Identification of Determinants of Ligand Binding Affinity and Selectivity in the Prostaglandin D\textsubscript{2} Receptor CRTH2*\S

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The chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) is a G protein-coupled receptor that mediates the pro-inflammatory effects of prostaglandin D\textsubscript{2} (PGD\textsubscript{2}) generated in allergic inflammation. The CRTH2 receptor shares greatest sequence similarity with chemoattractant receptors compared with prostanooid receptors. To investigate the structural determinants of CRTH2 ligand binding, we performed site-directed mutagenesis of putative mCRTH2 ligand-binding residues, and we evaluated mutant receptor ligand binding and functional properties. Substitution of alanine at each of three residues in the transmembrane (TM) helical domains (His-106, TM III; Lys-209, TM V; and Glu-268, TM VI) and one in extracellular loop II (Arg-178) decreased PGD\textsubscript{2} binding affinity, suggesting that these residues play a role in binding PGD\textsubscript{2}. In contrast, the H106A and E268A mutants bound indomethacin, a nonsteroidal anti-inflammatory drug, with an affinity similar to the wild-type receptor. HEK293 cells expressing the H106A, K209A, and E268A mutants displayed reduced inhibition of intracellular cAMP and chemotaxis in response to PGD\textsubscript{2}, whereas the H106A and E268A mutants had functional responses to indomethacin similar to the wild-type receptor. Binding of PGE\textsubscript{2} by the E268A mutant was enhanced compared with the wild-type receptor, suggesting that Glu-268 plays a role in determining prostanooid ligand selectivity. Replacement of Tyr-261 with phenylalanine did not affect PGD\textsubscript{2} binding but decreased the binding affinity for indomethacin. These results provided the first details of the ligand binding pocket of an eicosanoid-binding chemoattractant receptor.

Prostaglandin D\textsubscript{2} (PGD\textsubscript{2})\textsuperscript{3} is the predominant prostanooid species produced by allergen-activated mast cells (1, 2) and has been implicated in the pathogenesis of allergic diseases such as allergic asthma and atopic dermatitis (1, 3). Increased production or exposure to PGD\textsubscript{2} leads to elevated Th2-type cytokines and eosinophilic inflammation in murine asthma models (4, 5). However, the molecular mechanism of PGD\textsubscript{2} action in the pathogenesis of allergic disease remains only partially characterized.

PGD\textsubscript{2} exerts its effects through two G protein-coupled receptors (GPCRs), the D prostanooid receptor (DP) and the recently discovered chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2). DP receptor signaling has been linked to NF-κB activation (6) and may influence dendritic cell function leading to skewing of the T cell response toward a Th2 phenotype (7). Mice deficient in the DP receptor display reduced Th2-mediated airway inflammation in the ovalbumin-induced asthma model (8), suggesting that PGD\textsubscript{2} signaling through the DP receptor plays a pro-inflammatory role in settings of allergic inflammation. On the other hand, PGD\textsubscript{2} has been hypothesized to exert anti-inflammatory effects by inhibiting dendritic cell migration and T cell activation (9).

The role of PGD\textsubscript{2} signaling through the CRTH2 receptor in allergic disease is less well established. In humans, the CRTH2 receptor is expressed on Th2 cells, eosinophils, basophils, and monocytes (7, 10, 11), which are known to play a role in the pathogenesis of allergic diseases such as asthma (12). Polymorphisms in the 3′ untranslated region of the CRTH2 receptor gene that confer greater mRNA stability have been linked to increased asthma severity (13), and increased numbers of circulating T cells expressing the CRTH2 receptor have been correlated with severity of atopic dermatitis (14, 15). CRTH2 receptor activation stimulates chemotaxis of human Th2 cells, eosinophils, and basophils both in vitro and in vivo (16, 17), suggesting that the CRTH2 receptor may directly mediate recruitment of inflammatory cells in response to PGD\textsubscript{2} generated in settings of allergic inflammation and thus play a pro-inflammatory role (18). Recently it was reported that ramatroban, a thromboxane receptor antagonist used clinically for the treatment of allergic rhinitis, also exhibits CRTH2 antagonist activity and inhibits PGD\textsubscript{2}-stimulated eosinophil migration (17, 19). Consistent with this finding, ramatroban had been observed previously to inhibit antigen-induced mucosal eosinophilia in sensitized guinea pigs (20). Ramatroban shares structural similarity with indomethacin, an arylacetic acid class nonselective cyclooxygenase inhibitor and widely used nonsteroidal anti-inflammatory drug (NSAID) that has also been shown to be a potent CRTH2 agonist (21). We recently performed structure-activity relationship analysis of arylacetic acid NSAIDs that revealed structural features of indomethacin and ramatroban that are required for binding to the mouse CRTH2 receptor (22).

The CRTH2 receptor does not share significant sequence homology with the DP or other prostanooid receptors but instead exhibits greatest sequence similarity to peptide chemoattractant receptors such as the formyl peptide receptor (16). Residues that have been identified as playing a role in prostanooid ligand binding by the DP and other prostanooid receptors are not conserved in the CRTH2 receptor sequence, suggesting that the CRTH2 receptor binds its prostaglandin ligand in a manner distinct from the other prostanooid receptors. In addition to peptide

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9 The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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12 The abbreviations used are: PGD\textsubscript{2}, prostaglandin D\textsubscript{2}; C5aR, C5a anaphylatoxin receptor; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; DK-PGD\textsubscript{2}, 13,14-dihydro-15-keto-PGD\textsubscript{2}; DP, D prostanooid; EC, extracellular loop domain; FPR, formyl peptide receptor; GPCR, G protein-coupled receptor; NSAID, nonsteroidal anti-inflammatory drug; TM, transmembrane domain; IP, prostacyclin receptor; HA, hemagglutinin; PE, phycoerythrin; ELISA, enzyme-linked immunosorbent assay; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FBS, fetal bovine serum.
Expression of HA-mCRTH2 and Mutant Receptors in HEK293 Cells—Two independently derived plasmids encoding the HA-mCRTH2 and each mutant mCRTH2 receptor construct were transiently transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen). Stable cell lines (HA-mCRTH2, H106A, K209A, K209R, and E268A) were selected in media containing 600 μg/ml G418, and clones were isolated by manual colony isolation using cloning rings. For the Y261F mutant, a stably transfected polyclonal population was generated. Cells were maintained at 37 °C in humidified air containing 5.5% CO2 in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin. Expression of HA-mCRTH2 and mutant receptors in both transiently and stably expressing cells was enhanced incubation with 5 mM sodium butyrate for 24 h prior to all experiments.

