CRTH2, an orphan receptor of T-helper-2-cells, is expressed on basophils and eosinophils and responds to mast cell-derived factor(s)

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Abstract **We have recently cloned a putative chemoattractant receptor, named CRTH2, which is preferentially expressed on human T-helper- (Th) 2 but not Th1 cells. In this study, we demonstrated that CRTH2 is also highly expressed on peripheral blood basophils and eosinophils. Our search for a CRTH2 ligand identified mast cells as the possible producers of a ligand. When stimulated with an anti-FcεR1 antibody, cord blood-derived mast cells secreted factor(s) that induced Ca^{2+} mobilization in CRTH2-expressing K562 cells but not in mock transfected cells. These findings implied the involvement of CRTH2 in mast cell-mediated immune responses such as allergic reactions.**

Key words: Orphan receptor; Th2; Basophil; Eosinophil; Ligand producer; Mast cell

1. Introduction

Interactions of receptors on leukocytes with chemoattractants play an important role in tissue- or site-specific recruitment of leukocytes under various physiological and pathological conditions [1–3]. A complete understanding of this process might allow the development of new therapies for inflammatory and allergic disorders [4]. We have recently cloned a new chemoattractant receptor-like molecule, named CRTH2, from a human Th2 clone [5]. Notwithstanding the paucity of information on the corresponding ligand(s), CRTH2 is intriguing in that it is preferentially expressed on Th2- but not Th1-type cells among peripheral blood $CD4^+$ T-lymphocytes [5]. The highest homology in the amino acid sequence is found with members of the *N*-formyl peptide receptor subfamily such as FMLP receptor but the similarity seems to be insufficient to classify CRTH2 as the members of the subfamily [5]. These findings suggest that CRTH2 utilizes an unknown ligand and thereby confers a particular property in the behavior and function of Th2 cells in vivo [6–8]. In this study, we examined the expression of CRTH2 in the entire population of leukocytes in order to understand the role of CRTH2 in the immune system. Our results demonstrated that CRTH2 is highly expressed on basophils and eosinophils, both of which, as well as Th2 cells, are known to be important in allergic diseases. Furthermore, we found that culture supernatants of mast cells treated with anti-high-affinity IgE receptor (FcεR1) antibody contain an agonist(s) for CRTH2 in a Ca^{2+} mobilization assay.

2. Materials and methods

2.1. Cells

Peripheral blood mononuclear cells (PBMCs) and granulocytes were isolated from heparinized peripheral blood of consented healthy subjects by density gradient centrifugation on a metrizoate-based supporting medium, Mono-Poly Resolving medium (Dainippon Pharmaceutical, Osaka, Japan). Mast cells were obtained by culturing $CD34^+$ cord blood mononuclear cells (CBMCs) for 12–18 weeks in the presence of recombinant human stem cell factor (R and D Systems, Minneapolis, MN, USA) and interleukin (IL)-6 (Intergen, Purchase, NY, USA), as described previously [9]. To facilitate mast cell maturation, recombinant human IL-4 (10 ng/ml, Genzyme, Cambridge, MA, USA) and human IgE (1 μg/ml, Cosmo Bio, Tokyo, Japan) were added to the cultures during the last 2–3 weeks before harvesting [10,11]. The purity of mast cells in the cultures was usually $>60\%$ as assessed by staining with alcian blue or an anti-FcεR1 mAb CRA1 (Kyokuto Pharmaceutical, Ibaraki, Japan) [12] and by expression of tryptase which was measured by flow cytometry as reported [12]. CRTH2-transfected line K562/B19 and its control line K562/neo were generated by stable introduction into K562 cells of the CRTH2-expression plasmid pRc/B19 [5] and its control plasmid pRc/CMV (Invitrogen, San Diego, CA, USA), respectively, and by selection with geneticin (400 μg base/ml, Sigma Chemical, St. Louis, MO, USA). K562/B19 cells expressing high level CRTH2 were selected by sorting with anti-CRTH2 mAb BM16 as described [5] (see Fig. 3A).

