

Utilizing Fcε-Bak chimeric protein for studying IgE–FcεRI interactions

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Abstract

We previously constructed a pro-apoptotic Fcε-Bak chimeric protein, targeted against cells expressing the IgE high affinity receptor (FcεRI). We demonstrated that the chimeric protein is internalized by target mast cells and kills them. These results, which constitute a promising basis for applying this approach to antiallergic therapy, raise some theoretical questions with respect to two major issues: (a) is the monomeric Fcε-Bak–FcεRI complex able to undergo endocytosis, and (b) does the receptor binding domain of human IgE (Fcε) react with rodent FcεRI? In an attempt to answer these questions, we have now thoroughly investigate the interaction of human (h) and mouse (m) Fcε-Bak with FcεRI-positive cells. Using established cultures of rodent and human origin, as well as a primary mouse mast cell culture, we demonstrate that binding of the chimeric protein to the membrane is followed by quick endocytosis, leading to the apoptosis of specific cells. We also confirm that this interaction depends on FcεRI and not on other IgE receptors. We found that the effect of Fcε-Bak on the cells depends on the level of surface FcεRI expression, but not on the origin of the target cells or of the Fcε moiety. We suggest that endocytosis of the monomeric Fcε-Bak–FcεRI complex results from the inability of Fcε-Bak to transduce signals, characteristic of the monomeric IgE–FcεRI complex due to the absence of the variable portion of the IgE molecule. Our results also indicate that at least the Fcε fragment of human IgE is able to interact with both human and rodent FcεRI.

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Introduction

Detailed investigation of the interaction between IgE and its high affinity receptor (FcεRI), the initial step in triggering the degranulation of mast cells and basophils, is important for understanding the mechanisms underlying allergic pathologies. Unraveling this process could facilitate the development of effective antiallergic treatments. IgE shows a high affinity (K_m of approximately 10^{-9}) for its receptor, and forms stable complexes on the surface of specific cells. Bridging of the IgE–FcεRI complexes, due to the interaction of IgE with a specific polyvalent antigen or anti-IgE antibodies, triggers degranulation, resulting in the rapid release of various mediators able to initiate allergic reac-

tions. This can eventually lead to anaphylactic shock or contribute to the pathology of asthma.

The pathological activity of mast cells and basophils could be vigorously curtailed by interfering with their interaction with IgE. One of the standard approaches for preventing FcεRI activation is the competitive saturation of IgE and FcεRI by polypeptides containing the binding sites of the receptor and the ligand molecule, respectively [1]. We recently proposed an additional approach for preventing allergy: the elimination of FcεRI-bearing cells by targeted chimeric proteins [2–4]. These are usually constructed by fusing cDNA encoding a ligand for a specific surface receptor with a bacterial or plant toxin, or a pro-apoptotic protein. Internalization of the killing moiety of the chimeric protein, provided by the specific binding of the targeting moiety to the unique receptor, enables the elimination of a particular cell population, sparing other cell types. In the past two decades, chimeric proteins were shown to be effective in the targeted therapy of human diseases, as well as useful experimental tools for investigating basic biological problems such as ligand–receptor interactions [5].

Abbreviations: BMMC, mouse bone marrow-derived mast cells; Cε-2-4, constant fragments of IgE; DNP-HSA, dinitrophenyl-human serum albumin; ECL, enhanced chemiluminescence; FcεRI, high affinity receptor to IgE; FITC, fluorescein-5-isothiocyanate; h, human; m, mouse.

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The present study focuses on the IgE–Fc ϵ RI interaction, using the human Fc ϵ -Bak chimeric protein. This chimera was constructed by fusing the C ϵ 3 fragment of the constant portion of human IgE as a targeting moiety, with the proapoptotic protein Bak as the killing component. We also constructed an analogous mouse Fc ϵ -Bak chimera. We investigated challenging properties of the hFc ϵ -Bak–Fc ϵ RI interaction: internalization of the chimeric protein by Fc ϵ RI-positive cells and the ability of human Fc ϵ to recognize the mouse Fc ϵ RI. We tested a panel of established mast and basophilic cell lines, as well as mouse primary bone marrow mast cells. Using FACS analysis, confocal microscopy and Western blot, we observed binding and internalization of the human Fc ϵ -Bak chimeric protein by both human and rodent target cells and confirmed the major role of Fc ϵ RI in this process. Endocytosis of the chimeric protein led to the apoptotic death of the cells due to the activity of the Bak moiety. However, cells lacking the Fc ϵ RI were unable to internalize the chimeric protein and, therefore, unaffected by it. We assume that binding of the monomeric Fc ϵ -based chimeric protein to Fc ϵ RI, although providing sensitization to degranulation, leads to endocytosis of the complex rather than to its stabilization on the cell surface.

It is generally accepted that human IgE does not recognize rodent Fc ϵ RI. However, working with mouse cell lines, as well as with mouse primary bone marrow mast cultures, we found that not only mouse, but also human Fc ϵ -based chimeras were able to internalize and kill these cells. Our results, suggesting that human IgE, at least its Fc ϵ fragment, reacts with rodent Fc ϵ RI, offer new possibilities for investigating the interaction of hIgE with Fc ϵ RI with the aid of rodent models.

Methods

Cell lines

The following Fc ϵ RI-positive cell lines originating in different species were used: MC-9, an IL-3-dependent mouse mast cell line of fetal liver origin; C57, a mast cell line of mouse bone marrow origin; the human chronic myeloid leukemia cell lines KU812 and LAMA-84; rat basophilic leukemia cell line RBL-2H3. Detailed information on the source of all the cell lines and their maintenance was reported previously [4]. Mouse bone marrow mast cells (BMMC) were generated from BALB/C mice as described [2] and cultured for 2–3 weeks in 20% conditioned medium obtained from WEHI-3 cells, as a source of IL-3.

Chimeric protein internalization

Confocal microscopy

C57 mouse mast cells were incubated with the hFc ϵ -Bak chimeric protein (10 μ g/ml, total protein concentration) at

4°C in the presence of 0.02% sodium azide, or at 37°C in the absence of azide for various time intervals. Slides were prepared for confocal microscopy as described [4]. Briefly, the cells were fixed on slides and incubated with sheep anti-human IgE antibody (Upstate Biotechnology, NY, USA), followed by incubation with second fluorescein-5-isothiocyanate (FITC)-conjugated antibody (Vector Laboratories, Burlingame, CA, USA) to detect hFc ϵ . Where indicated, slides were also stained with rabbit anti-human Bak antibody (PharMingen International, San Diego, CA, USA) and second Cy5-conjugated antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA.) to detect hBak. Nuclei were counterstained with DAPI (1 μ g/ml). The slides were examined and photographed using a Zeiss LSM 410 confocal laser scanning system attached to a Zeiss Axiovert 135 M-inverted microscope with a 63 $\times$ 1.2 C-apochromate water immersion objective lens (Carl Zeiss, Thornwood, NY, USA). A krypton/argon laser at 488 and 633 nm was used for fluorescence excitation of FITC and Cy5, respectively. A 364 Innova Enterprise Ion Laser was used for DAPI staining.