Flow Cytometric Analysis of Receptor Expression—HA-mCRTH2 and mutant receptor expression in transiently and stably transfected HEK293 cells was monitored by flow cytometry. After brief trypsinization, cells were resuspended in media containing 10% FBS and immediately placed on ice. Cells were incubated with 1:100 of the 262K monoclonal anti-HA antibody for 1 h at 4 °C, washed twice with ice-cold PBS, and incubated with 1:100 of a PE-conjugated goat anti-mouse antibody for 30 min at 4 °C. Cells were washed once with ice-cold PBS, resuspended in PBS, and analyzed for PE fluorescence on a FACScan flow cytometer (BD Biosciences).

Cell Surface ELISA Analysis of Receptor Expression—Cells stably expressing HA-mCRTH2 or mutant receptors were plated in poly-lysine-coated 96-well plates (BD Biosciences) at a density of 4 × 10^4/well 2 days prior to experiment to ensure confluency at the time of ELISA. Receptor expression was enhanced by addition of sodium butyrate (5 mM) 24 h prior to the ELISA. Cells transiently expressing HA-mCRTH2 or mutant receptors were plated at a density of 6 × 10^4/well in the presence of sodium butyrate 1 day prior to experiment. Cells were fixed by incubation with 4% paraformaldehyde containing 0.12 μM sucrose in PBS containing 1 mM MgCl2, and 0.5 mM CaCl2 (PBS-CM) for 20–30 min at room temperature. Cells were washed twice with PBS-CM and incubated with 3% BSA in PBS-CM for 30 min at 37 °C. Cells were incubated with 1:500 of the 3F10 rat anti-HA antibody in PBS-CM for 30 min at 37 °C. Cells were incubated with 1:100 of the 262K monoclonal anti-HA antibody in 3% BSA in PBS-CM for 1 h at 37 °C, washed three times with PBS-CM for 5 min each, and incubated with 1:100 of a horseradish peroxidase-conjugated goat anti-rat antibody in 3% BSA in PBS-CM for 1 h at 37 °C. Cells were washed three times with PBS-CM, and the chromogenic substrate o-phenylenediamine dihydrochloride (1 mg/ml, Pierce) was added. After color development (20–30 min), the reaction was stopped with the addition of an equal volume of 2.5 M sulfuric acid, and the absorbance at 490 nm was determined. In some experiments, cells were permeabilized by incubation with 0.2% Triton X-100 for 15 min prior to blocking with BSA.

Radioligand Binding—Membranes for radioligand binding experiments were harvested from HEK293 cells expressing HA-mCRTH2 wild-type or mutant receptors as described (24). Membranes (30 μg of membrane protein) were incubated with [3H]PGD2 and unlabeled ligands for 1.5 h at 4 °C in binding buffer (25 mM Hepes (pH 7.4), 1 mM EDTA, 5 mM MgCl2, 140 mM NaCl, 5 mM KCl). These conditions were sufficient to achieve apparent equilibrium of binding while ensuring ligand stability and minimizing nonspecific binding. The binding reaction was terminated by the addition of 3 ml of ice-cold binding buffer and rapidly filtered under vacuum over Whatman GF/F filters. Filters were washed three times with 3 ml of ice-cold binding buffer, dried and counted in 4 ml of Ultima Gold scintillation fluid (Packard Biosciences, Groningen, The Netherlands). For saturation isotherm experiments, the [3H]PGD2 concentration ranged from 2.5 to 30 nM. Specific binding did not exceed 5% of total radioligand concentration present in the sample.

Materials—[3H]PGD2 was purchased from Amersham Biosciences, and unlabeled prostaglandin ligands were from Cayman Chemical (Ann Arbor, MI). Indomethacin, forskolin, isobutylmethylxanthine, and sodium butyrate were from Sigma. Ramotrabam was a kind gift from K. Bacon (Bayer AG, Kyoto, Japan). DMEM and Opti-MEM were from Invitrogen. FBS was obtained from Atlanta Biologicals (Lawrenceville, GA). G418 was purchased from Mediatech (Herndon, VA). t-Glutamine and penicillin/streptomycin were from BioWhittaker (Walkersville, MD). The 262K monoclonal anti-HA antibody was purchased from Cell Signaling Technologies (Beverly, MA); the PE-conjugated goat anti-mouse antibody was from Jackson ImmunoResearch (West Grove, PA); the 3F10 rat anti-HA antibody was from Roche Applied Science, and the horseradish peroxidase-conjugated goat anti-rat antibody was from Amersham Biosciences.

