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IgE-mediated activation of mast cells and basophils underlies allergic diseases such as asthma. Histamine-releasing factor (HRF; also known as translationally controlled tumor protein [TCTP] and fortilin) has been implicated in late-phase allergic reactions (LPRs) and chronic allergic inflammation, but its functions during asthma are not well understood. Here, we identified a subset of IgE and IgG antibodies as HRF-interacting molecules in vitro. HRF was able to dimerize and bind to Igs via interactions of its N-terminal and internal regions with the Fab region of Igs. Therefore, HRF together with HRF-reactive IgE was able to activate mast cells in vitro. In mouse models of asthma and allergy, Ig-interacting HRF peptides that were shown to block HRF/Ig interactions in vitro inhibited IgE/HRF-induced mast cell activation and in vivo cutaneous anaphylaxis and airway inflammation. Intranasally administered HRF recruited inflammatory immune cells to the lung in naive mice in a mast cell– and Fc receptor–dependent manner. These results indicate that HRF has a proinflammatory role in asthma and skin immediate hypersensitivity, leading us to suggest HRF as a potential therapeutic target.
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IgE-mediated activation of mast cells and basophils underlies allergic diseases such as asthma. Histamine-releasing factor (HRF; also known as translationally controlled tumor protein [TCTP] and fortilin) has been implicated in late-phase allergic reactions (LPRs) and chronic allergic inflammation, but its functions during asthma are not well understood. Here, we identified a subset of IgE and IgG antibodies as HRF-interacting molecules in vitro. HRF was able to dimerize and bind to Igs via interactions of its N-terminal and internal regions with the Fab region of Igs. Therefore, HRF together with HRF-reactive IgE was able to activate mast cells in vitro. In mouse models of asthma and allergy, Ig-interacting HRF peptides that were shown to block HRF/Ig interactions in vitro inhibited IgE/HRF-induced mast cell activation and in vivo cutaneous anaphylaxis and airway inflammation. Intranasally administered HRF recruited inflammatory immune cells to the lung in naive mice in a mast cell– and Fc receptor–dependent manner. These results indicate that HRF has a proinflammatory role in asthma and skin immediate hypersensitivity, leading us to suggest HRF as a potential therapeutic target.

Introduction

Mast cells and basophils are key effector cells for IgE-dependent allergic inflammatory reactions (1). Upon activation, these cells secrete preformed proinflammatory chemical mediators (e.g., histamine, proteases, proteoglycans, and nucleotides) as well as de novo synthesized lipids (e.g., leukotrienes and prostaglandins) and polypeptides (e.g., cytokines and chemokines). These substances lead to the development of allergic inflammation.

Since Thueson et al. first described an activity from cultured peripheral blood mononuclear cells that induced the release of histamine from basophils (2), histamine-releasing activities have been studied for more than 30 years (3). In addition to several cytokines and chemokines with this activity, an unrelated protein termed histamine-releasing factor (HRF) was purified and molecularly cloned in 1995 (4). HRF, also known as translationally controlled tumor protein (TCTP) and fortilin, is a highly conserved protein with both intracellular and extracellular functions (4–8). HRF is secreted by peripheral blood mononuclear cells that induced the release of histamine and IL-4 and IL-13 production from IgE-sensitized basophils and mast cells (9). HRF-like activities were found in nasal, skin blister, and bronchoalveolar lavage (BAL) fluids during late-phase allergic reactions (LPRs), implicating HRF in the LPR and chronic allergic inflammation (10–12). However, definitive evidence for the role of HRF in allergic reactions has been elusive (8, 9, 13).

