 5B, 5C). HK-1, hBD3, and icatibant induced normal degranulation in cells transiently expressing N16H, but they failed to activate this response in cells expressing W243R or H259Y variant (Fig. 6).

## Discussion

The unique features of MRGPRX2 that distinguish it from other GPCRs are its predominant expression on human MCs and its activation by a diverse group of cationic ligands (6, 7, 12, 16). Emerging evidence suggests that MRGPRX2 contributes to

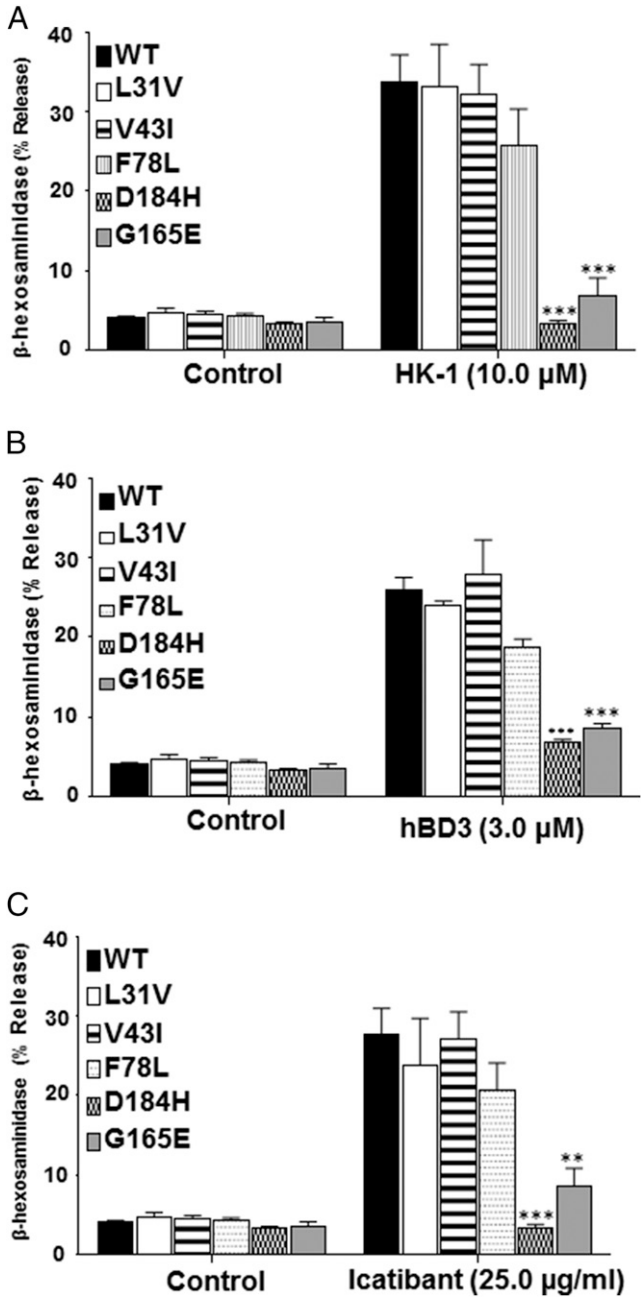



**FIGURE 3.** Effects of naturally occurring MRGPRX2 mutations (L31I, V43I, F78L, G165E, and D184H) on HK-1, hBD3, and icatibant-induced degranulation in stably transfected RBL cells. Cells stably expressing WT and MRGPRX2 variants were exposed to buffer (control) or stimulated with (**A**) HK-1 (10 μM), (**B**) hBD3 (3 μM), or (**C**) icatibant (25 μg/ml) for 30 min, and β-hexosaminidase release was determined. All the points expressed as mean ± SEM of three experiments in triplicate. Statistical significance was determined by two-tailed unpaired *t* test. $^{**}p < 0.01$, $^{***}p < 0.001$.

pseudoallergic drug reactions and a number of chronic inflammatory diseases (16, 27, 28). Although activation of MRGPRX2 or FcεRI on MCs elicits ICL $Ca^{2+}$ mobilization and comparable MC degranulation, MRGPRX2 stimulation triggers little to no cytokine or $PGE_2$ generation (16, 29, 30). Thus, the effects on MRGPRX2 on MC-mediated disorders likely reflects $Ca^{2+}$ mobilization and degranulation. In the current study, we have used eight naturally occurring missense MRGPRX2 variants and identified rare mutations that display loss of function phenotype for MC degranulation in response to ligands that participate in

