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Autoimmune Disease-Associated Histamine Receptor H₁ Alleles Exhibit Differential Protein Trafficking and Cell Surface Expression¹

Rajkumar Noubade,* Naresha Saligrama,* Karen Spach,* Roxana del Rio,* Elizabeth P. Blankenhorn,† Theodoros Kantidakis,‡ Graeme Milligan,‡ Mercedes Rincon,* and Cory Teuscher²*§

Structural polymorphisms (L263P, M313V, and S331P) in the third intracellular loop of the murine histamine receptor H₁ (H₁R) are candidates for Bphs, a shared autoimmune disease locus in experimental allergic encephalomyelitis and experimental allergic orchitis. The P-V-P haplotype is associated with increased disease susceptibility (H₁R⁵) whereas the L-M-S haplotype is associated with less severe disease (H₁R⁶). In this study, we show that selective re-expression of the H₁R⁵ allele in T cells fully complements experimental allergic encephalomyelitis susceptibility and the production of disease-associated cytokines while selective re-expression of the H₁R⁶ allele does not. Mechanistically, we show that the two H₁R alleles exhibit differential cell surface expression and altered intracellular trafficking, with the H₁R⁶ allele being retained within the endoplasmic reticulum. Moreover, we show that all three residues (L-M-S) comprising the H₁R⁶ haplotype are required for altered expression. These data are the first to demonstrate that structural polymorphisms influence cell surface expression of a G protein-coupled receptor in T cells regulates immune functions and autoimmune disease susceptibility. The Journal of Immunology, 2008, 180: 7471–7479.

Multiple sclerosis (MS) is the major demyelinating disease of the CNS in humans, affecting >2.5 million people worldwide (1). Both environmental and genetic factors contribute to the immunopathologic etiology of the disease. A genetic component in disease susceptibility is supported by the 20–30% concordance rate among monozygotic twins and 3–5% for dizygotic twins. Compared with the general population, MS is 20–40 times more common in first-degree relatives and there is no excess risk in adopted relatives of patients with MS (2). Evidence of an environmental etiology in MS comes primarily from migration studies and geographic distribution data. Migration studies indicate that individuals moving from high-risk areas before puberty tend to adopt the lower risk of the native population and vice versa (3). Thus, susceptibility to MS is likely the result of environmental triggers acting on a susceptible genetic background at the population level. Experimental allergic encephalomyelitis (EAE), the primary animal model of MS, is also a genetically determined inflammatory disease of the CNS (4). EAE can be actively induced in genetically susceptible animals by immunization with either whole spinal cord homogenate or encephalitogenic proteins/peptides and adjuvants (5). EAE, like MS, is a complex polygenic disease (6), with multiple genes exerting a modest effect, thus making it difficult to study the contribution of individual loci to overall disease pathogenesis. However, reduction of complex disease states into intermediate or subphenotypes that are under the control of a single locus has the potential to facilitate mechanistic studies and gene identification (6). One such phenotype associated with EAE is Bordetella pertussis toxin-induced histamine sensitization, which is controlled by the single autosomal dominant locus known as Bphs (7). Previously, we identified Hrh1/H₁R as the gene underlying Bphs (7) and as a shared autoimmune disease susceptibility gene in EAE (8) and experimental allergic orchitis (9). H₁R is a seven-transmembrane spanning, G protein-coupled receptor (GPCR). Generally, ligand of H₁R with histamine is believed to couple to second messenger signaling pathways via the activation of the heterotrimeric Gα₁₁ family of G proteins and leads to a variety of signaling cascades depending on the cell type involved (10).

Compared with wild-type (WT) mice, H₁R-deficient (H₁RKO) mice exhibit significantly reduced EAE susceptibility (7). As a disease susceptibility gene, Hrh1/H₁R can exert its effect in multiple cell types involved in the disease process including endothelial cells, APCs, and T cells. Moreover, H₁R may function at critical checkpoints during both the induction and effector phases of the disease. In this regard, we recently demonstrated that selective re-expression of the H₁R⁵ allele in T cells is sufficient to complement EAE in H₁RKO mice and that H₁R signals are important during priming of naïve T cells rather than during the effector phase of the disease (11).
HEK293T cells were plated at 1.25 × 10^6 cells/plate and cultured in DMEM-F12 containing 10% FBS. When the cells were ~50–80% confluent, they were transfected with 5 μg of pEGZ-HA-H1R,S, pEGZ-HA-H1R,A, or the empty pEGZ vector using the calcium phosphate method. After 16–24 hr, cells were scrapped off the plate by rigorous pipetting with 1% calf serum in PBS and stained with anti-HA mAb conjugated to PE (Miltenyi Biotec) according to the manufacturer’s guidelines. Cells were analyzed by flow cytometry using a FACSAria instrument (BD Pharmingen) and the data were further analyzed using FlowJo flow cytometry analysis software (Tree Star).