Construction and Expression of HA-tagged Wild-type mCRTH2 and HA-mCRTH2 Mutants—The HA-mCRTH2 expression plasmid was generated by ligation of fragments containing the HA epitope tag (SmaI/Ndel) from the 77AHA pRC/CMV plasmid (23) and the mCRTH2 coding region (NdeI/XbaI) from the p77AHA pRC/CMV plasmid (23) and we evaluated the effects of these mutations on ligand binding and receptor function. To investigate the structure of the mouse CRTH2 receptor ligand binding pocket, we performed site-directed mutagenesis of putative ligand-binding residues, and we evaluated the effects of these mutations on ligand binding and receptor function. These studies reveal that PGD2, likely binds in the CRTH2 binding pocket with an orientation that is distinct from that proposed for other prostaglandin receptors. Furthermore, these data demonstrate that PGD2 and indomethacin interact with distinct but overlapping sets of residues within the ligand binding pocket and suggest specific ligand-receptor interactions that may play a role in determining ligand binding affinity and selectivity.

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TABLE ONE

| Residue | Rationale for selection of mCRTH2 residues to mutate |
|---------|-----------------------------------------------------|
| TM I    | His-38 Based on mCRTH2 receptor model (22), oriented toward putative ligand binding pocket region; position corresponds to residues in the DP and IP receptor implicated in ligand recognition (32) |
| TM II   | Thr-87 Based on mCRTH2 receptor model (22), oriented toward putative ligand binding pocket region |
| TM III  | His-106 Represents gain of charge/polarity compared with chemotactic receptors; C5aR tolerates only hydrophobic residues at this position (30) |
| Ser-107 | Phenylalanine at this position in FPR is part of a cluster of residues that has been shown to be involved in ligand binding (26) |
| Ser-108 | Threonine at this position in FPR is part of a cluster of residues that has been shown to be involved in ligand binding (26) |
| Phe-110 | Based on mCRTH2 receptor model (22), preliminary ligand docking simulations suggested an interaction between the phenylalanine aromatic ring and the p-chlorobenzoyl moiety of indomethacin |
| EC II   | Arg-178 Based on mCRTH2 receptor model (22), positively charged side chain forms a “lid” on the putative ligand binding pocket |
| TM V    | Leu-205 Arginine in FPR has been involved in ligand binding (27) |
| Ser-208 | Represents gain of polarity compared with chemotactic receptors; alanine substitution for isoleucine in FPR leads to decrease in ligand binding (27) |
| Lys-209 | Conserved arginine in FPR and C5aR has been shown to be involved in ligand binding (27, 29, 31) |
| TM VI   | Tyr-261 Based on mCRTH2 receptor model (22), oriented toward putative ligand binding pocket region |
| Ser-265 | Based on mCRTH2 receptor model (22), oriented toward putative ligand binding pocket region |
| Glu-268 | Based on mCRTH2 receptor model (22), oriented toward putative ligand binding pocket region |
| TM VII  | Ser-290 Prostanoid subfamily GPCRs have conserved arginine that has been shown to play a role in ligand binding |

**Ligand-Receptor Interactions of the Mouse CRTH2 Receptor**

Binding reaction. For competition experiments, 3 nM [³H]PGD₂ was used.

[cAMP] Assay—HEK293 cells stably expressing HA-mCRTH2 or mutant receptors were plated at a density of 9 × 10⁵/well in 6-well plates 2 days prior to the experiment, and 5 mM sodium butyrate was added for the final 24 h. Thirty minutes prior to addition of ligands, media were replaced with Opti-MEM I containing 0.5 mM isobutylmethylxanthine. Cells were incubated with ligands for 10 min and washed once with PBS, and the reaction was terminated by the addition of 0.1 N HCl. Cells were scraped free, and the resulting cell suspension was centrifuged for 10 min at 1000 × g. Supernatants were assayed for protein content by BCA assay (Pierce). After normalization to protein content, [cAMP] levels were determined by an enzyme-linked immunosassay according to the manufacturer’s instructions (Cayman Chemical).

Transwell Migration Assay—Cells stably expressing HA-mCRTH2 or mutant receptors were incubated with 5 mM sodium butyrate prior to harvesting. Cells were trypsinized, washed three times in PBS, and resuspended in DMEM. Cells (1 × 10⁵) were added to the upper chamber of 24-well 8.0-μm polycarbonate transwell inserts (Costar, Cambridge, MA) that had been previously treated overnight with 5 μg/ml Matrigel (BD Biosciences) in PBS at 4 °C and blocked in the presence of 2% BSA in PBS for 1 h at 37 °C. Ligands were diluted in DMEM and added to the lower chamber. After incubating for 4 h at 37 °C, inserts were removed, and cells adhering to the top of the membrane were removed with a cotton swab. Cells on the bottom of the membrane were fixed with 3.7% formaldehyde for 1 h, washed twice with PBS, and stained overnight with crystal violet. For each insert, five independent fields were counted in blinded fashion at ×200 magnification.

Molecular Modeling—The mCRTH2 model was constructed as described previously (22). Briefly, the transmembrane-spanning α-helical bundle of mCRTH2 was constructed with homology modeling methods, using a β₂-adrenergic receptor model as a template (25). The extracellular and cytoplasmic loops were generated de novo by attaching the loops as extended polypeptides to the appropriate helix and applying weak harmonic constraints during low temperature (30 K) molecular dynamics to connect appropriate loop segments with a trans-peptide bond. A putative disulfide cross-link between the extracellular region of transmembrane helix III and extracellular loop II was generated by applying additional constraints during the generation of extracellular loop II. The N and C termini were generated using similar techniques in the absence of constraints. To simplify model construction, the C-terminal tail was truncated at Val-321. The intact mCRTH2 receptor model was then refined further with limited energy minimization and low temperature molecular dynamics simulation to relieve any peptide backbone conformational strain or residual bad steric interactions. Ligands were docked into the putative ligand-binding site using both manual (PGD₂) and automated (indomethacin) ligand docking algorithms. Receptor-ligand complexes that were consistent with mutagenesis data were refined by using limited energy minimization and low temperature molecular dynamics simulations in which weak harmonic positional restraints were placed on backbone atoms to prevent overcompaction of the receptor. All structural refinement calculations were performed *in vacuo* with a distance-dependent dielectric model, using standard AMBER all-atom potential functions. Energy minimization and molecular dynamics calculations were performed with the AMBER package. Automated ligand docking was performed using the automated docking module in MOE (Chemical Computing, Inc.).