Confounding the research, HRF has a wide range of intracellular functions, including cell cycle progression, proliferation, survival, and malignant transformation of a variety of cell types (8). HRF is ubiquitously expressed in all tested eukaryotic cells; its expression is active in mitotically active tissues (14, 15) and subject to both transcriptional and translational control (16). In tumor cells, HRF is highly expressed and downregulated upon tumor reversion (17). It is involved in the elongation step of protein synthesis by interacting with both eEF1A (a small GTPase) and eEF1Bβ (a guanine nucleotide exchange factor) (18–20). Drosophila and human HRFs act as the guanine nucleotide exchange factor for the Ras superfamily GTPase, Rheb, which regulates the TSC1-TSC2-mTOR pathway (21, 22). These studies implicate this protein in the regulation of growth and proliferation as well as in the control of organ size. HRF interacts with Mcl-1 (23, 24) and Bcl-xL (25), antipaprotic members of the Bcl-2 family, and antagonizes apoptosis by inserting into the mitochondrial membrane and inhibiting Bax dimerization (26). HRF also interacts with p53 tumor suppressor and suppresses p53-mediated apoptosis (27). Other HRF-interacting molecules include tubulin (28), NEMO (29) and vitamin D3 receptor (30). Phosphorylation of HRF by the protein kinase Pkl decreases the microtubule-stabilizing activity of HRF (31).

The extracellular function of HRF is considered a cytokine-like activity toward IgE-primed mast cells and basophils (9). Despite considerable efforts, researchers have failed to identify an HRF receptor. Unfortunately, HRF knockout mice are embryonic lethal (32, 33) and cannot provide meaningful information on HRF function. Because of the lack of reagents that can distinguish between HRF’s intracellular and extracellular functions, it is particularly dif-
ficult to dissect extracellular functions in complex in vivo settings. In this study, we sought to identify HRF-interacting molecules and inhibitors of interactions of HRF with HRF-reactive molecules.

Results

HRF binds to Fab fragments of a subset of IgE and IgG antibodies. Despite a previous study implying that IgE does not interact with HRF (34), we reexamined this possibility first by using an ELISA and supplementary Figure 2, C and E), and the C38-2–mHRF interaction was not inhibited by TNP-lysine, whereas the IGELa2–mHRF interaction was inhibited by TNP-lysine. These results suggest that the HRF-binding site in IgE overlaps at least in part with the antigen-binding sites. Collectively, these results suggest that a considerable proportion of antibodies in immunized mice interact with HRF. In addition, 1 of the 5 tested human IgEs (i.e., HE-1) bound GST-mHRF (data not shown).

Figure 1

A subset of IgE and IgG molecules binds HRF. (A) IgE molecules were incubated in GST-mHRF–coated wells. HRF-bound IgE was quantified by ELISA, as detected by color development with HRP. OD450 values with GST-mHRF subtracted from those with GST control are shown. OD450 ≤ 0.1 was used as an arbitrary cutoff value. Data represent at least 3 experiments. (B) IgEs were incubated with GST- or GST-mHRF–agarose beads. Bead-bound IgEs were pulled down. IgE and GST proteins were detected by immunoblotting. Lanes were run on the same gel but were noncontiguous (white lines). Representative of 2 experiments. (C) BMMCs preincubated with (black line) or without (gray shading) the indicated IgE (see Supplemental Table 3) were incubated with mHRF-His6, and bound mHRF-His6 was detected with rabbit anti-His tag antibody and Alexa Fluor 647–conjugated anti-rabbit IgG. HRF binding was detected by flow cytometry. Insets show IgE binding: the same cells were incubated with FITC-labeled anti-mouse IgE. Representative of 2 experiments. (D) HRF-bound IgGs were detected by ELISA. Representative of 3 experiments. HRF binding was independent of IgG isotype, as the tested IgG1, IgG2a, and IgG2b molecules contained both HRF-reactive and -nonreactive molecules. The Kd values for HRF binding were 0.685 μM (JK17), 2.78 μM (JK31), and 5.78 μM (JK96). Black bars, IgG1; white bars, IgG2a; gray bars, IgG2b.
Peptides corresponding to the Ig-binding sites within HRF inhibit HRF-Ig interactions. We next mapped the Ig-binding sites within HRF. IgE and IgG binding assays using a panel of truncated GST-mHRF proteins gave similar binding patterns (Figure 3, A and B, and Supplemental Figure 3). A major Ig-binding site was mapped to the N-terminal 19-residue peptide (N19), as GST-tagged N19 (referred to herein as GST-N19), but not GST fusion proteins containing shorter N-terminal fragments, bound Igs. Another binding site was mapped to internal residues 79–142 (Figure 3, A and B). Further fine mapping localized the latter binding site to the H3 region (residues 107–135, termed GST-H3; Figure 3D and data not shown).