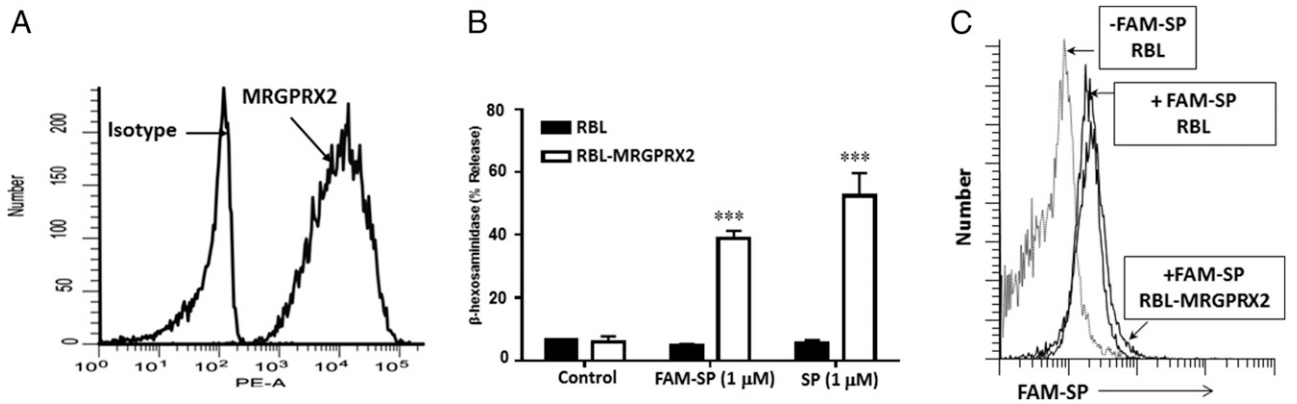



**FIGURE 4.** FAM-SP–induced degranulation and binding in RBL-MRGPRX2. (**A**) RBL-MRGPRX2 were exposed to PE-conjugated anti-MRGPRX2 or PE-conjugated isotype-matched Ab, and cell surface receptor expression was determined by flow cytometry. (**B**) RBL cells and RBL-MRGPRX2 were exposed to buffer (control), SP, or FAM-SP for 30 min, and β-hexosaminidase release was determined. (**C**) RBL cells or RBL-MRGPRX2 were exposed to FAM-SP (1 μM, 60 min, 4°C), washed, and fluorescence was determined by flow cytometry. Data presented for flow are representative of three similar experiments, and degranulation data are expressed as mean ± SEM of three experiments in triplicate. Statistical significance was determined by two-tailed unpaired *t* test. ***$p < 0.001$.

pseudoallergy (icatibant) (16), itch/chronic urticaria (SP) (5, 7), asthma (HK-1) (10–12), and periodontitis (hBD3) (6).

Molecular modeling and docking approaches have recently been used to identify $Glu^{164}$ (E164) and $Asp^{184}$ (D184) in MRPGRX2's fourth and fifth TM domains as the negatively charged residues that make ionic contact with cationic opioid ligands (18). Accordingly, E164Q or D184N substitution that retains the steric property of the WT residue but removes the negative charge resulted in loss of receptor activation by dextromethorphan, morphine, and related opioids ligands. These findings suggest that both $Glu^{164}$ and $Asp^{184}$ are important for the binding of opioids to MRGPRX2, and that removal of one negatively charged residue results in loss of ligand–receptor interaction. However, MRGPRX2 activation by a metabolite of an endogenous opioid peptide, dynorphin A (1–13), is lost in the D184N but not the E164Q mutation (18). Modeling studies predict that $Arg^7$ and $Phe^4$ of dynorphin interact with $Asp^{184}$ but not $Glu^{164}$. These findings suggest that one or both anionic $Glu^{164}$ and $Asp^{184}$ participate in the binding of cationic opioids and endogenous opioid peptide metabolites. Using publicly available Web portals, Reddy et al. (17) also predicted that SP-binding pocket in MRGPRX2 consists of a number of structurally conserved hydrophilic residues along with a buried glutamic acid residue ($Glu^{164}$, E164). Accordingly, replacement of $Glu^{164}$ with a positively charged Arg (E164R) results in loss of MRGPRX2 activation by SP. However, this mutant responds normally to the HDP LL-37 (17). These findings suggest that different ligands interact with different amino acid residues on MRGPRX2's predicted ligand-binding pocket to induce MC degranulation (17, 18).