**RESULTS**

To predict putative CRTH2 receptor ligand-binding residues, we performed multiple sequence alignments to compare the transmembrane domains of the human and mouse CRTH2 receptors with those of related chemotactic receptors, including the formyl peptide receptor (FPR), the C5a anaphylatoxin receptor (C5aR), and the leucotriene B₄ receptors (BLT1 and BLT2). The ligand binding pockets of the FPR and C5a receptors have been extensively studied and ligand-binding residues identified (26–31). We reasoned that CRTH2 receptor residues with the highest probability of interacting with ligand are as follows: 1) those that are conserved between the CRTH2 and chemotactic receptor that have been shown to play a role in chemotactic ligand binding or 2) those charged or polar residues that lie within the regions of conservation but are not themselves conserved. Based on these criteria (TABLE ONE), an initial set of candidate residues (His-106, Ser-107, Ser-108, Leu-205, Ser-208, and Lys-209) was selected and
TABLE TWO

Expression level (B_{max}) and [^{3}H]PGD_{2} binding affinities (K_{d}) of HA-mCRTH2 mutants

| Mutant | K_{d} (nM) | B_{max} (pmol/mg) |
|--------|------------|-------------------|
| Wild type | 16 ± 3 | 4.8 ± 0.7 |
| TM I H38A | 29 ± 6 | 6.7 ± 1.3 |
| TM II T87A | 20 ± 4 | 6.1 ± 1.4 |
| TM III H106A | >150 | NE |
| S107A | 15 ± 2 | 6.9 ± 1.2 |
| S108A | 38 ± 11* | 9 ± 5 |
| S208A | 61.1 | 7.7 |
| S290A | 40 | 40 |
| F110A | NE | |
| EC II R178A | 80 ± 10* | 2.7 ± 0.8 |
| R178K | 40 ± 3* | 4.6 ± 0.4 |
| R178H | 90 ± 40* | 2.4 ± 0.4 |
| TM V L205A | 100 ± 20 | 3.4 ± 0.8 |
| S208A | 38 ± 5* | 1.5 ± 0.5 |
| K209A | >150 | |
| K209R | >150 | |
| TM VI Y261A | NE | |
| Y261F | 35 ± 4* | 6.6 ± 0.6 |
| S265A | 40 ± 12* | 10 ± 3 |
| E268A | >150 | |
| E268Q | >150 | |
| E268D | >150 | |
| TM VII S290A | 40 ± 5* | 2.7 ± 0.9 |

* K_{data}.

FIGURE 1. Mutant HA-mCRTH2 receptors show variable PGD_{2} binding affinities. A and B, membranes isolated from HEK293 cells transiently transfected with HA-mCRTH2 or mutant receptors were incubated with varying concentrations of [^{3}H]PGD_{2} in the absence (total binding) or presence (nonspecific binding) of 10 μM OK-PGD_{2}, as described under “Experimental Procedures.” Specific binding was determined to be the difference between total and nonspecific binding. For HA-mCRTH2 and all mutants, bound [^{3}H]PGD_{2} represented 0.5% of total specific activity. Each data point was determined in duplicate; these data are representative of 3–4 independent experiments utilizing two independently derived mutant receptor plasmids. A, the S107A (open squares), S108A (open circles), S208A (open inverted triangles), and S290A (open triangles) mutants exhibited [^{3}H]PGD_{2} binding affinities (<5-fold change) similar to the wild-type HA-mCRTH2 receptor (closed squares). B, the H106A (open squares), L205A (open circles), K209A (open inverted triangles), and K209R (open triangles) mutants had decreased binding (>5-fold change) compared with the HA-mCRTH2 receptor (closed squares). C, the cell surface expression level of transiently expressed mutant receptors with decreased ligand binding (black) was determined by flow cytometry as described under “Experimental Procedures.” PE fluorescence of vector-transfected cells is shown for comparison (gray), with the percentage of PE-positive cells expressing the HA-mCRTH2 or mutant receptor indicated.

individually mutated to Ala. Additionally, Ser-290 was selected because its position corresponds to that of a highly conserved arginine that has been shown to play a role in ligand binding for members of the prostanoid receptor GPCR subfamily. The wild-type and mutant mCRTH2 receptor constructs possessed an N-terminal hemagglutinin (HA) epitope tag, which had ligand binding and signaling indistinguishable from that observed for the wild-type untagged receptor (data not shown).

The effect of each mutation on PGD_{2} binding was initially assessed by saturation isotherm radioligand binding experiments utilizing total membrane fractions collected from transiently transfected HEK293 cells. The S107A, S108A, S208A, and S290A mutants exhibited [^{3}H]PGD_{2} binding affinities (K_{d}) and expression levels (B_{max}) similar to the wild-type HA-mCRTH2 receptor (<5-fold decrease in binding affinity), whereas the L205A and H106A mutants exhibited decreased [^{3}H]PGD_{2}-specific binding (>5-fold decrease in binding affinity; Fig. 1A and TABLE TWO). For the K209A mutant, no [^{3}H]PGD_{2}-specific binding was detected. K_{d} values greater than the highest concentration of radioligand used (30 nM) are an estimation and are reported as K_{data}. For each mutant, two independent constructs were tested to confirm that the observed phenotype was not caused by introduction of unintended mutations. The loss of PGD_{2} binding by the H106A and K209A mutants was not caused by introduction of a failure in receptor expression or trafficking, as both mutants were expressed at the cell surface at levels similar to wild-type levels (Fig. 1C). Preservation of the positive charge at position 209 by a more conservative lysine substitution (K209R) resulted in detectable but reduced [^{3}H]PGD_{2} binding compared with HA-mCRTH2 (Fig. 1B). This suggests that the mCRTH2 ligand binding pocket is similar to chemoattractant but not prostanoid receptors and that His-106 and Lys-209, and to a lesser degree Leu-205, may interact with PGD_{2} within the binding pocket.