Confocal microscopy

HEK293T cells were transfected with pEGZ-HA-H1R,S, pEGZ-HA-H1R,A, or empty pEGZ control vector (5 μg of total DNA) using the calcium phosphate method. Cells were fixed, permeabilized, and stained using an anti-HA mAb (Cell Signaling Technologies) followed by an incubation with Alexa-568 anti-mouse Ab (Molecular Probes), TOPRO-3 nuclear stain (Molecular Probes) was used as a nuclear marker. For nonpermeabilized cells, the transfected HEK293T cells were stained with the anti-HA mAb and were then fixed. Cells were examined by confocal microscopy using the Zeiss LSM 510 META Confocal Laser Scanning Imaging System (Carl Zeiss Microimaging).

Cell lysates and Western blotting

Whole-cell lysates were prepared from HEK293T cells transfected with various pEGZ constructs in Triton lysis buffer and were then separated via SDS-PAGE and transferred to nitrocellulose membranes as described previously (11). Anti-HA mAb (Abcam) was used as primary Ab. Anti-actin (Santa Cruz Biotechnology) was used as a loading control.

Site-directed mutagenesis

pEGZ-HA-H1R,S was used as template to generate single H1R,S mutants with each of the polymorphic residues replaced with the corresponding HA-H1R,S alleles using the Quickchange (Stratagene) site-directed mutagenesis kit, according to the manufacturer’s guidelines. The forward primers used for the mutagenesis were: for P263L, 5′-GACACAGCCGCCTGAGGGAGATGCCAGG-3′; for P330S, 5′-GACACAGCCGCCTGAGGGAGATGCCAGG-3′; for P330L, 5′-GACACAGCCGCCTGAGGGAGATGCCAGG-3′. Reverse primers were the complementary sequences of these primers. The altered nucleotides are shown in bold and underlined. The mutants were sequence confirmed and were used as template for the generation of different combinations of double H1R,S mutants.

Conventional and quantitative real-time RT-PCR

Total RNA was extracted from CD4 T cells using RNeasy RNA isolation reagent (Qiagen) as recommended by the manufacturer. cDNA generated from 1 μg of total RNA was used in conventional and quantitative real-time RT-PCR as described earlier (11).
MOG35–55-CFA induction protocol, mice are injected s.c. with an emulsion of 100 μg of MOG35–55 and an equal volume of CFA containing 200 μg of Mycobacterium tuberculosis H37RA (Difco Laboratories) in the posterior right and left flank; 1 wk later, all mice were similarly injected at two sites on the right and left flank anterior of the initial injection sites. Animals immunized using the MOG 35–55-CFA plus PTX single-inoculation protocol received an emulsion of 200 μg of MOG35–55 and equal volume of CFA containing 200 μg of M. tuberculosis H37RA by s.c. injections distributed equally in the posterior right and left flank and scruff of the neck. Immediately thereafter, each animal received 200 ng of PTX (List Biological Laboratories) by i.v. injection. Mice were scored daily starting at day 5. Regression analysis revealed that the disease course elicited fits a Sigmoidal curve and that the clinical disease course of the animals was significantly different among the strains. The clinical disease courses of WT and H1RKO-TgR5 mice were both significantly more severe than those of H1RKO-TgR6 and H1RKO mice (p < 0.0001 for all comparisons). D. WT (n = 18), H1RKO (n = 33), H1RKO-TgR5 (n = 23), and H1RKO-TgR6 (n = 14) mice were immunized with 2× MOG35–55-CFA. EAE severity was significantly different among the strains. The clinical disease courses of WT and H1RKO-TgR5 mice were both significantly more severe than those of H1RKO-TgR6 and H1RKO mice (p < 0.0001 for all comparisons).