2.2. Antibodies and flow cytometry

Biotinylated BM16 (rat anti-CRTH2) and control rat IgG2a were described previously [5]. A CRTH2-specific rat mAb BM7 (IgG2a), which was derived from the same fusion experiment as BM16 [5], was selected and used for its antagonistic activity in this study. BM7 and BM16 compete with each other for binding to CRTH2-expressing cells. For flow cytometric analysis, CRA1 was labeled with fluorescein isothiocyanate (FITC) (Molecular Probes, Eugene, OR, USA). The following materials were obtained from Becton Dickinson (San Jose, CA, USA): FITC-conjugated mAbs to CD3 (clone Leu-4), CD4 (Leu-3a), CD8 (Leu-2a), CD14 (Leu-M3), CD19 (Leu-12) and HLA-DR (L243); phycoerythrin (PE)-labeled mAbs to CD4, CD8 and CD56 (Leu-19); allophycocyanin (APC)-coupled mAbs to CD3, CD4, CD14 and CD19; and appropriate isotype-matched controls. FITC-conjugated mAbs to CD9 (M-L13), CD16 (3G8) and CD33 (HIM3-4), and RED670-labeled streptavidin were purchased from PharMingen (San Diego, CA, USA) and GIBCO BRL (Long Island, NY, USA). For multiple staining, cells were first pre-incubated at room temperature for 30 min in washing buffer [0.5% bovine serum albumin (BSA)/2 mM EDTA/0.05% NaN_3/PBS, pH 7.3] containing 10% normal rat serum, then biotinylated BM16 or control rat IgG2a (final 10 μg/ml) was added to the cells along with labeled mAbs to surface markers. The cells were incubated at 4°C for 30 min, washed with the washing buffer and incubated with RED670-labeled streptavidin at 4°C for 30 min in the washing buffer. Stained cells were analyzed on a

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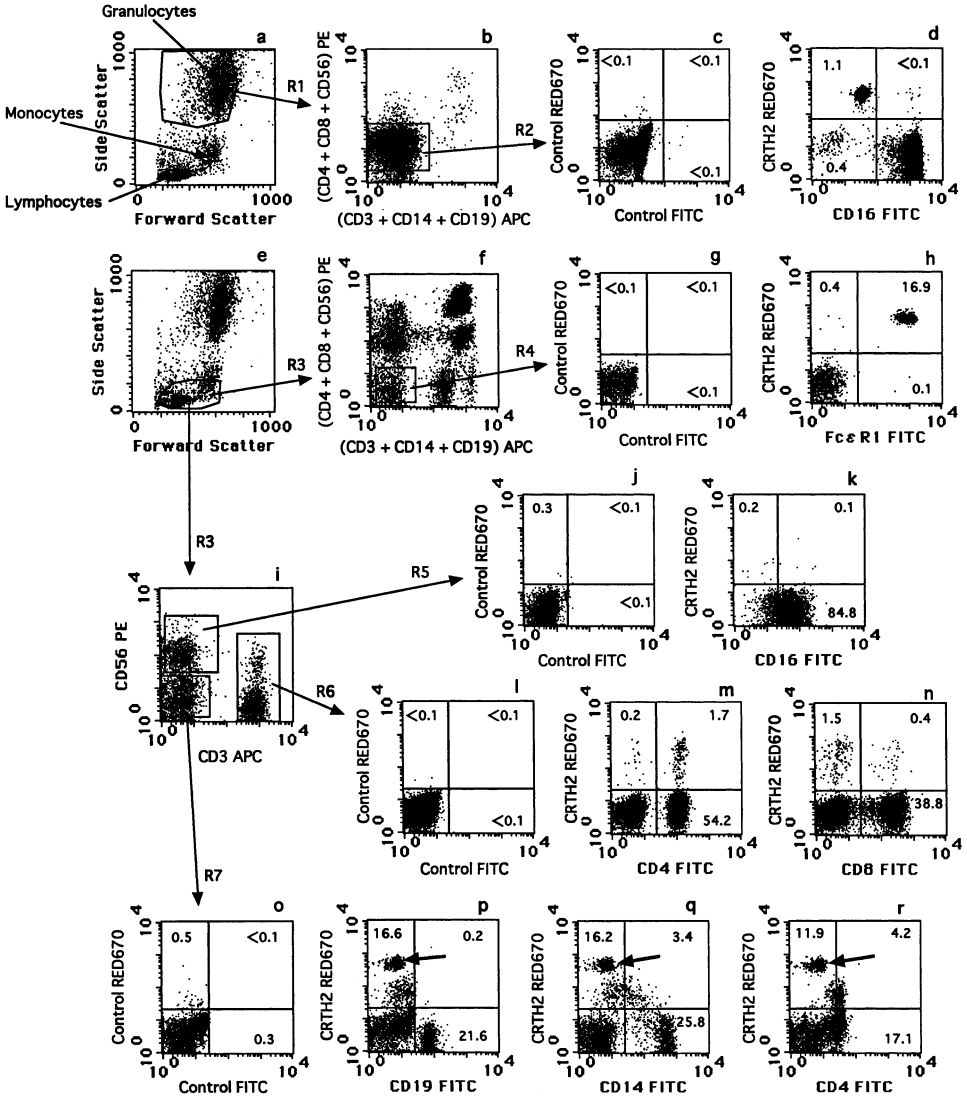