To further predict residues that may play a role in mCRTH2 ligand binding, we used a molecular model of the mCRTH2 receptor that has been described previously (22) to identify additional residues (His-38, Thr-87, Phe-110, Arg-178, Tyr-261, Ser-265, and Glu-268) in the putative ligand binding pocket region (TABLE ONE). Of particular interest was His-38 in TM I, which corresponds to a position in the mouse IP (Ser-50) and DP (Gly-22) receptors that has been implicated in ligand recognition (32). The H38A, T87A, and S265A mutants exhibited [^{3}H]PGD_{2} binding similar to HA-mCRTH2, whereas the F110A, R178A, Y261A, and E268A mutants had reduced binding (Fig. 2A, data not shown). Although the R178A and E268A mutants were expressed at the cell surface similar to HA-mCRTH2, cell surface expression levels of the F110A and Y261A mutants were greatly reduced (Fig. 2, B and C). The reduced cell surface expression is likely a result of both decreased total expression and intracellular retention, suggesting that these mutations introduced structural changes leading to misfolding. The mutant receptor bearing the more conservative Y261F substitution was expressed at the cell surface and exhibited [^{3}H]PGD_{2} binding similar to the wild-type receptor (TABLES TWO and THREE). The more conservative substitutions of Gln or Asp at position 268 did not lead to improved [^{3}H]PGD_{2} binding compared with the E268A mutant receptor.
FIGURE 2. A, membranes isolated from HEK293 cells transiently transfected with HA-mCRTH2 or mutant receptors were incubated with varying concentrations of [3H]PGD2 in the absence (total binding) or presence (nonspecific binding) of 10 μM DK-PGD2, as described under "Experimental Procedures." Specific binding was determined to be the difference between total and nonspecific binding. Each data point was determined in duplicate; these data are representative of 3–4 independent experiments utilizing two independently derived mutant receptor plasmids. The H38A (open squares), T87A (open circles), and S265A (open diamonds) exhibited [3H]PGD2 binding affinities (≈5-fold change) similar to the wild-type HA-mCRTH2 receptor (closed squares), whereas the R178A (open triangles) and E268A (×) mutants had decreased binding (≈5-fold change). B, the cell surface expression level of transiently expressed mutant receptors with decreased ligand binding (black) was determined by flow cytometry as described under "Experimental Procedures." PE fluorescence of vector-transfected cells is shown for comparison (gray), with the percentage of PE-positive cells expressing the HA-mCRTH2 or mutant receptor indicated. C, expression of the F110A and Y261A mutants transiently expressed in HEK293 cells was evaluated by cell surface ELISA and flow cytometry as described under "Experimental Procedures." For the ELISA, cells were incubated in the absence (solid bars) or presence (open bars) of Triton X-100 to detect extracellular only or intracellular plus extracellular receptors, respectively. Data are representative of six independent experiments; similar expression profiles were observed for two independently derived mutant receptor plasmids.

| Mutant | PGD<sub>2</sub> | DK-PGD<sub>2</sub> | Indomethacin | Ramatroban |
|--------|----------------|-----------------|--------------|------------|
| Wild type | 32 ± 6 | 30 ± 4 | 1900 ± 300 | 51 ± 11 |
| TM I H38A | 40 ± 1 | ND | 700 ± 200 | ND |
| TM II T87A | 14 ± 2 | ND | 5000 ± 1000 | ND |
| TM III H106A | 800 ± 300<sup>a</sup> | 140 ± 30<sup>a</sup> | 2700 ± 700<sup>a</sup> | 38 ± 13<sup>a</sup> |
| S107A | 11 ± 1 | ND | 1000 ± 100 | ND |
| S108A | 37 ± 4 | ND | 3400 ± 500 | ND |
| EC II R178A | 300 ± 100 | 160 ± 40 | 10,000 ± 5000 | 190 ± 40 |
| R178K | 250 ± 50 | 60 ± 10 | 5600 ± 600 | ND |
| R178H | 269 ± 70 | 80 ± 20 | 4000 ± 1000 | ND |
| TM V L205A | 70 ± 20 | 21 ± 5 | 8000 ± 1000 | ND |
| S208A | 76 ± 7 | ND | 7000 ± 2000 | ND |
| K209R | 900 ± 400<sup>a</sup> | 510 ± 60<sup>a</sup> | 22000 ± 8000<sup>a</sup> | 400 ± 200<sup>a</sup> |
| TM VI Y261F | 22 ± 5 | 25 ± 2 | 20,000 ± 1000 | 440 ± 40 |
| S265A | 40 ± 4 | ND | 2700 ± 600 | ND |
| E268A | 700 ± 200<sup>a</sup> | 600 ± 200<sup>a</sup> | 1800 ± 300<sup>a</sup> | 22 ± 12<sup>a</sup> |
| E268Q | 900 ± 300<sup>a</sup> | ND | 1400 ± 400<sup>a</sup> | ND |
| E268D | 1200 ± 800<sup>a</sup> | ND | 7000 ± 2000<sup>a</sup> | ND |
| TM VII S290A | 50 ± 10 | ND | 2300 ± 200 | ND |

<sup>a</sup> Ki<sub>apparent</sub>

Ki values determined for the low affinity mutant receptors are estimates due to limitations of the experimental technique; therefore, PGD<sub>2</sub> binding affinity constants (Ki) for all mutants except F110A, K209A, and Y261A (which displayed insufficient radioligand binding) were more precisely determined via competition binding experiments. Consistent with the saturation binding isotherm results, the H106A, R178A, and...
K209R, E268A, E268Q, and E268D mutants exhibited decreased affinity for PGD2 compared with the wild-type receptor (TABLE THREE). Most unexpectedly, the $K_i$ value calculated for the L205A mutant was only slightly increased and not significantly different from that of the wild-type HA-mCRTH2 receptor.