Identification of the chimeric protein within treated cells by Western Blot

C57, MC-9, LAMA-84 or RBL-2H3 cells (10⁶ cells/ml) were incubated for various time intervals with the hFc ϵ -Bak chimeric protein (10 μ g/ml, total protein concentration). The cells were washed twice with PBS and the proteins were extracted using Tri-Pure isolation reagent (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. The chimeric protein within the cells was detected by Western Blot, using anti-Bak antibody (PharMingen International) and goat anti-rabbit horseradish peroxidase-conjugated antibody (Jackson ImmunoResearch Laboratories, Inc.) or anti-human IgE-horseradish peroxidase-conjugated antibody (Serotec Ltd., Kidlington, Oxford, UK). The bands were visualized by enhanced chemiluminescence (ECL) (Amersham Corp., Arlington Heights, IL, USA).

Internalization of hFc ϵ -Bak into target cells, determined by FACS

Mouse BMMC, C57 and J-774 cells were used in these experiments. Control cells or cells treated with hFc ϵ -Bak for 2 h (1 to 2 $\times$ 10⁶ cells/ml) were washed twice with cold 3% FCS/PBS, followed by centrifugation at 300 $\times$ g for 5 min. The pellets were resuspended in 0.5 ml cold methanol and incubated for 15 min at 4°C, washed twice and resuspended in (50 μ l of the buffer. The BMMC cells were then incubated at 4°C for 30 min with anti-human Bak antibody (PharMingen International), the C57 and J-774 cells (with anti-IgE antibody (Upstate Biotechnology). The washed cells were incubated with the appropriate FITC-conjugated IgG (Jackson ImmunoResearch Laboratories, Inc.) for 30 min at 4°C. After repeated washing, the cells were analyzed using the FACScan and CELLQuest programs.

Cell viability and apoptosis measurements

Viability

Cells were seeded onto 24-well plates (5×10^4 cells in 1-ml culture medium). Chimeric proteins, partially purified as described [4], were then added to a final concentration of up to 20 $\mu\text{g/ml}$ (an equal volume of PBS was added to the control cells). The cultures were harvested after various incubation periods and counted in a hemocytometer. Viability was assessed by trypan blue exclusion.

Annexin V

Nontreated cells and cells incubated with hFc ϵ -Bak (10 $\mu\text{g/ml}$, total protein concentration) for 5 h were washed with cold PBS and double-stained with propidium iodide and annexin V (Genzyme, Cambridge, MA, USA), according to the manufacturer's instructions. The cells were analyzed using the FACSscan and CELLQuest programs (Becton Dickinson, Immunocytometry System, San Jose, CA, USA).

Mitochondrial depolarization

To confirm the apoptotic effect of hFc ϵ -Bak on target cells, we traced one of the major apoptotic consequences, mitochondrial depolarization, using Mitotracker, a selective red fluorescent dye that accumulates in active mitochondria, depending on membrane potential. C57 mouse mast cells and KU812 human basophilic cells were incubated with the hFc ϵ -Bak chimeric protein (10 $\mu\text{g/ml}$, total protein concentration) or with PBS at 37°C for 6 h. At 30 min before the end of incubation, 250 nM of a mitochondrion-specific dye were added (Mitotracker Red CMXRos, Molecular Probes Inc., Eugene, OR, USA). The cells were then washed with PBS and allowed to adhere to glass coverslips pretreated with 10% poly-L-lysine (Sigma Chemical Co., St. Louis, MO, USA). The slides were treated as described previously [4] and incubated with rabbit anti-human Bak antibody (PharMingen International) and with goat anti-rabbit FITC-conjugated antibodies (Jackson ImmunoResearch Laboratories, Inc.) to detect internalized hBak. Confocal microscopy was performed using a krypton/argon laser at 488 and 568 nm lines for fluorescence excitation of FITC and Mitotracker Red CMXRos, respectively.

Determination of Fc ϵ RI and Fc γ RIII/II expression

C57 or J-774 cells (1×10^6 cells/ml) were washed twice with 3% FCS/PBS, centrifuged at $300 \times g$ for 5 min, resuspended in 50 μl of the buffer and incubated for 30 min at 4°C with anti-human Fc ϵ RI antibody or anti-mouse Fc γ RIII/II antibody 2.4G2 (PharMingen International). The cells were washed twice with 3% FCS/PBS containing 0.02% sodium azide, resuspended in 50 μl of the same buffer and incubated with 1 μl of the appropriate FITC-conjugated IgG antibody (Jackson ImmunoResearch Laboratories, Inc.) for 20 min at 4°C. The cells were washed twice with 3% FCS/PBS, resuspended in 0.5 ml PBS and FACS-analyzed.

Degranulation assay

The extent of degranulation was determined by measuring the release of β -hexosaminidase. Degranulation was induced using three methods: (a) treatment with hFc ϵ -Bak (20 $\mu\text{g/ml}$, total protein concentration) following triggering with anti-Bak; (b) sensitization with 20 $\mu\text{g/ml}$ of human IgE-anti-nitrophenyl (NP) (Serotec) following triggering with sheep anti-human IgE antibodies; and (c) triggering with dinitrophenyl-human serum albumin (DNP-HSA; Sigma Co.) of cells passively activated with mouse IgE-anti-DNP (1 $\mu\text{g/ml}$) (clone SPE-7, Sigma Co.). The cells were washed twice with Hanks Balanced Salt Solution (HBSS) containing 1% gelatin. Cell suspensions were incubated for 1 h at 4°C with hFc ϵ -Bak or human or mouse IgE. They were then washed with the same buffer, resuspended in 250 μl of HBSS containing 1% gelatin, 1 mM MgCl_2 and 1.6 mM CaCl_2 and incubated with the appropriate triggering agent at 37°C. The cells were collected 1 h after triggering, and β -hexosaminidase activity was measured, as described [4], in 200 μl aliquots of the supernatant.