Fig. 1. Expression of CRTH2 on normal blood leukocytes. Whole leukocytes (PBMCs+granulocytes) from a representative healthy adult were stained with the indicated antibodies as described in Section 2. Staining patterns with control antibodies are shown in panels c, g, j, i and o. Percentages of positive or negative cells in each gated region are presented in each panel. Arrows in panels p–r indicate basophils.

FACSCalibur flow cytometer using CellQuest software (Becton Dickinson).

2.3. Reverse transcription (RT)-PCR analysis

Basophils were purified to homogeneity (>97% pure as determined by May-Grünwald Giemsa staining) from pooled PBMCs of healthy volunteers by negative selection using mAbs to CD3, CD4, CD8, CD14, CD19 and CD56, and anti-mouse IgG-coated magnetic beads (Miltenyi Biotec, Bergisch Gladback, Germany), followed by positive selection using anti-FcεR1 mAb CRA1 and the magnet beads [13–15]. Eosinophils (>99% pure) were similarly obtained from pooled granulocytes by negative selection with mAbs to CD3, CD4, CD8, CD14, CD16, CD19, CD56 and FcεR1, followed by positive selection with anti-CD9 [15,16]. RT-PCR was performed as described previously [5] except for primers for CRTH2 which were 5′-AATCCTGTG-CTCCCTCTGTGCCCA (sense) and 5′-GGGAAGCAGAGGCCAA-CAGGTCG (antisense).

2.4. Ca^{2+} mobilization assay

Cells were loaded with 5 μM Fura-2AM (Dojindo, Kumamoto, Japan) at 37°C for 1 h in RPMI 1640 medium supplemented with 10% fetal bovine serum, washed with Hanks' balanced salt solution (HBSS), then resuspended in HBSS containing 0.1% BSA and 10 mM