In addition to PGD2, the CRTH2 receptor binds indomethacin, a structurally unrelated arylacetic acid class NSAID. The indomethacin binding affinity of HA-mCRTH2 mutant receptors was determined by competition binding experiments. The H106A and E268A mutants, which have disrupted PGD2 binding, exhibited indomethacin binding affinities not significantly different from wild type (Fig. 3 and TABLE THREE). Both PGD2 and indomethacin binding affinities were decreased by the K209R mutation. This suggests that PGD2 and indomethacin possess distinct pharmacophores with His-106 and Glu-268 forming interactions only with PGD2, and Lys-209 participating in binding both ligands. To test this hypothesis, we evaluated the binding affinities of the PGD2 analog 13,14-dihydro-15-keto-PGD2 (DK-PGD2) and ramatroban, a CRTH2 antagonist that shares structural similarity to indomethacin (22). Similar to PGD2, the H106A, K209R, and E268A mutants exhibited decreased DK-PGD2 binding affinity compared with HA-mCRTH2 (Fig. 3 and TABLE THREE). Similarly, the H106A and E268A mutations had little effect on ramatroban binding affinity.

To investigate further the distinction between residues interacting with PGD2 versus indomethacin, we evaluated the functional characteristics of the H106A, K209A, K209R, and E268A mutants. HEK293 cell lines stably expressing each of the mutant receptors were established, and clones expressing similar levels of receptor were selected for further evaluation (supplemental Fig. 1). In response to PGD2, the H106A and E268A mutants inhibited forskolin-induced [cAMP], to a similar magnitude as the wild-type HA-mCRTH2 receptor but required ~10–fold greater concentrations (Fig. 4A and TABLE FOUR). In contrast, the H106A and E268A mutants had EC50 values for inhibition of [cAMP] by indomethacin similar to HA-mCRTH2. No effect on [cAMP], was observed at nanomolar concentrations of PGD2 for the K209A mutant; at concentrations $\geq 1\mu M$, PGD2 treatment results in an increase in [cAMP] in HEK293 cells, presumably by activation of the endogenous $\gamma$-coupled EP4 receptor (data not shown). Inhibition of [cAMP], by the K209A mutant was observed at micromolar concentrations of indomethacin, demonstrating that the receptor exists in or is capable of achieving a properly folded functional conformation. Thus the binding defect observed for the K209A mutant may be due to the loss of a key ligand-receptor interaction rather than a disruption in the folded conformation of the receptor, although we cannot rule out the theoretical possibility of rescue from a misfolded state driven by the presence of ligand. Finally, the K209R mutant had increased EC50 values for inhibition of [cAMP], in response to both PGD2 and indomethacin.

**Table Four**

|       | EC50 PGD2 (nM) | EC50 Indomethacin (nM) |
|-------|----------------|------------------------|
| HA-mCRTH2 | 0.7 ± 0.3       | 2.0 ± 0.7               |
| H106A   | 6.6 ± 0.8       | 2.1 ± 0.7               |
| K209A   | $>1000^a$       | $>1000^a$               |
| K209R   | 5 ± 1$^a$       | 6.4 ± 0.6$^a$           |
| E268A   | 8 ± 2$^a$       | 3.6 ± 0.5               |

$^a$ p < 0.05 compared with HA-mCRTH2, unpaired t test (Instat).
We have reported previously that the mCRTH2 receptor mediates chemotaxis of ER293/mCRTH2 cells in response to PGD$_2$ and indomethacin (24). Consistent with the observed ligand binding and signaling properties, HEK293 cells stably expressing H106A and E268A mutants displayed impaired chemotaxis toward PGD$_2$ but not indomethacin compared with cells expressing the wild-type HA-mCRTH2 receptor (Fig. 4B). Minimal response to PGD$_2$ or indomethacin was observed at concentrations tested for the K209A mutant, and the K209R mutant had reduced chemotaxis in response to both PGD$_2$ and indomethacin.

In contrast to the H106A and E268A mutant receptors, the Y261F mutant displayed reduced indomethacin but not PGD$_2$ binding affinity (Fig. 5A; TABLE THREE), suggesting that the Tyr-261 hydroxyl interacts with indomethacin but not PGD$_2$. A decrease was also observed in the binding affinity for ramatroban but not DK-PGD$_2$. Consistent with the differentiation between prostanoid and arylacetic acid ligand binding, HEK293 cells expressing the Y261F mutant receptor had a reduced chemotactic response to indomethacin compared with PGD$_2$ (Fig. 5B).

Of those residues investigated, Arg-178 is unique in that it lies in an extracellular loop region. The R178A mutant displayed a modest reduction in binding affinity for both PGD$_2$ and indomethacin (TABLE THREE). Although the side chain positive charge was preserved in the R178K and R178H mutants, a similar decrease in PGD$_2$ and indomethacin binding was observed, suggesting that geometric constraints may play a significant role in the interaction between Arg-178 and ligands within the binding pocket.