Results

Binding and internalization of chimeric proteins into target cells

Mouse and human Fc ϵ -based chimeric proteins (mFc ϵ -Bak and hFc ϵ -Bak, respectively) were designed to recognize and interact solely with the Fc ϵ RI. To confirm that human Fc ϵ -Bak binds to the mouse Fc ϵ RI, we followed the initial steps of its interaction with target mouse mast cells. Binding of hFc ϵ -Bak to the cell surface was traced by confocal microscopy of C57 mouse mast cells treated with the chimeric protein for 1.5 h at 4°C in the presence of 0.02% sodium azide, and stained with anti-hIgE antibody. Under these conditions, hFc ϵ -Bak is unable to enter the cells, and its binding to the Fc ϵ RI is detected as a green layer on the cell membrane (Fig. 1A1).

Internalization of hFc ϵ -Bak, following its binding to the Fc ϵ receptor, was demonstrated using three approaches:

(I) Following the fate of hFc ϵ -Bak within the target cells with the aid of confocal microscopy. A significant increase in hFc ϵ -Bak was detected within mouse C57 mast cells after incubation with the chimeric protein for 3 h at 37°C and staining with anti-IgE antibody (Figs. 1A2 and A3). When cells treated with hFc ϵ -Bak for 6 h were double-stained with anti-Bak antibody and anti-hIgE antibody (Figs. 1A4 and A5), superimposition of the stains resulted in a yellow color (Fig. 1A6), showing that the intact chimeric protein was present within the target cells. It should be pointed out that we used specific anti-human Bak antibody that does not cross-react with the endogenous mouse Bak protein.

(II) Western Blot analysis of proteins extracted from treated cells. A Bak-positive band, corresponding to the molecular weight of the full-length hFcε-Bak chimeric protein (38 kDa), was detected in the lysates of C57 and MC-9 mouse cells treated with hFcε-Bak (Fig. 1B). This band was also observed in extracts of rat (RBL-2H3) and human (LAMA-84) target cells treated with the chimeric protein (Fig. 1B).

(III) FACS analysis. Since fresh mouse mast cells may react differently from established mouse cell lines, we next investigated the internalization of hFcε-Bak by a primary mast cell culture originating in normal mice (BMMC). This

experiment was preceded by characterization of the degree of maturation of the BMMC. Mouse bone marrow cells cultivated for 2 weeks in medium containing IL-3 were stained with anti-FcεRI antibody and FACS-analyzed (Fig. 1C). About 55% of the cells expressed FcεRI. FACS analysis of BMMC treated with hFcε-Bak for 2 h and stained with anti-Bak antibody revealed an increase in the Bak-positive cell population, which accounted for 16.3% of the total cell number (Figs. 1D and E). When parallel experiments were performed on mouse C57 cells using anti-IgE antibody, a similar effect was observed, although of greater magnitude: 65% of the cells were IgE-positive (Fig. 4B). The difference between the two experiments may be attributed to the higher level of FcεRI expression in C57 cells (97%) [4].

All three unrelated methods revealed internalization of hFcε-Bak into mouse mast cells.

Cytotoxic and apoptotic effects of human Fcε-Bak chimeric proteins on target cells

mFcε-Bak and hFcε-Bak were designed for the targeted killing of mast cells and basophils. The mouse chimeric protein was shown to specifically eliminate mouse C57 and MC-9 mast cells by apoptosis [4]. We also demonstrated that mouse mast cell lines were sensitive to treatment with the human Fcε-based chimera [4]. This preliminary observation led us to investigate in detail the effects of human Fcε-Bak on target mouse cells. An FcεRI-positive mouse C57 mast cell line, as well as nonrelevant cells, were incubated with the