Intracellular HRF might contribute to allergic inflammation by controlling cell cycle progression, proliferation, and survival of immune and structural cells (8, 21, 32). Therefore, it is essential to find an inhibitor of HRF-Ig interactions to dissect HRF’s extracellular functions, separate from HRF’s intracellular functions. We tested whether the Ig-interacting HRF sequences might serve as specific inhibitors of HRF binding to Igs. Indeed, GST-N19 inhibited IgE binding to mHRF with potency similar to full-length GST-mHRF (Figure 3C). However, shorter mHRF peptides tested (residues 1–6, 1–12, 1–16, 5–19, and 9–19) or a scrambled peptide (KYI-N16) did not inhibit HRF-IgE binding (Supplemental Figure 4). Control experiments showed that GST-N19 did not affect the viability or proliferation (Supplemental Figure 5, A–D), nor did it affect apoptosis induced by growth factor withdrawal in BMMCs or by H2O2 in CHO-K1 cells (Supplemental Figure 5, E and F). These concentrations of HRF were higher than what has previously been shown to stimulate basophils (1.6–5 μM; ref. 35). Importantly, GST-N19 did not enter BMMCs (Figure 4A). A synthetic N19 peptide also inhibited IgE binding to mHRF and did not alter the growth or survival of various cells (Supplemental Figure 6). Similar to GST-N19, GST-H3 also inhibited IgE binding to mHRF (Figure 3D); GST-H3 neither affected cell growth or apoptosis nor entered the cells (Supplemental Figure 7 and data not shown). These results indicated that the HRF N19 and H3 peptides can be used to probe extracellular functions of HRF in vitro and in vivo.

N19 and H3 peptides block mast cell activation. Analysis of purified recombinant mHRF-His6 on reducing and nonreducing SDS-PAGE yielded direct evidence for disulfide-linked dimerization of HRF (Supplemental Figure 8A). Both monomeric and dimeric forms of HRF could bind to IgE (data not shown). Consistent with this, monomeric mHRF mutant 2CA, with 2 cysteine residues at positions 28 and 172 substituted with alanine, also bound Igs (Supplemental Figure 9, A and B). The dimerizing ability of HRF with 2 Ig-binding sites suggests the potential of HRF to crosslink Ig-bound Fc receptors (Supplemental Figure 8B). This notion was supported by activation of mast cells by
cotreatment with mHRF and HRF-reactive, but not HRF-nonreactive, IgEs, as evidenced by histamine release and cytokine production from BMMCs (Figure 4, B and C) and by β-hexosaminidase release from peritoneal mast cells (C38-2 IgE, 17.8% ± 4.6% release; C48-2 IgE, 2.4% ± 0.1% release; P < 0.0001). These reactions were inhibited by GST-N19 and GST-H3 (Figure 4, D and E, and data not shown). Consistent with mast cell activation, tyrosine phosphorylation of several proteins was observed in C38-2 IgE/HRF-treated cells (data not shown).