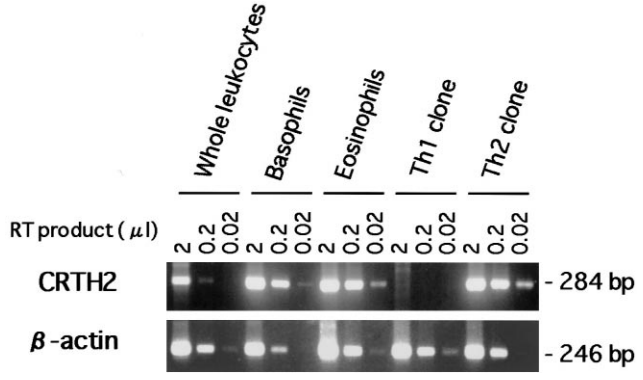

Fig. 2. Preferential expression of CRTH2 mRNA in basophils, eosinophils and Th2 cells. Total RNAs (0.4 μg) from the indicated cells were analyzed for relative levels of CRTH2 mRNA by RT-PCR as described previously [5]. The amounts of RT reaction product subjected to PCR are indicated. Th1 and Th2 clones used in this analysis were MID3 and 6L21, respectively [5]. DNA was stained with ethidium bromide.

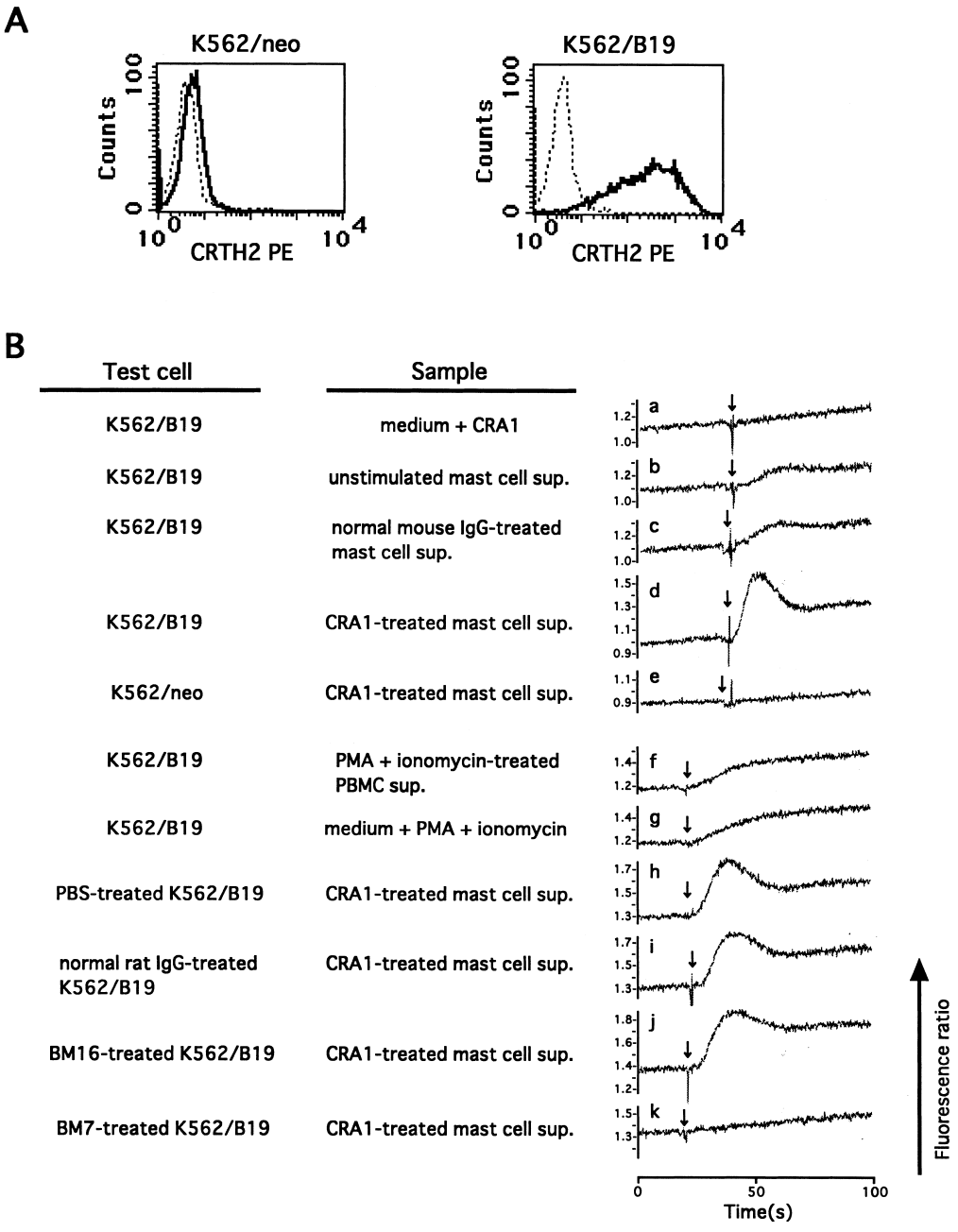

Fig. 3. Ca^{2+} mobilization in K562/B19 cells. A: Expression levels of CRTH2 on K562/neo and K562/B19 cells. Cells indicated at the top of each panel were stained with biotinylated BM16 (solid line) or control IgG2a (dotted line) as described in Section 2. B: Ca^{2+} mobilization assay. Cell suspensions of Fura-2-loaded test cells were either directly subjected to Ca^{2+} mobilization assay (experiments a–g) or treated with the indicated antibody (final 300 μg/ml) at room temperature for 20–30 min, then subjected to the assay (experiments h–k). Arrows indicate the time of stimulant (sample) addition. Fluorescence ratios induced by the addition of ionomycin (final 2 μM) or Triton X-100 (final 0.4%, Sigma) were 2.5–4.6 or 7.2–10.5, respectively.

HEPES (pH 7.3) at 10^6 cells/ml. Cells were transferred into quartz cuvettes (400 μl) that were placed in a fluorospectrometer LS50B (Perkin-Elmer, Norwalk, CT, USA). The test stimulant (10 μl) was then added to the cuvettes at indicated time points. The ratio of fluorescence intensities (emission wavelength, 510 nm) at excitation wavelengths 340 nm and 380 nm were calculated using FL-Winlab software (Perkin Elmer) as reported [17].

3. Results and discussion

We first sorted CRTH2-expressing cells from whole peripheral blood leukocytes (PBMCs+granulocytes) by using anti-CRTH2 mAb BM16, as described previously [5], then characterized CRTH2 positive cells microscopically. The May-Grünwald Giemsa staining illustrated that basophils and eosinophils were greatly enriched by the sorting. The combined percentages of these two cell types rose from $<5\%$ of unsorted whole leukocytes to $>60\%$ of BM16-sorted leukocytes with several normal adults. These results suggested that, in addition to Th2-type T-cells, basophils and eosinophils highly expressed CRTH2.