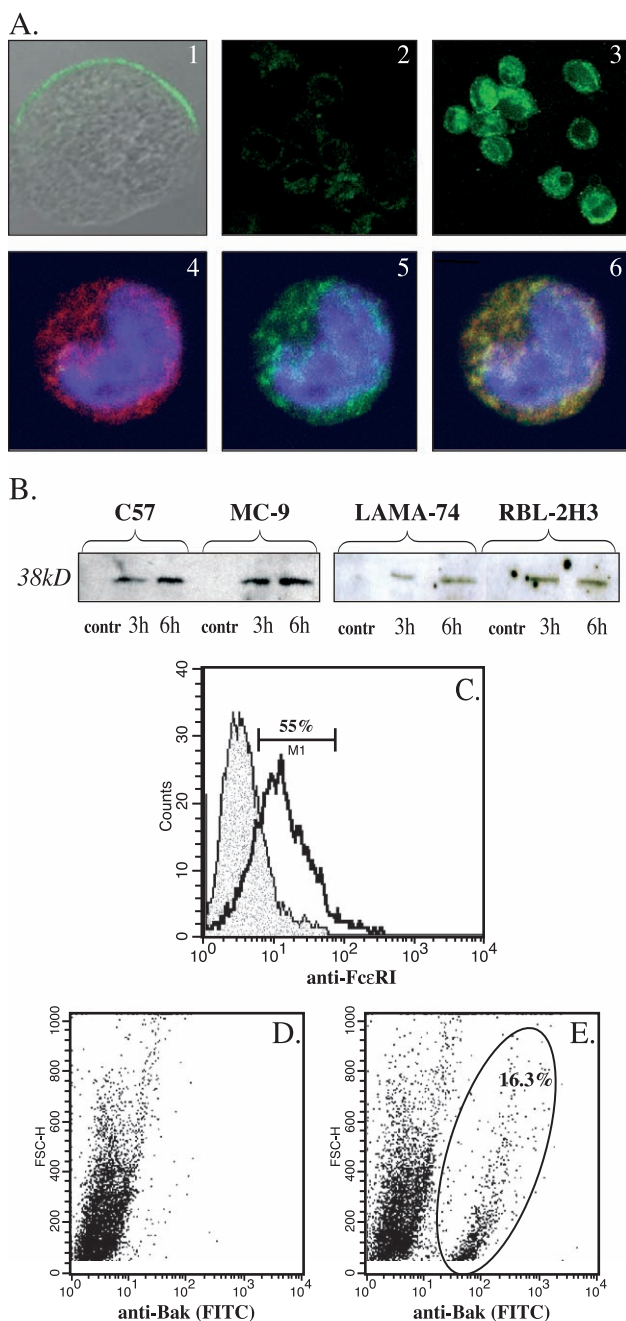


Fig. 1. Binding and internalization of hFcε-Bak by target cells. (A) Binding and internalization of hFcε-Bak by C57 cells was studied by confocal microscopy of C57 cells exposed to the chimeric protein. Slides were examined by laser fluorescence confocal microscopy, as described in Methods. Where indicated, hFcε-Bak was added at 10 μg/ml (total protein concentration). (1) Incubation of C57 cells with hFcε-Bak for 1.5 h at 4°C in the presence of NaN₃ prevents internalization of the chimeric protein. (2 and 3) C57 cells incubated at 37°C for 3 h: (2) control cells, not treated with chimeric protein; (3) cells exposed to hFcε-Bak. Slides 1–3 were incubated with anti-hIgE antibody and stained with the appropriate FITC-conjugated antibody. The fields were photographed with a 63 × 1.2 objective lens, ×3. (4–6) C57 mast cell after 6 h of treatment with hFcε-Bak at 37°C were incubated with both anti-Bak antibody and anti-IgE antibody and the appropriate second antibody conjugated with Cy5 and FITC, respectively: blue indicates nuclear staining; red (4)—staining for anti-hBak antibody; green (5)—staining for anti-hIgE antibody; (6)—superimposition of the three colors. (B) Western blot analysis of total protein fractions from control cells and cells treated with hFcε-Bak (10 μg/ml, total protein concentration) using anti-Bak antibody. (C–E) Internalization of hFcε-Bak by primary BMMC cells. (C) Characterization of BMMC maturation. Cells were incubated with anti-human FcεRI antibody stained with second FITC-conjugated antibody and FACS-analyzed. Cells stained with second antibody alone: gray; cells treated with both antibodies: blank. M1: percentage of FcεRI-positive cells. One representative experiment out of three is shown. (D and E) FACS analysis of hFcε-Bak internalization by BMMC. All cells were stained with anti-Bak antibody and the appropriate FITC-conjugated antibody: (D) nontreated cells; (E) cells treated with hFcε-Bak for 2 h; the number indicates the percentage of Bak-positive cells. One representative experiment out of three is shown.

human chimeric protein for 48 h, and the viable cells were counted. Treatment with the hFc ϵ -Bak chimera caused a dramatic decrease in the viability of the C57 cells in a dose-dependent manner. Similar results were obtained in control experiments using the mFc ϵ -Bak chimeric protein (Fig. 2A). This response is specific as we found that human and mouse Fc ϵ -Bak did not influence the survival of the nontarget cell lines HUT-102 (T-cell lymphoma), J-774 (mouse monocyte-macrophage cell line) and HMC-1 (a human mast cell line that does not express Fc ϵ RI) (Fig. 2B and results not shown). We also tested the effect of another Bak-based chimeric protein, GnRH-Bak [6]. This protein, containing an irrele-

vant targeting moiety, was expressed and prepared exactly as Fc ϵ -Bak. We found that this control chimeric protein had no cytotoxic effect on Fc ϵ RI-positive cell lines (results not shown). Thus, it appears that Fc ϵ -Bak chimeric proteins of both human and mouse origin specifically recognize and kill mast cells and basophils.

Apoptotic death due to internalization of h- and mFc ϵ -Bak by the mouse mast cells was followed by annexin V staining. C57 cells treated for 5 h with hFc ϵ -Bak exhibited an approximately 25% increase in the apoptotic population, as compared with nontreated cells (Figs. 3A and B). A similar effect was observed upon treating C57 cells with mFc ϵ -Bak (Fig. 3C). It should be pointed out that application of the annexin V assay for measuring apoptosis in mast cells is limited, since cells exposing phosphatidylserine due to degranulation are also stained [7]. However, our previous experiments clearly show that treatment with Fc ϵ -Bak does not lead to cell degranulation [4].

Another means of measuring apoptosis is to follow mitochondrial depolarization by staining with Mitotracker Red CMXRos, a selective red-fluorescent dye that accumulates in active mitochondria, depending on their membrane potential. We compared the effects of 6-h incubation with hFc ϵ -Bak on mouse (C57) and human (KU-812) target cells. In both cases, the mitochondria of nontreated cells were intensively stained red (Figs. 3D and G), while treatment with hFc ϵ -Bak led to a pronounced decrease in red staining due to depolarization (Figs. 3E, H, and F, I). In parallel, we observed the internalization of hFc ϵ -Bak, as reflected by an increase in green staining by anti-Bak antibody (Figs. 3F and I). The decline in the red staining may be attributed to the apoptotic changes caused by the internalization of Fc ϵ -Bak.

Specificity of hFc ϵ -Bak interaction with Fc-receptors

We next investigated whether the hFc ϵ -Bak chimeric protein indeed enters the cell via the Fc ϵ RI. Because IgE recognizes a number of immunoglobulin receptors, we tested the possible involvement of receptors other than Fc ϵ RI (all specific for IgE) in the Fc ϵ -Bak interaction with mouse mast cells. At least four other receptors for IgE have been described: Fc ϵ RII (CD23), ϵ BP (Mac-2), Fc γ RII and Fc γ RIII. On the basis of theoretical considerations, these receptors should not interact with monomeric Fc ϵ . The Fc ϵ -Bak chimeric molecule is a monomer and differs from the native IgE molecule, which contains two heavy chains. Therefore, the involvement of receptors other than Fc ϵ RI in the interaction of the chimeric protein with C57 mouse cells is unlikely (see also Discussion). However, because C57 cells express high levels of Fc γ RIII and Fc γ RII, we tested the possible interaction of Fc ϵ -Bak with these receptors. Mouse J-774 cells, known to express both Fc γ RIII and Fc γ RII [8], were unaffected by treatment with the hFc ϵ -Bak chimeric protein and remained viable for at least 72 h (Fig. 2B). We also investigated the internalization of hFc ϵ -Bak into J-774 cells. After treating the cells with the chimeric