The goal of the current study was to use publicly available databases to identify naturally occurring missense MRGPRX2

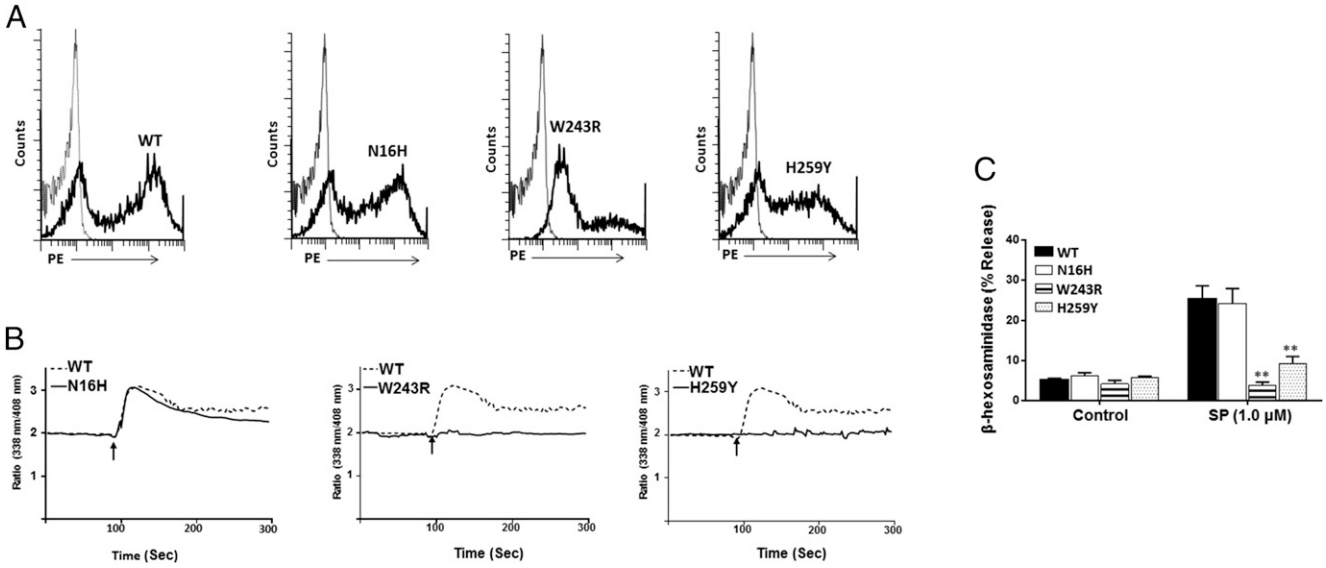



**FIGURE 5.** Effects of naturally occurring MRGPRX2 mutations (N16H, W243R, and H259Y) on cell surface expression, $Ca^{2+}$ mobilization, and SP-induced degranulation in transiently transfected RBL cells. (**A**) Flow cytometry was performed using PE–anti-MRGPRX2 Ab to determine cell surface expression of WT and variants in transiently transfected RBL cells. Representative histograms for WT/variant (thick line) and control untransfected cells (thin line) are shown. (**B**) Cells expressing WT and MRGPRX2 variants were loaded with Indo-1, and ICL calcium mobilization in response to SP (1 μM) was determined. Data shown are representative of three independent experiments. (**C**) Cells were exposed to buffer (control) or SP (1 μM) for 30 min, and β-hexosaminidase release was determined. All data points are expressed as mean ± SEM of three experiments performed in triplicate. Statistical significance was determined by two-tailed unpaired *t* test. **$p < 0.01$.