Taken together, these results suggest that His-106, Arg-178, Lys-209, and Glu-268 play a role in binding PGD$_2$. An overlapping set of residues Arg-178, Tyr-261, and Lys-209 appears to be critical for binding indomethacin. Ligand docking studies with PGD$_2$ and indomethacin suggest that Lys-209 may interact with the carboxylate of each ligand (Fig. 6). For PGD$_2$, this would position the cyclopentyl ring between His-106 of TM III and Glu-268 of TM VI, with the potential for formation of a hydrogen bond interaction between the 9-hydroxy of the cyclopentyl ring and Glu-268. To test whether His-106 or Glu-268 interacts with cyclopentyl ring substituents and plays a role determining prostanoid ligand binding selectivity, we examined the ability of PGE$_x$, a low affinity CRTH2 ligand (24, 33), to bind to the H106A and E268A mutants. Although the H106A exhibited a relative decrease in PGE$_x$ binding affinity similar to that observed for PGD$_2$ (data not shown), the E268A mutant had a 5-fold increase in PGE$_x$ binding affinity compared with the wild-type receptor ($K_I = 0.8 \pm 0.2$ and $4.0 \pm 0.3$ µM, respectively; Fig. 7). This suggests that Glu-268 plays a key role in determining prostanoid ligand binding selectivity.

**DISCUSSION**

PGD$_2$ is the major prostanoid species produced by allergen-challenged mast cells and binds with high affinity to two distinct GPCRs, the DP and CRTH2 receptors. The studies presented here demonstrate that the CRTH2 receptor more closely resembles classic chemoattractant receptors compared with the DP and other prostanoid receptors in the determinants of prostanoid ligand binding. The DP receptor is related to other members of the prostanoid GPCR subfamily such as the prostaclin/IP receptor and PGE$_x$, EP$_2$ receptor. The CRTH2 receptor shares greatest sequence identity (~30%) with classic chemoattractant receptors such as FPR and C5aR (10), which is approximately the same level of homology as that shared between DP and the prostanoid receptor subfamily members. Although the DP and CRTH2 receptors share little sequence homology, the presence of specific motifs shared by virtually all rhodopsin-like GPCRs (34) permit alignment of the transmembrane regions. Sequence comparison suggests that the CRTH2 receptor differs in the manner in which it binds ligand compared with the other prostanoid receptors. For instance, studies utilizing DP/IP receptor chimeras and mutants have suggested that high affinity binding of PGD$_2$ by the mouse DP receptor requires Lys-75 in TM II (32), and His-81 at the equivalent position in the rat FP receptor has been suggested to play a role in binding PGF$_{2\alpha}$ (35). For the CRTH2 receptor, the corresponding residue, alanine (mouse and rat) or serine (human), is unlikely to participate in ligand binding in a similar manner. Also, a conserved LAXARXA(S/T)XN motif in TM VII has been shown to be important for ligand binding by several prostanoid receptors (reviewed in Ref. 36), and we have shown previously (23) that the conserved arginine in this motif is critical for PGE$_x$ binding to the rabbit EP3 receptor. In the present study, we observed little effect on PGD$_2$ binding when the corresponding mCRTH2 residue Ser-290 was mutated to alanine. However, replacement of Lys-209 in TM V with Ala dramatically reduced PGD$_2$ binding. Lys-209 corresponds to Arg-205 in FPR and Arg-206 in C5aR, which have been shown to be involved in ligand binding (27, 29, 31).

Although the CRTH2 receptor shares some similarity with peptide chemoattractant receptors, it also possesses key differences. For instance, Thr-103 and Ser-114 of the human FPR and C5aR, respectively, have been implicated in ligand binding, although our results indicate that the corresponding Ser-107 of the CRTH2 residues does not.

**FIGURE 5.** The Y261F mutant receptor has impaired binding and functional response to indomethacin but not PGD$_2$. A, membranes isolated from HEK293 cells transiently transfected with HA-mCRTH2 (closed symbols) or the Y261F mutant receptor (open symbols) were incubated with 3 nM [3H]PGD$_2$ in the presence of varying concentrations of PGD$_2$, DK-PGD$_2$, indomethacin, or ramatroban, and the binding was determined as described under “Experimental Procedures.” Binding curves were generated using a one-site competition model (Prism). Each data point was determined in duplicate; these data are representative of 3–5 independent experiments (HA-mCRTH2 data is duplicated from Fig. 4). B, migration of HEK293 cells stably expressing HA-mCRTH2 (solid bars) or the Y261F mutant receptor (open bars) toward PGD$_2$, or indomethacin was assessed as described under “Experimental Procedures.” Data are expressed as a percentage of the maximal response to PGD$_2$ (100%) for each receptor type and are shown as the mean ± S.E. of 3–4 independent experiments.

Conversely, the residues corresponding to Glu-268 (Ala-264 and Met-265 in FPR and C5aR, respectively) have not been shown to be involved in ligand binding. Thus the present results validate the initial criteria used for predicting ligand binding, which were based on the assumption that CRTH2 possesses both similarity with the peptide chemoattractant residues but also differences responsible for the different ligand binding profiles. The chemoattractant GPCR subfamily also includes receptors that bind nonprostanoid eicosanoid ligands such as leukotriene B4 (BLT1 and BLT2) and lipoxin A4 (ALX/FPRL1). It is possible that the ligand binding pocket of these receptors shares similar features with the CRTH2 receptor because they bind ligands of similar chemical classes. However, little is known about how these receptors bind their ligands. Most interestingly, several residues that we identified to be important for binding are conserved in these receptors. For instance, the BLT1 and ALX receptors possess a histidine residue at the position corresponding to His-106 (His-96 and His-102 in the mouse BLT1 and ALX receptors, respectively). The BLT1 receptor also possesses a glutamic acid residue (Glu-245) at the position corresponding to Glu-268. Lys-209, which was originally identified because it corresponds to Arg-205 and Arg-206 in FPR and C5aR, also corresponds to charged residues in the BLT1, BLT2, and ALX receptors (Glu-186, Glu-185, and Arg-201, respectively).