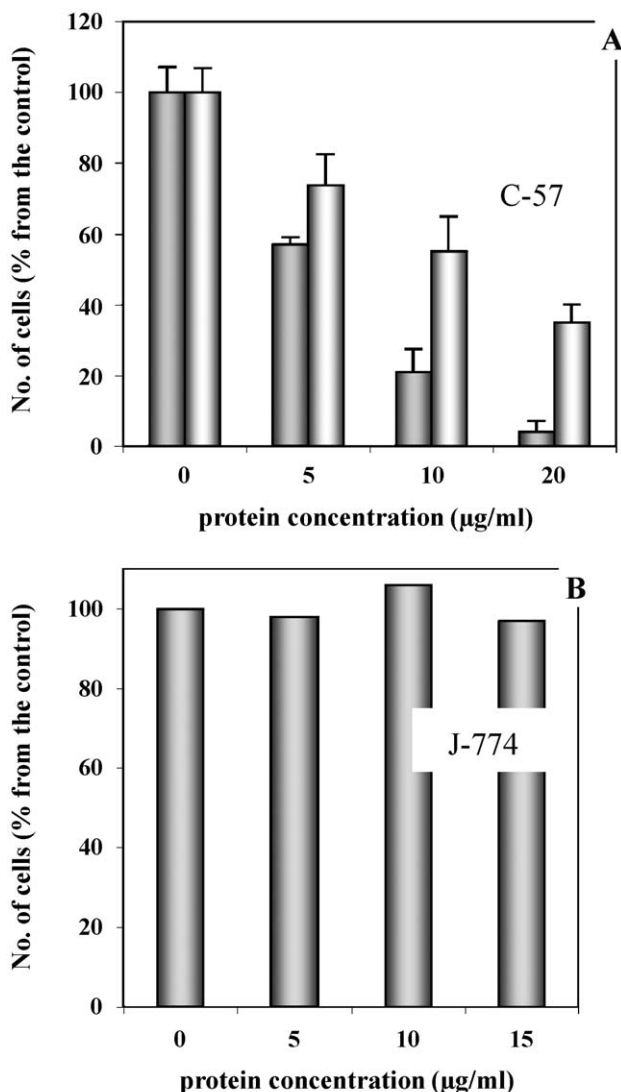


Fig. 2. Effect of Fc ϵ -based chimeric protein concentration on survival of target C57 cells (A) and nontarget J774 cells (B). C57 and J774 cells (5×10^4 cells/ml) were incubated for 48 h with increasing concentrations of hFc ϵ -Bak (■) or mFc ϵ -Bak (□) (μg/ml of total protein) and counted. The number of cells is presented as the mean of triplicates of treated cells compared with that of the cells in the control samples. Each histogram is the mean of three separate experiments.

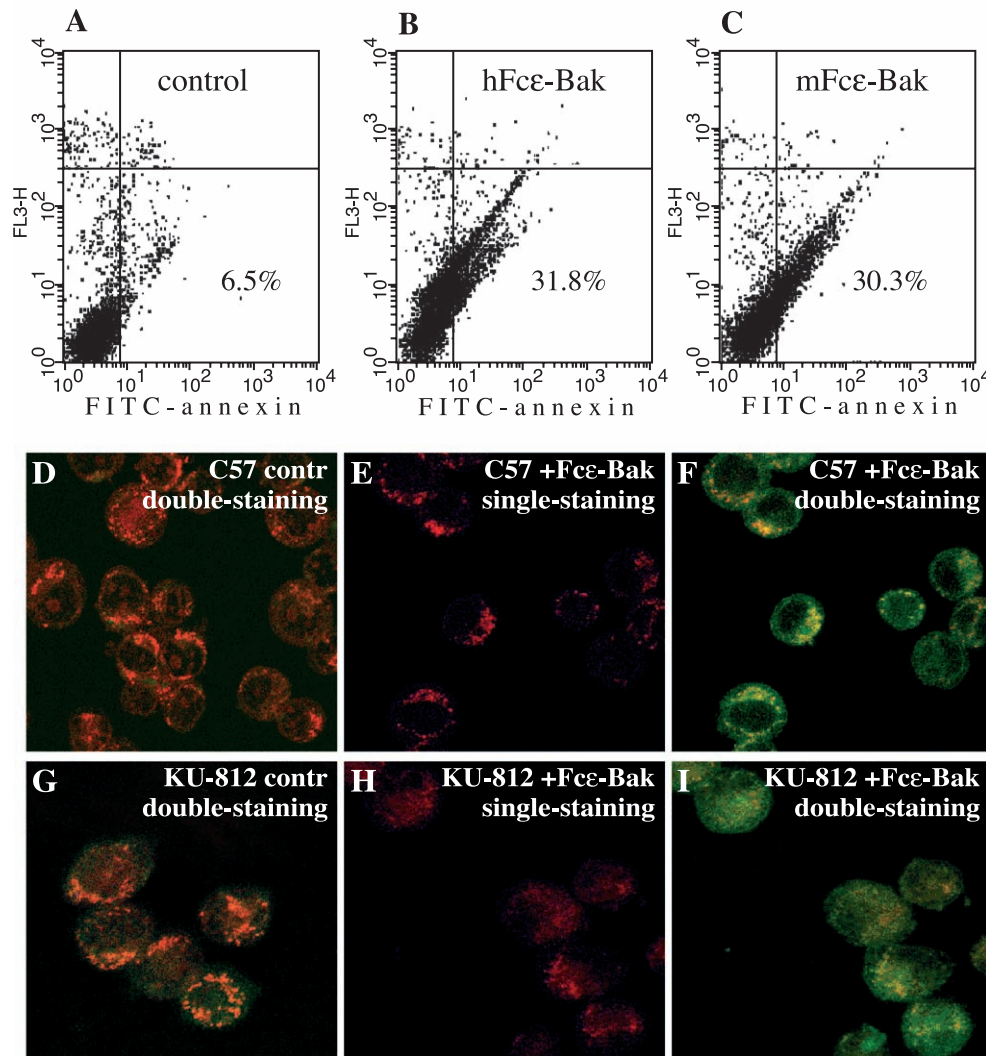


Fig. 3. Apoptotic effects of hFcε-Bak on mouse and human target cells. (A–C) FACS analysis of C57 mouse cells stained with annexin V-FITC following treatment with hFcε-Bak or mFcε-Bak. (A) Nontreated cells; (B) cells treated with hFcε-Bak for 5 h; (C) cells treated with mFcε-Bak for 5 h. One representative experiment out of three is depicted. The numbers indicate the percentage of apoptotic cells. Both chimeric proteins were added at 10 µg/ml (total protein concentration). (D–I) Mitochondrial depolarization correlates with internalization of hFcε-Bak into C57 and KU-812 cells. Nontreated mouse (C57) and human (KU-812) target cells and cells treated with hFcε-Bak for 6 h were stained with Mitotracker Red CMXRos (red) for mitochondrial activity and with anti-Bak antibody (green) to visualize the internalization of hFcε-Bak. Panels D, F, G and I show overlaying of the two stainings; panels E and H, Mitotracker Red staining only. Intensive staining with Mitotracker in the control cells (panels D and G) strongly decreased following 6 h treatment with hFcε-Bak (panels E and H), while anti-Bak staining increased (panels F and I). One representative experiment out of three is shown.

protein for 2 h under standard growth conditions, the cells were fixed and FACS-analyzed, using anti-IgE antibody. No increase in the staining of J-774 cells with anti-IgE antibody was detected following such treatment (Fig. 4A). This was also confirmed in experiments with anti-Bak antibody (results not shown). When the same experiment was performed using C57 mouse mast cells, which express FcγRIII as well as FcεRI, the IgE-positive cell population increased by approximately 60% (Fig. 4B). To compare the level of FcγRIII expression on J-774 and C57 cells, we used 2.4G2 antibody, specific for FcγRIII/FcγRII. Both cell lines were FcγRIII-positive (Figs. 4C and D), and the level of receptor expression was similar (approximately 75%). However, C57 cells, as opposed to J774 cells, express FcεRI. The finding

that only C57 cells, and not J-774 cells, were sensitive to treatment with hFcε-Bak, corroborates that FcγRIII/FcγRII are not involved in the interaction and that, most probably, it is the mFcεRI that is targeted by hFcε-Bak.