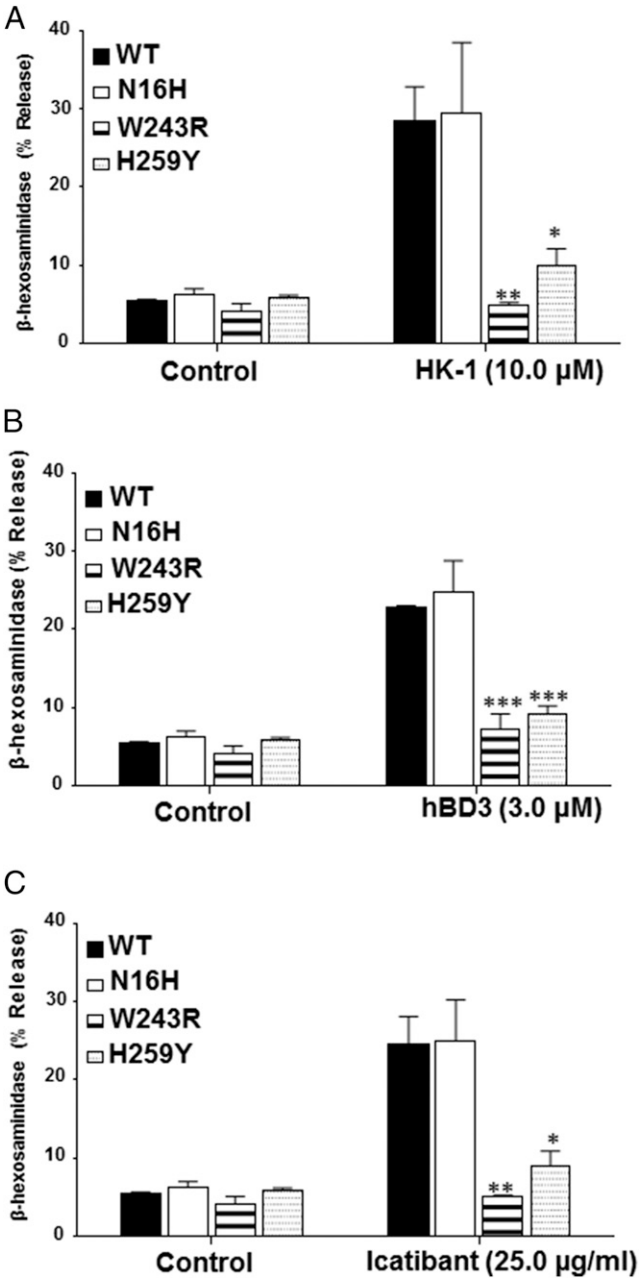



**FIGURE 6.** Effects of MRGPRX2 mutations (N16H, W243R, and H259Y) on degranulation in response to HK-1, hBD3, and icatibant. RBL cells transiently expressing WT, N16H, W243R, and H259Y variants were stimulated with (**A**) HK-1 (10 μM), (**B**) hBD3 (3 μM), and (**C**) icatibant (25 μg/ml), and β-hexosaminidase release was determined. All the points expressed as a mean ± SEM of three experiments in triplicate. Statistical significance was determined by two-tailed unpaired *t* test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

variants that display gain or loss of function phenotype for MC degranulation by a diverse group of cationic ligands. There are a number of interesting features regarding the rare MRGPRX2 variant G165E (MAF <0.01%) that warrant discussion. First, it is present within the predicted binding pocket for opioids and SP, as determined from modeling studies (17, 18). Second, although we were unsuccessful in demonstrating specific binding of FAM-SP to RBL-MRGPRX2 because of high background binding, it is likely that this mutation interferes with SP's binding to MRGPRX2. This contention was supported by the finding that cells expressing G165E variant did not respond to SP for $Ca^{2+}$ mobilization despite normal cell surface expression. Third, and most importantly, none of the other MRGPRX2 ligands tested caused degranulation in cells expressing this variant. It is noteworthy that G165E mutation results in the replacement of an aliphatic side chain with a positively charged residue next to a negatively charged amino ($Glu^{164}$, E164) required for ionic interaction with SP and opioids (17, 18). It is therefore likely that the presence of two adjacent negatively charged side chains (E164, E165) in the variant G165E interferes with the integrity of the binding pocket, thus preventing MC activation by SP and all other cationic ligands used in this study.

Studies by Reddy et al. (17) did not consider $Asp^{184}$ as a possible site for SP interaction with MRGPRX2 despite the fact this amino acid is critical for receptor activation of all opioid ligands tested, including dynorphin A (18). It is interesting to note that the naturally occurring D184H variant results in the switching of a negatively charged side chain to a positively charged one. An important finding of the current study was that none of the MRGPRX2 agonists tested stimulated degranulation in RBL cells stably expressing either G165E or D184H variant. These results suggest that SP and opioid-binding pocket identified within MRGPRX2's fourth and fifth TM domains (17, 18) is shared by all MRGPRX2 agonists that induce MC degranulation, and that G165E or D184H mutation disrupts the integrity of the binding pocket, preventing receptor activation.

We found that two additional mutations, W243R and H259Y, in the sixth and seventh TM domains outside MRGPRX2's predicted ligand-binding pocket also rendered the receptor unresponsive to all ligands tested. The mechanism by which these mutations lead to loss of degranulation is unknown. Interestingly, we found that although cells expressing these variants do not survive the stable transfection procedure, they express normally in transiently transfected cells. It is therefore possible that replacement of the bulky Trp with a positively charged Arg in variant W243R and His with Tyr in variant H269Y influences both the receptor's expression status and its ability to interact with diverse ligands. In future studies, it will be interesting to determine the MC status of individuals harboring these mutations and their responsiveness to MGRPRX2 ligands.

In summary, we have shown that naturally occurring rare MRGPRX2 variants with single amino acid substitution in the receptor's predicted ligand-binding pocket (G165E and D184H) renders it unresponsive to SP, HK-1, hBD3, and icatibant for MC degranulation. Although the naturally occurring variants W243R and H259Y in the receptor's sixth and seventh TM domain also rendered the receptor unresponsive to all ligands tested for degranulation, the mechanisms of their actions are unknown and remain to be determined. An important clinical implication of the current study is that individuals harboring any of the missense MRGPRX2 mutation (G165E, D184H, W243R, or H259Y) may be protected from MC-mediated drug-induced pseudoallergy and chronic inflammatory diseases such as itch, chronic urticaria, asthma, and periodontitis. A goal for our future studies is to determine the responsiveness of MCs isolated from these individuals to MRGPRX2 ligands and to test whether these individuals develop skin reactions to pseudoallergic drugs.

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## Disclosures

The authors have no financial conflicts of interest.

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