Statistical analysis

Statistical analyses, as detailed in the figure legends, were performed using GraphPad Prism 4 software (GraphPad Software). A p value of 0.05 or less was considered significant.

Results

Expression of H1R<sup>R</sup> does not complement EAE in H1R-deficient mice

Using transgenic complementation, we recently showed that expression of the H1R<sup>R</sup> allele only in T cells of H1RKO mice was sufficient to restore EAE severity to WT levels in these mice (11). To understand whether the H1R<sup>R</sup> allele would also complement EAE in H1RKO mice, we generated transgenic mice expressing the N-terminal HA-tagged H1R<sup>R</sup> allele under the control of the distal lck promoter, which drives expression in peripheral T cells (14). The transgenic founders were generated directly on the C57BL/6J background and were crossed to H1RKO mice to obtain H1RKO-TgR mice expressing the H1R<sup>R</sup> allele selectively in T cells. The expression of the transgene in CD4 T cells was assessed by RT-PCR using transgene-specific primers (Fig. 1A) and by real-time RT-PCR using primers that recognize H1R (Fig. 1B). The two established lines of H1R<sup>R</sup> (H1RKO-TgR1 and H1RKO-TgR2) expressed the transgene mRNA at levels comparable to one of the H1R<sup>S</sup> allele transgenic mice (H1RKO-TgS) that we reported previously (11).

We then examined the susceptibility of these transgenic mice to MOG35–55 induced EAE. We used two protocols to induce disease: MOG35–55-CFA plus PTX (Fig. 1C) and 2× MOG35–55-CFA (Fig. 1D). Regression analysis revealed that the clinical disease courses elicited by both induction protocols fit a Sigmoidal curve and that the clinical course of disease in two independent lines of H1RKO-TgR mice was not different from that in H1RKO mice. However, as reported previously (11), the clinical course of EAE in H1RKO-TgR5 mice was significantly more severe than that of H1RKO mice.
and was equivalent to the disease course observed in WT mice. These results indicate that, unlike the $H_{R}R_{k}^{c}$ allele, expression of the $H_{R}R_{k}^{a}$ allele by H1RKO T cells does not complement EAE susceptibility. An analysis of EAE-associated clinical quantitative trait variables from the two transgenic cohorts revealed that the mean day of onset (DO), cumulative disease score (CDS), overall severity index (SI), and the peak score (PS) were significantly different among the strains immunized with either MOG$_{35-55}$-CFA plus PTX or 2×MOG$_{35-55}$-CFA (Table I). Post hoc multiple comparisons of each trait variable revealed that H1RKO-Tg$^{R}$ mice were equivalent to WT mice while H1RKO-Tg$^{S}$ mice were equivalent to H1RKO mice. Furthermore, for each trait, H1RKO-Tg$^{S}$ and WT mice were significantly greater than H1RKO-Tg$^{R}$ and H1RKO mice.

We next analyzed the ex vivo MOG$_{35-55}$-specific proliferative response of spleen and draining lymph node (DLN) cells from mice immunized with 2×MOG$_{35-55}$-CFA. Significant differences in proliferative responses were not detected among WT, H1RKO, H1RKO-Tg$^{S}$, and H1RKO-Tg$^{R}$ mice (data not shown). Because MOG$_{35-55}$-stimulated splenocytes from immunized-H1RKO mice exhibit an immune deviation from Th1 to Th2 response in ex vivo recall assays (7), we analyzed cytokine production by MOG$_{35-55}$-stimulated spleen and DLN cells from mice immunized with both EAE-induction protocols. With the classical MOG$_{35-55}$-CFA plus PTX protocol, as we observed previously (11), Ag-stimulated spleen and DLN cells from H1RKO-Tg$^{S}$ mice produced significantly greater amounts of IFN-$\gamma$ compared with H1RKO mice and at levels comparable to WT mice (Fig. 2A). In contrast, the levels of IFN-$\gamma$ produced by Ag-stimulated spleen and DLN cells from the two lines of H1RKO-Tg$^{R}$ mice were equivalent to those produced by H1RKO mice. Similarly, Ag-stimulated spleen and DLN cells from H1RKO-Tg$^{S}$ mice produced IL-4 at levels comparable to WT mice while those from H1RKO-Tg$^{R}$ mice were similar to H1RKO mice (Fig. 2B). Similar results for IFN-$\gamma$ (Fig. 2D) and IL-4 (Fig. 2E) were observed for 2×MOG$_{35-55}$-CFA-immunized mice.