Artificial degranulation of mouse C57 mast cells passively activated by hFcε-Bak or recombinant human IgE

Bridging of IgE bound to its high affinity receptor, provided by IgE interaction with a specific polyvalent antigen or anti-IgE antibodies, triggers cell degranulation. To confirm that binding of hFcε-Bak to FcεRI produces an active complex that can trigger degranulation upon bridging, we sensitized mouse C57 mast cells with hFcε-Bak (20 µg/

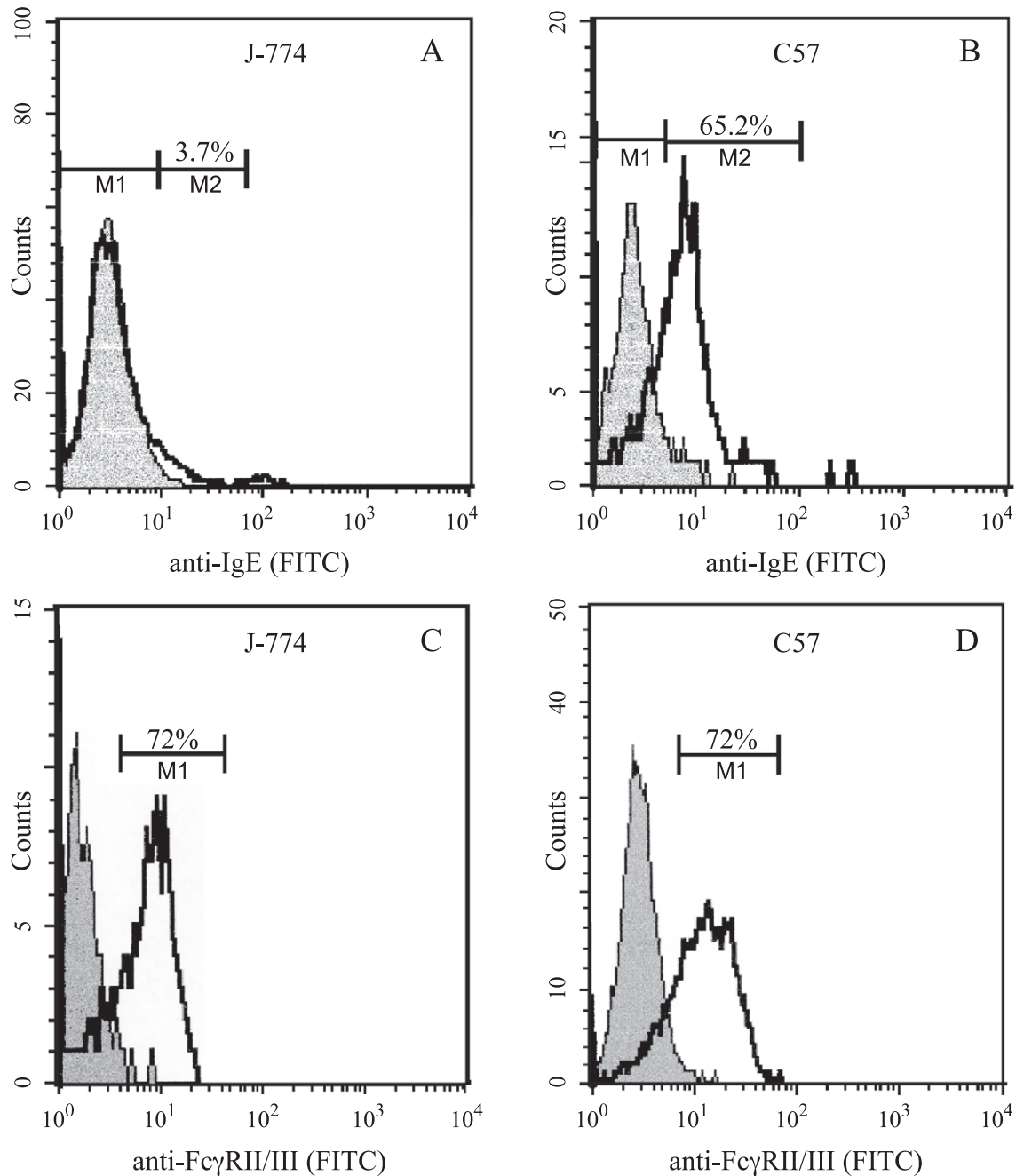


Fig. 4. Both J-774 and C57 mouse cells express high levels of Fc γ RIII/II, but internalization of hFc ϵ -Bak occurs only in the C57 cells. FACS analysis of J-774 (A) and C57 (B) cells. Cells were treated with hFc ϵ -Bak and stained with anti-IgE antibodies. Control cells: gray; cells treated with hFc ϵ -Bak for 2 h: blank. M1, population negative for anti-IgE staining; M2, positive population. One representative experiment out of three is shown. Levels of Fc γ RIII/II expression on J-774 (C) and C57 (D) cell lines measured by FACS analysis. Cells were incubated with anti-mouse Fc γ RIII/II antibody stained with second FITC-antibody and FACS-analyzed. Cells stained with second antibody alone: gray; cells treated with both antibodies: blank. M1 represents the percentage of Fc γ RIII/II-positive cells. One representative experiment out of three is shown.

ml, total protein concentration) and artificially triggered them with anti-Bak antibody. Indeed, we observed an increase of approximately 45% in degranulation, as compared with spontaneous degranulation in the control cells. This degranulation was about 4-fold lower than that triggered by mouse IgE-anti-DNP and DNP-albumin in a parallel experiment, (Fig. 5), albeit significant. Mouse C57

cells were also passively activated with 20 μ g/ml of human IgE-anti-NP, following triggering with anti-hIgE. Degranulation increased by approximately 50% (Fig. 5), a value similar to that obtained by activation with the hFc ϵ -Bak chimeric protein. In both cases, secondary antibodies alone did not cause degranulation (Fig. 5). The ability of hFc ϵ -Bak and hIgE to activate mFc ϵ RI to degranulate further confirms