HRF inhibitors suppress passive cutaneous anaphylaxis. Acute passive cutaneous anaphylaxis (PCA) reactions induced by antigen in IgE-sensitized mice are mediated mainly by histamine released from activated mast cells (36). LPRs in the skin are mediated in part by mast cell–derived TNF-α (37, 38) and IL-33 (39). Strikingly, when HRF was injected i.d. 24 hours after IgE injection, both acute reactions and LPRs were induced by HRF-reactive, but not HRF-nonreactive, IgE (Figure 5, A and B). The HRF-reactive C38-2 IgE induced increased vascular permeability after HRF injection (Figure 5A). Interestingly, the LPRs induced by HRF, as measured by increased ear swelling at 6 hours, were as high as those induced by antigen (Figure 5B). Both acute reactions and LPRs induced by IgE/HRF were prevented by pretreatment with GST-N19 (Figure 5C and data not shown) and appeared to be mast cell mediated, as the reactions were abolished in mast cell–deficient KitW/W–sh mice (40) and restored in KitW/W–sh mice engrafted with WT BMMCs (Figure 5A). Furthermore, loss of FcεRI abolished PCA reactions (Figure 5D). Control experiments showed little effect of GST or GST-H3 in place of GST-N19 treatment. Similar results were observed using GST-H3 in place of GST-N19 and GST-N19 plus GST-H3 alone (Supplemental Figure 10). These results suggest that HRF is required for maximal IgE/antigen-induced PCA reactions. Consistent with this, HRF was dramatically increased in the dermis during LPRs (Supplemental Figure 11).

HRF inhibitors suppress mast cell–dependent airway inflammation. Asthma is a chronic lung disease characterized by airway inflammation, airway hyperresponsiveness (AHR), and reversible airway obstruction (41). We used a mast cell–dependent, OVA-induced airway inflammation model (42). In addition to the increased HRF levels in lungs and blood (Figure 6A), immunofluorescence microscopy showed increased levels of HRF staining in nonpermeabilized lung tissues (Supplemental Figure 12A), which indicates that HRF is secreted into lung tissues in OVA-challenged mice. Levels of HRF-reactive IgG were also increased in plasma and BAL fluids of these mice (Supplemental Figure 12B). Pretreatment with GST-N19 before the OVA challenges abrogated airway inflammation, as evidenced by reduced eosinophils and neutrophils in BAL fluids (Figure 6B) and by reduced inflammatory cells and goblet cell hyperplasia in the lung (Figure 6C). Production of IL-13 (the cytokine essential for AHR, eosinophilia, and mucus production; refs. 43–45) and IL-5 (the cytokine critical for eosinophilia and...
AHR; ref. 46) in lung tissues was drastically decreased in GST-N19–treated mice (Figure 6D). Consistent with these observations, GST-N19 treatment inhibited AHR ($P < 0.05$ vs. GST at 48 mg/ml methacholine, Bonferroni correction; Figure 6E). Circulating systemic HRF was reduced by GST-N19 (Supplemental Figure 12C), probably reflecting an antiinflammatory effect of GST-N19. In contrast, HRF-reactive plasma IgG levels and OVA-specific IgE, IgG1, and IgG2a levels were not affected by GST-N19 (Supplemental Figure 12B and data not shown). Administration of a synthetic N19 peptide or GST-H3 abrogated airway inflammation with similar potency to that of GST-N19 (Supplemental Figure 13 and data not shown).

We confirmed the efficacy of GST-N19 in a second model of asthma: partially IgE-dependent airway inflammation induced by *Aspergillus fumigatus* allergens (47). HRF inhibition resulted in substantial reduction of allergic airway inflammation and inflammatory cells in BAL fluids (Supplemental Figure 14). Interestingly, HRF inhibition failed to reduce airway inflammation in a mast cell–independent OVA-alum model (ref. 48 and Supplemental Figure 15).