**FIGURE 2.** Transgenic expression of $H_{R}R_{k}^{R}$ in H1RKO T cells fails to complement cytokine production by H1RKO mice. A–C, Spleen and DLN cells were isolated from MOG$_{35-55}$-CFA plus PTX-immunized WT, H1RKO, H1RKO-Tg$^{S}$, and H1RKO-Tg$^{R}$ mice 10 days postimmunization and stimulated with 50 μg/ml MOG$_{35-55}$ for 72 h ($n=4–8$ mice/group). Supernatants were collected and analyzed for the production of IFN-$\gamma$ (A), IL-4 (B), and IL-17 (C). Significance of differences in cytokine production were assessed using the nonparametric Kruskal-Wallis test followed by Dunnett’s post hoc multiple comparisons ($H=25.73; p<0.0001$ for IFN-$\gamma$, $H=31.34; p<0.0001$ for IL-4, $H=5.041; p>0.5$ for IL-17, *$p<0.05$; **$p<0.01$; and ***$p<0.001$). D–F, Spleen and DLN cells from 2×MOG$_{35-55}$-CFA immunized mice were collected on day 10 postimmunization and were activated with 50 μg/ml MOG$_{35-55}$ for 72 h, supernatants were collected and analyzed for IFN-$\gamma$ (D), IL-4 (E), and IL-17 (F) by ELISA in triplicate. Significance of differences in cytokine production were assessed using the nonparametric Kruskal-Wallis test followed by Dunnett’s post hoc multiple comparisons ($H=52.23; p<0.0001$ for IFN-$\gamma$, $H=23.88; p<0.0001$ for IL-4, $H=35.22; p<0.0001$ for IL-17, *$p<0.05$; **$p<0.01$; and ***$p<0.001$). Data are presented as the mean ± SEM and are representative of two independent experiments.
Because IL-17 is considered to be an important effector cytokine in EAE (19), we examined IL-17 production by spleen and DLN cells following ex vivo stimulation with MOG35–55. IL-17 production by WT, H1RKO, H1RKO-TgS, and H1RKO-TgR mice immunized with MOG35–55-CFA and PTX was not significantly different among strains (Fig. 2C). However, compared with WT mice, H1RKO mice produced significantly less IL-17, indicating that H1R signaling regulates IL-17 production by T cells. Moreover, production of IL-17 by H1RKO-TgR8 mice was not significantly different from WT mice and IL-17 production by H1RKO-TgR8 mice was not significantly different from H1RKO mice (Fig. 2F). Taken together, like EAE, H1R expression in H1RKO T cells does not complement cytokine production by these cells.

**H1R alleles activate Goq and Gα11 equally well in vitro**

To understand the mechanism by which the polymorphic residues of the H1R<sup>R</sup> and H1R<sup>R</sup> alleles influence H1R function, we examined the predicted structural location for the three residues within H1R. The three polymorphic residues reside within the third intracytoplasmic loop of H1R (Fig. 3A), which is the region frequently associated with recruitment and activation of downstream G proteins (12). We, therefore, examined whether the polymorphic residues distinguishing the H1R<sup>R</sup> and H1R<sup>R</sup> alleles might result in significant alterations in G protein activation. Because H1R is normally coupled to G<sub>0</sub> and/or G<sub>11</sub> proteins, we generated fusion proteins of the two H1R alleles with both G<sub>0</sub> and G<sub>11</sub> by linking in-frame the N terminus of G<sub>0/11</sub> with the C-terminal tail of H1R<sup>R</sup> or H1R<sup>R</sup>. HEK293 cells were transfected with the H1R<sup>R</sup>-Go<sub>q</sub> or H1R<sup>R</sup>-Go<sub>q</sub>/11 fusion proteins, lysed and membrane fractions prepared from these cells. These were used initially to measure the levels of expression of each construct via the specific binding of the H1R antagonist [3H]mepyramine. There were no differences in the levels of specific binding of [3H]mepyramine between the various constructs, indicating that the polymorphisms did not alter total protein expression. Also, the binding affinity of [3H]mepyramine was not different between the two alleles (Fig. 3B). To study their differential capacity to activate Go<sub>q</sub> and Gα<sub>11</sub>, membrane amounts containing exactly the same number of copies of each construct were used in [35S]GTPγS-binding assays. A maximally effective concentration of histamine stimulated binding of [35S]GTPγS equally to Go<sub>q</sub> or Gα<sub>11</sub> when each G protein was linked to either the H1R<sup>R</sup> or H1R<sup>R</sup> variants (Fig. 3, C and D). The dose-response curves to histamine indicated that the potency of histamine is equivalent for each receptor variant (data not shown). These data indicate that the H1R<sup>R</sup> and H1R<sup>R</sup> alleles can activate these G proteins equally well and that the phenotypic difference associated with the H1R alleles is not inherently a function of differential capability to activate Go<sub>q</sub> or Gα<sub>11</sub>.