Neutralization of the negatively charged carboxylate of PGD₂ has been demonstrated to negatively impact binding (16, 33). We showed recently that neutralization of the carboxylate of the arylacetic acid class NSAID and the CRTH2 agonist indomethacin also reduced binding, and we speculated that this moiety may interact with Lys-209 (22). The data presented here support this hypothesis and suggest that a similar interaction between Lys-209 and the carboxylate of PGD₂ may occur. This would further differentiate the CRTH2 receptor from other prostanoid receptors, for which it has been suggested that TM VI and VII are responsible for recognition of the prostaglandin α-chain and terminal carboxylate by the DP and IP receptors (37) and that a conserved arginine in TM VII in the TP (38) and EP3 receptors (23, 39) interacts with the prostaglandin carboxylate. Interaction between the carboxylate of PGD₂ and Lys-209 of CRTH2 would position PGD₂ in the ligand binding pocket with an opposite orientation compared with what is proposed for the other prostanoid receptors.

Interactions between the receptor and cyclopentyl ring substituents of prostaglandin ligands are thought to determine the ligand selectivity of each receptor. For the DP receptor, residues in TM I and II have been demonstrated to confer high affinity and selectivity of PGD₂ binding, suggesting that the cyclopentyl ring of PGD₂ is positioned next to TM I and II (32). In contrast, we propose that for the CRTH2 receptor, the cyclopentyl ring of PGD₂ lies between TM III and VI. First, interaction between the prostaglandin carboxylate and Lys-209 of TM V, which is positioned ~25 Å from TM I, would not allow the cyclopentyl ring to be positioned near TM I and II. Second, replacement of His-38 and Thr-87, which contribute charged and polar side chains to the putative ligand binding pocket in the vicinity of TM I and II, had no effect on ligand binding. It is worth noting that His-38 corresponds to Ser-50 and Gly-22 in the mouse IP and DP receptors, respectively, which have been implicated in ligand recognition (32). Third, mutation of Glu-268 of TM VI resulted in decreased PGD₂ binding, yet the E268A mutant exhibited increased PGE₂ binding affinity compared with the wild-type receptor, suggesting that this residue may play a role in recognition of the cyclopentyl ring. Our molecular modeling studies suggest that Glu-268 is in proximity to form a hydrogen bond with the 9-hydroxyl of PGD₂ when the carboxylate is positioned to interact with Lys-209.

FIGURE 6. Model of mCRTH2 depicting residues mutated in this study. Side (A and C) and extracellular (B and D) views of PGD₂ (A and B) or indomethacin (C and D) docked into the putative ligand-binding site. Residues for which alanine substitution affected ligand binding (≥5-fold decrease in binding affinity) are shown in red; residues for which alanine substitution had a minimal effect of ligand binding (<5-fold decrease in affinity) are shown in green, and residues for which replacement resulted in decreased expression are shown in yellow. The carboxylate of both PGD₂ and indomethacin is positioned to form a hydrogen bond with Lys-209. The cyclopentyl ring of PGD₂ lies between TM III and VI. Note the different pattern of ligand-binding residues for PGD₂ compared with indomethacin.
Bolsters isolated from HEK293 cells transiently transfected with HA-mCRTH2 (open symbols) or the E268A mutant receptor (open symbols) were incubated with 3 nM [3H]PGD$_2$ in the presence of varying concentrations of PGD$_2$ (squares) or PGE$_2$ (circles), and the binding was determined as described under “Experimental Procedures.” Binding curves were generated using a one-site competition model (Prism). Each data point was determined in duplicate; these data are representative of three independent experiments (PGD$_2$). The numbering of the cyclopentyl ring carbon atoms is indicated; hydrogen atoms are not depicted in this view.

Because PGD$_2$ and indomethacin lack obvious structural similarity, it is not surprising that they interact with different residues within the CRTH2 ligand binding pocket. These data suggest that His-106, Lys-209, and Glu-268 play a role in binding PGD$_2$ and DK-PGD$_2$, whereas Lys-209 and Tyr-261 but not His-106 or Glu-268 are involved in binding indomethacin and ramatroban. This pattern was also observed in the functional responses of these mutants to PGD$_2$ and indomethacin.

The effects of the mutations on ligand binding and receptor function might be due to nonspecific changes in receptor conformation rather than disruption of specific ligand-receptor interactions. However, the fact that indomethacin binding affinity of the H106A and E268A mutants was similar to the wild-type receptor indicates that the decrease in PGD$_2$ binding affinity is not because of gross structural changes. Furthermore, PGE$_2$ binding was modestly improved for the E268A mutant. Conversely, the Y261F mutant had decreased indomethacin binding affinity despite binding PGD$_2$, similar to the wild-type receptor. In addition, signaling studies demonstrated that the H106A, K209R, and E268A receptors were maximally functional, and activity of the K209A mutant was detected at high ligand concentrations. These studies cannot conclusively discriminate between the loss of a direct receptor-ligand interaction and local conformational changes in a nearby residue that interacts with ligand, however.

The mCRTH2 receptor model was generated via a combination of homology modeling based on a recently described 4-hydroxy cyclopropyl ring substituent of PGD$_2$ (blue) docked in the putative ligand binding pocket is in proximity to interact with Glu-268 (red). The numbering of the cyclopentyl ring carbon atoms is indicated; hydrogen atoms are not depicted in this view.

FIGURE 7. Glu-268 may play a role in prostaglandin ligand binding selectivity. A membranes isolated from HEK293 cells transiently transfected with HA-mCRTH2 (closed symbols) or the E268A mutant receptor (open symbols) were incubated with 3 nM [3H]PGD$_2$ in the presence of varying concentrations of PGD$_2$ (squares) or PGE$_2$ (circles), and the binding was determined as described under “Experimental Procedures.” Binding curves were generated using a one-site competition model (Prism). Each data point was determined in duplicate; these data are representative of three independent experiments (PGD$_2$). The numbering of the cyclopentyl ring carbon atoms is indicated; hydrogen atoms are not depicted in this view.

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