**Figure 4**

GST-N19 does not enter the cell interior and serves as an HRF inhibitor. (A) BMMCs were incubated with TAT-GST, GST, or GST-N19 protein for the indicated time periods at 37°C. Washed cells were fixed, permeabilized, and stained with anti-GST followed by Alexa Fluor 488–conjugated anti-mouse IgG. Nuclei were stained with DAPI. Fluorescence was observed by confocal microscopy. DIC, differential interference contrast. Original magnification, ×150. Percentages of the cells similar to the representative images are shown (≥150 cells scored). (B and C) BMMCs were incubated with 5 μg/ml of the indicated IgE and 100 μg/ml mHRF for 45 minutes (histamine release) or 20 hours (IL-6 production). (D and E) BMMCs were incubated with IgE and mHRF in the presence of 100 μM GST or GST-N19. *$P < 0.05$, **$P < 0.01$ versus respective control, Student’s t test. Data are representative of 3 (A) or 2 (B–E) experiments.
FcRy is shared by multiple Fc receptors, including FcεRI, FcγRI, FcγRIII, and FcγRIV (51, 52). Among the Igs and Fc receptors, IgE and FcεRI were the predominant contributors to the effects of HRF, as HRF-induced lung inflammation was almost abrogated in naive FcεR1γ−/− mice (Figure 7A). Since FcεRI is expressed on mast cells and basophils in mice (53), these results were consistent with the effectiveness of N19 and H3 peptides in mast cell–dependent asthma models (Figure 6 and data not shown).

Importantly, the absence of inflammatory cell responses to HRF in μMT or FcRγ−/− mice corroborated our finding that HRF bound Igs (Figure 1). To further evaluate the target range of HRF, we performed global gene expression analysis. Expression of 196 genes was up- or downregulated more than 3-fold by HRF in the lungs of naive WT mice, with 90 genes up- or downregulated more than 5-fold (Figure 7C). Upregulated genes included those encoding Th1-, Th2-, and Th17-associated cytokines and various chemokines, potentially accounting for the recruitment of monocytes/macrophages, neutrophils, eosinophils, and other immune cells (Supplemental Figure 16). Expression of some Th1 and Th2 cytokines were confirmed by real-time PCR analysis (data not shown). Other upregulated genes included the previously reported genes in mouse asthma models, such as Agr2, Ccl8, Cdl11, Fcg2b, Scin, Serpina3g, Serpina3n, and Temp1. However, only a small fraction of these genes (39 of 196) fluctuated more than 3-fold in FcεR1γ−/− mice; furthermore, fewer genes (11 of 196) were changed in FcRγ−/− mice (Figure 7C). These results suggest that HRF executes its action largely, if not exclusively, by engaging IgE- and IgG-bound Fc receptors and promotes airway inflammation.

Discussion

Despite considerable efforts in the last 15 years since the cloning of HRF (4), the receptor for HRF has not been identified. Using functional assays on RBL-2H3 rat mast cells expressing human FcεRI, Wantke et al. indirectly suggested that human recombinant HRF does not bind to IgE (34). However, we clearly demonstrated that a subset of IgE and IgG can interact with HRF. Our study differs from that of Wantke et al., as theirs used human FcεRI-expressing RBL-2H3 cells, which in our hands were difficult to activate. Importantly, we used more than a dozen IgE mAbs in an ELISA-based binding assay, compared with 2 types of polyclonal IgE used in the prior study. Furthermore, HRF binding of some IgE mAbs does not bind to IgE (34). However, we clearly demonstrated that mHRF-Hisγ−/− mice that HRF-reactive IgEs and IgGs contain unique V(D)J sequences of a limited number of IgE and IgG molecules indicates that HRF-reactive IgEs and IgGs contain unique V(D)J sequences.
In contrast, these IgEs and IgGs use different V\textsubscript{H} family members. The Ig-bind-ing N19 peptide forms 2 antiparallel \(\beta\)-sheets (positions 3–5 and 14–15), which, together with the C-terminal \(\beta\)-sheet, form the 3-stranded sheet B (54, 55). The structure consisting of sheet B, the 4-stranded sheet A, and the small helix is similar to that of the human protein Mss4, which binds to Rab proteins and is proposed to be a guanine nucleotide-free chaperone (56). The other Ig-interacting H3 domain is a long \(\alpha\)-helix packed against part of sheet A.