**H1R alleles are differentially expressed on the cell surface**

Specific mutations in the signaling domain of several GPCRs (e.g., vasopressin V2 receptor, rhodopsin) can interfere with their cell surface expression and are associated with disease (20). To determine whether the polymorphisms in H1R influence cell surface
FIGURE 4. 

**HₐRᵦ** and **Hᵦ⁺** alleles are differentially expressed on the cell surface. A. HEK293T cells were transfected with empty pEGZ, pEGZ-HA-Hᵦᵦ, or pEGZ-HA-Hᵦ⁺ plasmids. Cells were collected 16–24 h later without trypsinization, stained with anti-HA mAb, and analyzed by flow cytometry. The thin line represents cells transfected with empty pEGZ whereas the thick line represents cells transfected with HA-Hᵦᵦ and the filled area represents cells transfected with HA-Hᵦ⁺. B and C. HEK293T cells were analyzed as in A and the percentage (B) and the MFI of anti-HA on Hᵦᵦ-positive cells (C) were determined. D. HEK293T cells transfected with HA-Hᵦᵦ or HA-Hᵦ⁺ plasmids and 24 h later cells were stained with anti-HA mAb (red) without permeabilization. Cells were visualized by confocal microscopy. GFP (green) is shown as a marker of transfected cells. Representative data from three independent experiments are shown.

expression of the receptor, HA-Hᵦᵦ or HA-Hᵦ⁺ expression vectors were used to transfected HEK293T cells. The expression of these receptors at the cell surface was then examined by flow cytometric analysis using an anti-HA mAb. HA-Hᵦᵦ was expressed at higher levels than HA-Hᵦ⁺ (Fig. 4A). The number of Hᵦᵦ-positive cells (Fig. 4B) and the mean fluorescence intensity of Hᵦᵦ were considerably higher than those of Hᵦ⁺ (Fig. 4C), indicating that the two Hᵦ⁺ alleles are differentially expressed on the cell surface. We observed similar results when the Hᵦᵦ and Hᵦ⁺ constructs were transfected into 721.221 B cells (data not shown).

In parallel, we examined the cell surface expression of Hᵦᵦ and Hᵦ⁺ by confocal microscopy using anti-HA mAb in cells stained before permeabilization. The results confirmed higher expression of Hᵦᵦ on the surface than Hᵦ⁺ (Fig. 4D). However, Western blot analysis of Hᵦᵦ and Hᵦ⁺ expression in lysates of transfected HEK293T cells showed no difference in the amount of total protein present (Fig. 4E). Taken together, these data indicate that the polymorphic residues associated with the Hᵦ⁺ and Hᵦ⁺ haplotypes result in differential translocation of the receptor to the cell surface.

**Hᵦ⁺** is retained in the ER

The Western blot results described above (Fig. 4E) suggest that the Hᵦᵦ and Hᵦ⁺ alleles are expressed at similar levels but that the Hᵦ⁺ allele is largely retained in intracellular compartments instead of being trafficked to the cell surface. To investigate this possibility, HEK293T cells were transfected with HA-Hᵦᵦ or HA-Hᵦ⁺ constructs. After 24 h, cells were fixed, permeabilized, stained with anti-HA mAb, and observed by confocal microscopy.