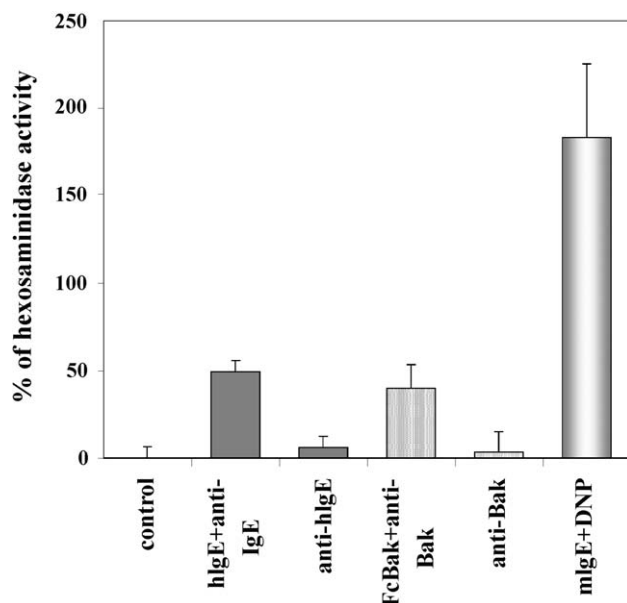


Fig. 5. Degranulation of C57 cells. Degranulation was triggered by preincubation with 20 μ g/ml of human IgE-anti-NP, followed by incubation with anti-hIgE antibody (■); treatment with 20 μ g/ml (total protein concentration) of hFc ϵ -Bak followed by the addition of anti-Bak antibody and anti-Bak antibody (□); or by preincubation with 1 μ g/ml of mouse IgE-anti-DNP, followed by incubation with 50 ng/ml of DNP-albumin (▨). Control experiments with anti-hIgE or anti-Bak alone are also presented. The histograms show the percentage of β -hexosaminidase activity in the medium of the activated cells as compared with that of the nonactivated cells. Spontaneous release served as the basic level. Each histogram is the mean of duplicates. One experiment out of three is shown.

the interaction between the human Fc ϵ moiety and mouse Fc ϵ RI. The fact that bridging hFc ϵ -Bak with antibodies stimulates mFc ϵ RI and triggers degranulation confirms that this complex transduces signals like the physiological complex composed of mFc ϵ RI, mIgE and antigen.

Discussion

We recently constructed a novel chimeric protein targeted against the human Fc ϵ RI by fusing a fragment of human Fc ϵ with an apoptosis-inducing protein Bak (hFc ϵ -Bak). The design of this protein was based on our results with the bacterial toxin-based chimeric protein Fc ϵ -PE, also targeted against Fc ϵ RI. The Fc ϵ -PE chimeric protein was shown to specifically enter and kill the target mast cells and basophils in vitro [2] and to prevent passive cutaneous anaphylaxis reaction in vivo [3]. In our novel construct, we replaced the bacterial toxin with the pro-apoptotic protein Bak, and introduced the Fc ϵ RI-binding fragment of human IgE. We demonstrated that the hFc ϵ -Bak chimeric protein enters the target cells and prompts apoptosis without triggering degranulation [4]. In the present study, we investigated the binding of hFc ϵ -Bak and its internalization by various mast and basophilic cell lines and nontarget cell lines, as well as the pro-apoptotic function of the chimeric

protein within the cell. Using FACS analysis, confocal microscopy and Western blot, we found that hFc ϵ -Bak binds specifically to mast cells and basophils of both human and rodent origin. This binding results in internalization of hFc ϵ -Bak within a few hours (Fig. 1), ultimately inducing the apoptotic death of the target cells (Figs. 2 and 3). We also demonstrated the ability of hFc ϵ -Bak to sensitize mast cells and to trigger artificial degranulation upon bridging with anti-Bak antibodies (Fig. 5).

We next examined whether internalization of the Fc ϵ -Bak chimeric protein depends mainly on Fc ϵ RI and not on other IgE-specific receptors. An additional IgE receptor, known as Fc ϵ R2 or CD23, is expressed mostly on lymphocytes and binds only disulfide-linked ϵ -chain dimers [9]. The monomeric C ϵ 3 does not bind Fc ϵ R2 [10]. We demonstrated earlier [2] that Fc ϵ R2 is not targeted by the mouse Fc ϵ -derived chimeric protein. In addition, it was shown that mouse C57 mast cells [8] and BMDC [11] do not express this receptor. Therefore, Fc ϵ R2 is most likely not involved in the effects of the chimeric protein on mouse mast cells. Another IgE receptor, ϵ BP, binds only glycosylated IgE [12]. Since Fc ϵ -Bak is expressed in bacteria, it is not glycosylated and is not reactive towards ϵ BP, as confirmed by our previous observation that ϵ BP is not targeted by mFc ϵ -PE [2]. It is known that Fc γ R2 and Fc γ R3 have an affinity for IgE-immune complexes [8]. As the monomeric structure of the Fc ϵ -Bak chimeric protein does not fulfill the binding requirements of these receptors, the probability that Fc γ R2 or Fc γ R3 is involved in chimeric protein binding is negligible. However, the level of Fc γ R3 expression by C57 cells is very high. Thus, to prove definitively that the interaction of hFc ϵ -Bak with mast cells does not depend on these receptors, we tested our chimera on the Fc γ R2/3-positive mouse cell line J-774. The cells were not affected by treatment with hFc ϵ -Bak (Figs. 2B and 4A). This observation is consistent with our previous results showing that other nontarget cells, HMC-1, HUT-102 and Jurkat, which lack Fc ϵ RI expression, were resistant to treatment with hFc ϵ -Bak [4]. Taking into consideration our previous finding that pretreatment of chimeric protein with anti-hIgE antibody prevents its apoptotic activity [4], we suggest that interaction of Fc ϵ -Bak with mast cells is due to specific recognition of Fc ϵ RI by the C ϵ 3 domain of the chimera. As demonstrated, this specific recognition leads to internalization of the Fc ϵ -Bak–Fc ϵ RI complex by the cells.