Our observations collectively suggest that 2 sites of HRF interact with V regions specifically. However, data on hapten inhibition of HRF-Ig interactions cannot rule out the possibility that HRF-Ig binding is enacted by relatively nonspecific ionic or other interactions of different parts of Igs.

As shown by others (57, 58), our bacterially expressed mHRF preparations can form a dimer. HRF has 2 Ig-binding sites at the N19 and H3 peptide regions. Our present biochemical analyses suggested that an HRF dimer can aggregate 2 or 4 Fc\(\varepsilon\)RI complexes preloaded with IgE (Supplemental Figure 8B). As a dimer is the minimal Fc\(\varepsilon\)RI complex required for cell activation (59), mHRF along with HRF-reactive IgE could induce mast cell activation. Consistent with these in vitro data, Fc\(\varepsilon\)RI-dependent PCA-like skin inflammation and lung inflammation were induced by WT mHRF, but not the monomeric 2CA mutant mHRF.

The progress in HRF research has been hindered by the lack of identification of an HRF receptor and the lack of tools to distinguish its extracellular from intracellular HRF functions. The peptides N19 and H3 corresponding to the Ig-interacting sites within mHRF turned out to be specific inhibitors that interfered with the interactions between extracellular HRF and IgE, but had no effect on HRF’s intracellular functions. The biologic activities of these peptides were shown by their suppression of in vitro mast cell activation and in vivo mast cell–dependent inflammation, i.e., PCA and airway inflammation. It should be emphasized that these peptides used as GST fusion proteins did not affect HRF’s intracellular functions, as they were not taken up intracellularly.

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**Figure 6**

GST-N19 blocks mast cell–dependent airway inflammation. C57BL/6 mice were sensitized with OVA (10 \(\mu\)g) and i.n. challenged with OVA (20 \(\mu\)g) or PBS. Some mice were i.n. pretreated with GST or GST-N19 (400 \(\mu\)g) before every OVA challenge. 24 hours after the last challenge, mice were subjected to invasive lung function testing, and BAL fluids and lung tissues were collected. (A) Increased HRF amounts in the lung and sera of OVA-sensitized and -challenged mice. SDS-PAGE was performed on lung homogenates and serum samples, and HRF amounts of 3 mice were evaluated by immunoblotting. ERK1/2 expression was used as a loading control. (B) Total and specific immune cell numbers in BAL fluids. Eos, eosinophils; Neu, neutrophils; Lym, lymphocytes; M\(\phi\), macrophages and monocytes. (C) H&E and periodic acid-Schiff (PAS) staining of lung tissues. Scale bars: 200 \(\mu\)m. (D) IL-5 and IL-13 in lung homogenates were measured by ELISA. (E) Airway resistance was measured using FlexiVent. *\(P\) < 0.05, **\(P\) < 0.01, ***\(P\) < 0.001. 3–6 mice were used for each cohort. All data are representative of 3 experiments.
A synthetic N19 peptide also did not affect the intracellular functions. Kim et al. have shown that N10 of HRF can work as a protein translocation domain (PTD) when fused with some proteins at their N termini (60). Protein internalization by this PTD was characterized by the high dose requirement (8–32 μM) and slow kinetics compared with that of TAT, an HIV-encoded peptide (61). However, we found that the PTD function of N10 peptide in conjunction with GST (both N10-GST and GST-N10) was very weak compared with TAT-GST, particularly when N10 was fused at the C-terminus of GST (compare Supplemental Figure 7 and Figure 4A). More importantly, we clearly showed that GST-N19 and GST-H3 did not enter the mast cell or other cells.