A predominantly plasma membrane-staining pattern was observed for the Hᵦᵦ allele (Fig. 5A). In contrast, a large fraction of the Hᵦ⁺ allele appeared to localize intracellularly (Fig. 5A, right panel), indicating that Hᵦ⁺ is retained in the intracellular compartments and fails to traffic efficiently to the cell surface. The network-like intracellular distribution of Hᵦ⁺ throughout the cell (Fig. 5A, right panel) resembled that of ER. Therefore, to determine whether the Hᵦ⁺ allele is retained in this compartment, we transiently cotransfected HEK293T cells with Hᵦᵦ or Hᵦ⁺ constructs and a plasmid expressing the dsRed fluorescent protein that targets the ER. Colocalization of the two proteins was examined by

FIGURE 5. Hᵦ⁺ is retained in the ER. A. HEK293T cells were transfected with HA-Hᵦᵦ or HA-Hᵦ⁺ plasmids. Twenty-four hours later, cells were fixed, permeabilized, stained with anti-HA mAb (red) and TOPRO-3 nuclear stain (green), and visualized by confocal microscopy. B. HEK293T cells were cotransfected with pdsRed plasmid that expresses ER-targeted fluorescent dsRed protein (red) and HA-Hᵦ⁺ or HA-Hᵦ⁺ constructs. Twenty-four hours later cells were fixed, permeabilized, stained with anti-HA mAb (green), and the colocalization of HA-Hᵦ⁺ with dsRed was visualized by confocal microscopy. Yellow color represents the colocalization of red and green colors. C. Quantification of HA-Hᵦ⁺ colocalization with dsRed. Using Zeiss LSM 510 META Confocal imaging software, the number of pixels expressing both colors were determined in a number of cells (n = 26) and the data are presented as the average of number of pixels that coexpress dsRed and HA-Hᵦ⁺. Error bars represent SEM. Data were analyzed using the nonparametric Mann-Whitney U test (U = 1330; ****, p < 0.00001).
confocal microscopy following staining the cells for HA-H1R. The majority of H1RR was again expressed intracellularly and colocalized with the dsRed protein, whereas the thick line represents cells transfected with HA-H1R3, and the filled area represents cells transfected with HA-H1R Rb. HEK293T cells were analyzed as in A and the mean fluorescence intensity of anti-HA on H1Rb-positive cells was determined. The data presented are the average of triplicate transfections. C, HEK293T cells were cotransfected with dsRed plasmid that express ER-targeted dsRed protein (red) and HA-H1R, mutants of HA-H1R3 or HA-H1Rb. Twenty-four hours later, cells were fixed, permeabilized, stained with anti-HA mAb (green), and the colocalization of HA-H1R with dsRed (red) was visualized by confocal microscopy. Yellow color represents the colocalization of red and green colors. D, Quantification of HA-H1R colocalization with dsRed protein. Using Zeiss LSM 510 META Confocal imaging software, the number of pixels expressing both the colors was determined in a number of cells (n = 16) and the data are presented as the average number of pixels that coexpress dsRed and HA-H1R. Error bars indicate SEM.

**FIGURE 6.** ER retention of the H1Rb allele requires the L-M-S haplotype. A. HEK293T cells were transfected with empty control, single HA-H1R3 mutants, or HA-H1Rb plasmids. Cells were collected 16–24 h later without trypsinization, stained with anti-HA mAb, and analyzed by flow cytometry. Cells transfected with HA-H1R3 are shown as positive controls in the far right panel. The thin line represents cells transfected with empty pEGZ, whereas the thick line represents cells transfected with HA-H1R3, and the filled area represents cells transfected with HA-H1Rb. B. HEK293T cells were analyzed as in A and the mean fluorescence intensity of anti-HA on H1Rb-positive cells was determined. The data presented are the average of triplicate transfections. C. HEK293T cells were cotransfected with dsRed plasmid that express ER-targeted dsRed protein (red) and HA-H1R, mutants of HA-H1R3 or HA-H1Rb. Twenty-four hours later, cells were fixed, permeabilized, stained with anti-HA mAb (green), and the colocalization of HA-H1R with dsRed (red) was visualized by confocal microscopy. Yellow color represents the colocalization of red and green colors. D. Quantification of HA-H1R colocalization with dsRed protein. Using Zeiss LSM 510 META Confocal imaging software, the number of pixels expressing both the colors was determined in a number of cells (n = 16) and the data are presented as the average number of pixels that coexpress dsRed and HA-H1R. Error bars indicate SEM.