To examine in detail the expression level of CRTH2 on whole blood leukocytes, a six-parameter flow cytometric analysis was performed with whole leukocytes from five healthy adults. All donors showed a similar pattern of CRTH2 expression, which was seen in at least four different cell types. Results from a representative donor are shown in Fig. 1. Among granulocytes, which were defined by signals in forward and side light scatter, and surface phenotypes (negative for lymphocyte/monocyte lineage markers CD3, CD4, CD8, CD14, CD19 and CD56), CRTH2 was preferentially expressed on $CD16^-$ leukocytes, which correspond to eosinophils (Fig. 1, region (R) 1 and R2, panels a–d) [15,16]. In the region containing mostly lymphocytes with some monocytes, a high expression of CRTH2 was typically observed on leukocytes with $CD3^-$, $CD4^-$, $CD8^-$, $CD14^-$, $CD19^-$, $CD56^-$ and $Fc\varepsilon R1^{bright}$ phenotypes, which are considered to be basophils (Fig. 1, R3 and R4, panels e–h) [13–15]. In the same region, a small portion of $CD3^+$ T-cells expressed various levels of CRTH2 as reported earlier [5] (Fig. 1, R3 and R6, panels i and l–n), most of which, including $CD8^+$ T-cells, showed the Th2-type cytokine pattern in separate experiments (data not shown). In addition, the same region contained cells that weakly expressed CRTH2 with phenotypes of $CD3^-$, $CD4^{weak}$, $CD14^{-\text{ to intermediate}}$, $CD19^-$, and $CD56^-$ (Fig. 1, R3 and R7, panels i and o–r). These leukocytes also expressed CD33, HLA-DR and often CD16 (data not shown), suggesting they consisted of monocytes [18] and/or dendritic cells [19]. Further studies are necessary to fully characterize these leukocytes. In this study, CRTH2 expression was not identified in other leukocyte populations including NK cells ($CD3^-$/$CD56^+$) and B-cells ($CD19^+$) (Fig. 1 panels k and p, respectively).