A recent study shows that an N-terminal deletion mutant of rat HRF (Del-N11) exhibits a stronger dimerizing propensity and a stronger cytokine activity than the full-length HRF (58), consistent with our results indicating that the dimer is the biologically active form of HRF. Consistent with these data, transgenic mice expressing HRF in a lung Clara cell–specific manner exhibit increased numbers of macrophages in BAL fluids in naive mice and increased airway inflammation in OVA-sensitized and OVA-challenged mice (62). However, the effect of HRF overexpression in this transgenic study could not be ascribed solely to the function of the secreted HRF molecule; the effect of the transgene could be due to the intracellular effect of HRF as well. Given our present data on the crucial role of HRF in asthma models, as well as the previous data of HRF-like activity in asthma and other allergic conditions, further studies of HRF and the utility of N19 and H3 inhibition of HRF are warranted in preclinical and clinical settings.

Airway inflammation by HRF in naive mice may be mediated predominantly by FcεRI expressed on mast cells, but not basophils, as there are normal numbers of basophils in KitW−/W− mice.

Figure 7
Lung inflammation is induced by HRF in naive mice in an Fc receptor–dependent manner. Naive WT C57BL/6 and mutant mice were treated i.n. with 40 μg mHRF-His6 3 times every third day. PBS served as a negative control. (A and B) HRF-induced lung inflammation required B and mast cells as well as FcεRI (and probably Fcγ receptors). BAL procedures were conducted 24 hours after the last HRF administration. Differential cell counting was performed on cytospin preparations stained with May-Giemsa. (C) Genes whose expression was up- or downregulated by HRF. Black symbols, ≤3-fold change; orange symbols, 3- to 5-fold change; red symbols, >5-fold change. Genes whose expression fluctuated ≤3-fold in WT, FcεRIα−/−, and FcγRI−/− mice are not shown in the plots for the mutant mice. Dashed and solid lines indicate 3- and 5-fold differences, respectively, in gene expression. *P < 0.05, **P < 0.01, ***P < 0.001, Student’s t test. Each cohort consisted of 3–5 mice. All data are representative of 5 (WT) and 2 (mutant) experiments.
(63). However, Fcγ receptors may also contribute to this inflammation. Abrogation of HRF-induced airway inflammation and gene modulation in naïve FcεRIα−/− and FcεRIγ−/− mice support the notion that IgE and IgG are the long-sought receptors for HRF in the lung. Based on the profile of up- or downregulated genes, we propose the following scenario: HRF crosslinks FcεRI-bound IgE (and Fcγ receptor–bound IgG) on mast cells in naïve mice and activates the cells; activated mast cells secrete various proinflammatory mediators; these mediators then initiate inflammation by directly or indirectly recruiting various inflammatory cells. In addition, a similar HRF-mediated mechanism promotes the amplification of allergen-induced inflammation by activating mast cells and basophils, in which FcεRI complexes are occupied suboptimally with allergen-specific IgE (or, rather, occupied with nonspecific IgEs) to respond to allergen. Potentially at odds with the above scenario, sera and BAL fluids from naïve mice contain HRF, which does not appear to induce inflammation under homeostatic conditions. Thus, there seem to be mechanisms to suppress inflammation potentially inducible by endogenous HRF. The endogenous amount of HRF might be lower than the threshold for HRF to induce inflammation. Alternatively, there might be endogenous inhibitors that inhibit HRF’s extracellular functions. These possibilities are worthy of investigation.

In summary, our study demonstrated that the bioactive HRF (i.e., dimers and oligomers) interacted with some IgE molecules and could crosslink that IgE-bound FcεRI. FcεRI aggregates activated mast cells in vitro. Inhibitors that prevent HRF-Ig interactions suppressed IgE/antigen-induced skin hypersensitivity and allergen-induced mast cell–dependent airway inflammation. Thus, we conclude that HRF promotes allergen inflammation in the skin and lung.

Methods

Mice. C57BL/6 and Balb/c mice were purchased from the Jackson Laboratory. FcεRIα−/−, FcεRIγ−/−, and μMT mice were also used.