Retention of H1Rb in the ER requires the L-M-S haplotype

To understand which of the three amino acids comprising the H1Rb L-M-S haplotype is responsible for the observed differential cell surface expression of the allele, we generated single H1R3 mutants, replacing each of the H1Rb haplotype-associated residues with the corresponding H1R3 allele (P263L, V312M, and P330S), by site-directed mutagenesis. HEK293T cells were transfected with H1R3, H1Rb, and each of the three H1R3 mutant constructs. Cells were stained with anti-HA mAb, without permeabilization, and cell surface expression of H1R was analyzed by flow cytometry. Each of the single H1R3 mutants was expressed at higher levels on the cell surface than the H1Rb allele (Fig. 6A) with the levels comparable to those observed with the H1R3 allele. This indicates that the presence of a single H1Rb polymorphism is not sufficient to induce its intracellular retention. We also generated double mutants of the H1Rb allele wherein we replaced two residues of the H1Rb haplotype with the corresponding H1R3 alleles (P263L and V312M, P263L and P330S, V312M and P330S). Similar to the single H1R3 mutants, the double H1R3 mutants were expressed on the cell surface at levels comparable to the H1R3 and at significantly higher levels than the H1Rb allele (Fig. 6B). We observed similar results in 721.221 B cells following transient transfection with H1R3, H1R3 mutants, and H1Rb constructs (data not shown). Furthermore, when HEK293T cells were cotransfected with double H1R3 mutants and the dsRed plasmid, each of the mutants showed a typical plasma membrane expression pattern with very little colocalization with the ER-targeted dsRed protein (Fig. 6C). Quantification of the number of pixels expressing dsRed protein and HA-H1R confirmed that each of the double H1R3 mutants behaved like H1R3 and only H1Rb was retained in ER (Fig. 6D), confirming the flow cytometry data that all the polymorphic residues are required for differential cell surface expression of the H1R alleles. Taken together, these data indicate that all three residues of the H1Rb L-M-S haplotype are required for its intracellular sequestration. Interestingly, we sequenced the H1R alleles from >100 different inbred laboratory and wild-derived mouse strains and did not identify any recombinant haplotypes.
suggesting that the two alleles are evolutionarily conserved and may have been selected functionally (data not shown).

Discussion
To date, Hrh1/H1R is the only murine EAE and experimental allergic orchitis susceptibility gene that has been positionally cloned (7). In this study, using transgenic mouse models, we show that polymorphic variants in H1R regulate cytokine production by T cells thereby influencing susceptibility to EAE. Furthermore, using HEK293T cells, we show that the polymorphisms in H1R affect its functions by modulating cell surface expression rather than inherently altering the capacity of the receptor to generate intracellular signals.

Hrh1/H1R has long been implicated in EAE susceptibility (7, 8). As H1R is widely expressed (10), this suggested that it might act in different cell types and at multiple checkpoints. We recently showed, however, that H1R expression in T cells is sufficient to complement EAE severity in H1RKO mice. In this study, we show that the polymorphic residues of the H1R allege allele interfere with its ability to complement EAE in H1RKO mice. This is in accordance with genetic complementation studies in F1 hybrids between H1RKO and strains of mice expressing the H1RS allele. Susceptibility to histamine sensitivity could be restored in F1 hybrids of H1RKO and SJL/J, 129X1/SvJ, or C57BL/6J that express the H1R allele but not in F1 hybrids between H1RKO and C3H/HeJ or CBA/J mice that express H1R (7).

Hrh1/H1R also controls delayed-type hypersensitivity (DTH) responses when PTX is used as an adjuvant. The DTH response is mediated by CD4 T cells that produce large amounts of IFN-γ (21–23). Using C3H.BphsS congenic mice expressing the H1R allele from SJL/J mice on the resistant C3H/HeJ background, Gao et al. (24) showed that polymorphisms in H1R regulate the OVA-specific DTH response elicited in mice immunized with OVA in CFA and PTX, indicating that the polymorphisms in H1R regulate IFN-γ production by CD4 T cells. This study confirms the role of H1R polymorphisms in regulating IFN-γ production by these cells. Furthermore, the complementation of IFN-γ production by splenocytes immunized using the 2× MOG35-55 model suggests that H1R regulation of IFN-γ production by T cells does not require PTX.