However, this conclusion is not consistent with many other observations regarding the internalization of the monomeric IgE–Fc ϵ RI complex. Since the 1980s, it is known that aggregation of the IgE–Fc ϵ RI complex by multivalent antigen at 37°C causes rapid endocytosis of the complexes, such that 50–60% of them are internalized with a half-time of about 5 min [13]. In contrast, until quite recently, very little was known about the internalization of monomeric complexes and nonoccupied Fc ϵ RI. In the last 2–3 years, a few laboratories have investigated this issue to understand how monomeric IgE stimulates

Fc ϵ RI overexpression [14–16]. They concluded that ligand–receptor interaction stabilizes the IgE–Fc ϵ RI complex at the cell surface, and prevents receptor internalization and degradation. They also suggested that regulation of Fc ϵ RI α expression on mast cells and basophils is mediated by a process of IgE-regulated loss concomitant with constant synthesis. Endocytosis occurs only in the case of unoccupied receptors, while the surface IgE–Fc ϵ RI complexes are very stable, with the half-life of about 7–10 days [15]. Our finding that Fc ϵ -Bak binding to the receptor leads to internalization rather than to surface stabilization of the complex calls for further investigation.

The different consequences of IgE and Fc ϵ -Bak interaction with the Fc ϵ RI can be explained in the context of the recently proposed theory of signaling by monomeric IgE. Recent observations suggest that the interaction of monomeric IgE with Fc ϵ RI not only completely prevents endocytosis of the ligand–receptor complexes but also stimulates cell survival [11,17,18], possibly leading to the secretion of pro-survival cytokines [17,19]. It is conceivable that the interaction triggers a number of signaling pathways other than degranulation [17,19]. It is believed that formation of small clusters via ligand–ligand interactions mediated through the IgE-variable regions results in prolonged intracellular signaling of low intensity through the Fc ϵ RI. In the framework of this theory, we assume that since our constructs lack the variable portion of IgE, loading of Fc ϵ RI with Fc ϵ -Bak would theoretically avert cluster formation by preventing the interaction between two ligand molecules. Thus, no signal will be transduced and the receptor will behave as though unengaged and will undergo endocytosis, as indicated by our results (Fig. 1). It should be noted that internalization of monomeric ligand–receptor complexes was indeed observed by Xu et al. [20] in experiments using FITC- and Cy3-labeled IgE. The labeling most probably abolished the ability of the Fab regions of IgE to cross-talk, thus preventing signal transduction and stabilization of these complexes. It should also be mentioned that IgE molecules differing in Fab region, produced by various hybridomas, showed varied signaling capacity, presumably due to the difference in intensity of ligand–ligand interactions [17].

However, it is also possible that binding of the chimeric protein to the Fc ϵ RI results in a nonproductive complex, incapable of any type of signaling. To check this possibility, we artificially bridged Fc ϵ -Bak with the aid of anti-Bak antibody. The bridging stimulated receptor cross-linking and triggered degranulation (Fig. 5). This artificial degranulation most probably suggests that Fc ϵ -Bak forms a productive complex with Fc ϵ RI, but, unlike certain monomeric IgE, does not allow a ligand–ligand interaction of low intensity leading to signaling. Due to the absence of signaling via the Fc ϵ -Bak–Fc ϵ RI monomeric complex, rapid internalization of the complex, rather than its surface stabilization, is observed.

In the present study, we also examined in depth the activity of the human Fc ϵ -Bak chimeric protein towards rodent mast cells and basophils. The species specificity of the IgE interaction with the high-affinity Fc ϵ receptor was the subject of extensive investigation, mainly some 20–30 years ago. However, the ability of human IgE to interact with rodent cells is not clear. It is commonly considered that interaction of this type is negligible. A review of the literature on the interaction of human IgE with rodent mast cells and basophils shows highly controversial results: some investigators failed to demonstrate the binding of human IgE to rat mast cells or basophils [21–23], others provided evidence of the interaction [24–33]. The discrepancy between the various findings is voiced in various reviews [34–36].

Our results demonstrate that a chimeric protein of human origin interacts with human target cell lines in the same way as with mouse cells (Figs. 1–3 and 5). We also provide evidence that the effects of hFc ϵ -Bak on the cell are due solely to the interaction via Fc ϵ RI (Fig. 4). To confirm the physiological relevance of these observations, we also tested the sensitivity of BMMC to treatment with the chimeric protein and obtained similar results (Figs. 1C–E). Thus, we conclude that the constant portion of human IgE is able to interact with rodent Fc ϵ RI and to adequately sensitize the cells to degranulation.

Chimeric proteins can be considered physiological vehicles for internalizing ligands into specific cells, utilizing ligand–receptor interactions for this purpose. Therefore, the activity of the two-headed chimeras, and consequently, ligand–receptor pathways can be followed readily and accurately both *in vitro* and *in vivo*. A case in point is IL-2-PE66^{4glu}, which was used to investigate the role of various IL-2 receptor subunits in receptor internalization [37]. In the present study, we utilized this unique approach to study the hFc ϵ interaction with mouse and human Fc ϵ RI. We found that at least the Fc ϵ fragment of human IgE readily interacts with mouse Fc ϵ RI. On the basis of our results, we suggest that the interaction of Fc ϵ 3-Bak (containing only the constant C ϵ -3 portion of IgE) with Fc ϵ RI does not provide signal transduction, and, the complex, therefore, undergoes rapid endocytosis. These conclusions appear to be correct not only as regards the C ϵ -3 portion of IgE but are also applicable to the constant fragments of IgE (C ϵ -2-4), since another chimeric protein, hFc ϵ 2-4-Bak, behaves quite similarly to hFc ϵ 3-Bak (results not shown).

Many laboratories are concentrating their efforts on designing IgE and Fc ϵ RI derivatives for the development of antiallergic drugs that could prevent the negative consequences of mast cell and basophil activation. Convenient experimental models for *in vitro* and especially *in vivo* investigation of hIgE interaction with Fc ϵ RI are not accessible. In contrast to rodent cell lines, native cell lines of human origin with a high level of Fc ϵ RI expression are not available. Production of highly purified primary cultures of human mast cells and basophils is not straightforward: cells

transfected with plasmids, encoding various subunits of Fc ϵ RI, do not represent the natural context of a mast cell. Moreover, to the best of our knowledge, transgenic mice carrying the α -subunit of human Fc ϵ RI [38,39], have not been used to investigate this issue. We now demonstrate that human Fc ϵ interacts with mouse and rat Fc ϵ RI. Thus, rodent Fc ϵ RI-positive cell lines could serve as convenient and readily available models in the search for molecules able to block the human IgE interaction with the Fc ϵ RI.

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