Preparation of recombinant mHRF. mHRF cDNAs were amplified by RT-PCR using the primers listed in Supplemental Table 2. GST fusion proteins were purified using glutathione-agarose (Sigma-Aldrich), mHRF-His6 expressed by pET-24a(+) plasmid was purified using ProBond resin (Invitrogen). All recombinants were further purified by Sephacryl S-100 and dialyzed against PBS. mHRF-His6 preparations contained less than 0.05 pg/μg protein of endotoxin, as measured by Limulus amebocyte lysate test.

ELISA. 96-well ELISA plates were coated overnight with GST, GST-mHRF, or mHRF-His6 (each at 10 μg/ml in 0.1 M carbonate buffer [pH 9.5]). The plates were washed and blocked with 10% FCS or 1% BSA. Next, mouse IgE and IgG molecules (10 μg/ml), plasma (1:100–1:200 dilution), and BAL fluids (1:10 dilution) were incubated in the coated wells, after which bound IgE was detected by incubation with biotinylated anti-mouse IgE followed with HRP-conjugated streptavidin. Bound IgG was detected by incubation with HRP-conjugated anti-mouse IgG. Dye was added 30 minutes after HRF challenge was measured by extracting ears in formamide. Engraftment of KIRγ−/−m−/− mice with BM-MSCs was performed 6 weeks before the experiments (66). In some experiments in which mice were stimulated without Evans blue, ear thickness was measured.

Asthma models. In the first model (42), C57BL/6 mice were sensitized with i.p. injection of OVA (10 μg) at days 0, 7, 14, 21, 28, and 35. At days 40, 43, and 46, mice were i.n. challenged with OVA (20 μg). Some mice were i.n. pretreated with 40–400 μg of GST or GST-N19 before each OVA challenge. In some experiments, 20 μg of synthetic N19 peptide or vehicle (2% DMSO) was used for pretreatment. 24 hours after the last challenge, lung function was tested using FlexiVent system (SCIREEQ). Mice were sacrificed, and BAL fluids as well as blood and lung tissues were collected. Cells in BAL fluids were enumerated after staining with May-Giemsa. Paraffin-embedded lung tissues were stained with H&E and periodic acid-Schiff. Cytokines in lung homogenates were quantified by ELISA.

In the second model of asthma (47), BALB/c mice were i.n. treated with Aspergillus fumigatus allergen (50 μl; Greer Laboratories) or PBS 3 times per week for 3 weeks. Some mice were i.n. pretreated with GST or GST-N19 (200 μg/50 μl) beginning at the second week for 30 minutes before each immunization. 24 hours after the last challenge, mice were sacrificed.

In the third model (48), C57BL/6 mice were i.p. immunized with OVA in the presence of alum on days 0 and 12. Mice were i.n. administered with OVA (20 μg/20 μl) on days 24, 26, and 28. Some mice were i.n. pretreated with GST or GST-N19 (400 μg/20 μl) before each OVA challenge. 24 hours after the last challenge, mice were sacrificed.

Oligonucleotide microarray. Total RNA was extracted from lungs using RNeasy Total RNA Mini Kit (Qiagen). A microarray analysis was performed using 200 ng of total RNA from each sample and SurePrint G3 Mouse Gene Expression 8x60K arrays (Agilent Technologies) according to the manufacturer’s instructions. The microarray data have been deposited in Gene Expression Omnibus (GEO; accession no. GSE34133; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE34133). Data analysis was performed with GeneSpring software (version GX 10.3). Because the expression levels of housekeeping genes (GAPDH and β-actin) did not differ among all samples, specific normalization was not performed. To eliminate genes containing only a background signal, genes were selected only if the raw values of “Expression” were more than 100. In addition, we focused on probes with reliable annotations (https://earray.chem.agilent.com/earray/) in the present study. A total of 16,374 genes met these criteria and were subjected to further analysis.
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