Recently, IL-17-producing Th17 CD4 T cells have been considered more pathogenic in EAE (19). We show here, for the first time, that H1R signaling regulates IL-17 production and that H1R polymorphisms influence IL-17 production by T cells. However, it is noteworthy that we did not observe differences in IL-17 production between WT and H1RKO mice immunized with MOG35-55-CFA plus PTX, nor in Th17 cells differentiated in vitro in the presence of excessive amounts of IL-6. PTX promotes the generation of Th17 cells, by inducing IL-6 production (25). Thus, it is possible that immunization with PTX (in vivo) or addition of exogenous IL-6 (in vitro) enables CD4 T cells to overcome the absence of H1R signals required for the optimal IL-6 production and generation of Th17 cells. Even though we observed significant differences in IL-17 production by spleen and DLN cells from transgenic mice selectively expressing either H1R or H1R in T cells, we believe, based on in vitro differentiation data, that the H1R regulation of IL-6 and IL-17 is independent of H1R signals in T cells. In this regard, compared with WT macrophages H1RKO macrophages produce significantly less IL-6 (our unpublished data) and treatment of lung parenchymal macrophages with H1R blockers results in decreased IL-6 production (26). Further studies are being conducted to elucidate the role of H1R in the generation of Th17 CD4 T cells.

GPCRs, despite the diversity of their polypeptide sequences, as a family retain enough structural information to allow them to be properly folded in the ER and adopt their highly conserved seven transmembrane confirmation (27). Several studies have identified critical residues and motifs in many of the functions of GPCRs including ligand binding, G protein coupling, internalization, down-regulation, and intracellular trafficking (28). However, the three polymorphic residues distinguishing the H1R allege and H1R allel alleles are located in the third intracytoplasmic loop and do not constitute any known motif. Even though the exact PXXP motif is not present, it is worth noting that two of the three polymorphic residues associated with the H1R allege haplotype are prolines, and that proline-rich motifs are known to mediate protein-protein interactions with Src homology 3 (SH3) domains (29). In this regard, polymorphic residues containing proline motifs in the third intracytoplasmic loop of the dopamine D4 receptor and β1-adrenergic receptor have been shown to interact with multiple SH3 domain-containing proteins (30) and affect the trafficking of these receptors. However, at this point, we do not have any evidence to suggest that H1R interacts with any of the known SH3 domain-containing proteins or that such interactions differ between H1R allege and H1R allel alleles. Future studies will address this issue.

GPCRs interact with numerous proteins that play a role in their cellular trafficking (12). H1R has an unusually long third intracytoplasmic loop, suggesting that the polymorphic residues may result in improper folding of the receptor to a non-native conformation in ER, which is then recognized by the quality control machinery of molecular chaperones and excluded from ER export. Several chaperone proteins (such as NINA (31, 32), ODR-4 (33, 34), and a variety of receptor activity modifying proteins (35, 36)) that support the trafficking of a range of GPCRs to their target site have been identified. Therefore, it is possible that polymorphic residue-induced misfolding of H1R allel could hinder its interaction with an essential chaperone thereby affecting its trafficking.

Proper cell surface expression of GPCRs is required to access the requisite ligands and signal transduction machinery (12). The functional importance of proper GPCR localization is emphasized by several human diseases that result from receptor mutation and mislocalization, including X-linked nephogenic diabetes, retinitis pigmentosa, and hypogonadotropic hypogonadism, which result from intracellular accumulation of mutant V2 vasopressin receptor, rhodopsin, and gonadotropin-releasing hormone receptor, respectively (20). In fact, mutations that lead to intracellular accumulation comprise the largest class of mutations in GPCRs that result in human diseases (12). Accordingly, our results are the first to demonstrate that structural polymorphisms influencing differential trafficking and cell surface expression of a GPCR in T cells can regulate immune functions and susceptibility to autoimmune disease.

Disclosures
The authors have no financial conflict of interest.

References