The preferential expression of CRTH2 in basophils and eosinophils as well as Th2 cells was further confirmed by the expression of CRTH2 mRNA, which was examined by RT-PCR analysis using highly purified cell populations (Fig. 2). However, the question whether all of basophils and eosinophils in blood express CRTH2 remains to be solved.

The finding that CRTH2 is commonly expressed by basophils, eosinophils and Th2 cells indicated the possible production of a physiological ligand for CRTH2 at the sites of allergic inflammation since all three cell types are known to selectively accumulate in such sites [20–22]. Mast cells also reside at these sites and play a central role in allergic inflammatory process through the release of various mediators [23,24]. Thus, in the next step we examined whether mast cells produce a CRTH2 ligand. Since it was difficult to obtain a sufficient number of pure mast cells from human tissues, we generated such cells in vitro from $CD34^+$ CBMCs. The cells were incubated at 2×10^5 cells/ml in the presence or absence of an anti-FcεR1 mAb CRA1 (1 μg/ml) at 37°C for 5–6 h, then the agonist activity in the culture supernatants was measured by a Ca^{2+} mobilization assay using Fura-2-loaded K562/B19 cells (Fig. 3A). As shown in Fig. 3B, a weak agonist activity was detected in supernatants from unstimulated mast cell cultures (panel b). Marked enhancement of the agonist activity was seen with cells stimulated with CRA1 (CRA1 supernatants), while control IgG (10 μg/ml) did not induce any enhancement and CRA1 itself was not active in the assay (Fig. 3B, panels a, c and d). These results indicated that the agonist activity was derived from $Fc\varepsilon R1^+$ cells, namely, mast cells, but not from other cell types in the cultures. The same CRA1 supernatants elicited no Ca^{2+} mobilization in K562/neo cells (Fig. 3B, panel e) not even in K562/B19 cells when they were pre-treated with an antagonistic anti-CRTH2 mAb BM7 (Fig. 3B, panels h–k), indicating that both spontaneously released and CRA1-induced agonist activities were mediated by CRTH2. In addition to mast cells, blood leukocytes produce various types of chemoattractants including cytokines and chemokines [24]. However, no significant CRTH2-specific agonist activity was detected in culture supernatants of PBMCs, granulocytes and IL-2-maintained T cells, each of which was treated with various combinations of stimuli; phorbol myristate acetate (PMA, 20 ng/ml, Sigma), ionomycin (0.5 μM, Sigma) and phytohemagglutinin (PHA, 5 μg/ml, Sigma) (Fig. 3B, panels f and g and data not shown). These results suggested that the natural ligand for CRTH2 is produced by restricted cell types including mast cells.

The above results demonstrated that CRTH2 is commonly expressed on three cell types (basophils, eosinophils and Th2-type T-cells) involved in allergic processes. Interestingly, the chemokine receptor shared by these three cell types is CCR3, a receptor for CC-chemokines, eotaxin, eotaxin 2, RANTES, MCP-2, -3 and -4, [8,25]. Our previous study showed, however, that CRTH2 does not respond to either eotaxin or RANTES [5], and the present study indicates that CRTH2 interacts with a mast cell-derived factor, which, from a series of preliminary experiments, has a molecular mass of <5 kDa (Hirai, H., unpublished data). Although extensive studies are required to characterize the ligand, our results presented here suggest that CRTH2 mediates the recruitment and/or activation of these three cell types at sites containing mast cells activated by invading allergens.

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