Mouse mast cell gp49B1 contains two immunoreceptor tyrosine-based inhibition motifs and suppresses mast cell activation when coligated with the high-affinity Fc receptor for IgE

(immunoglobulin superfamily/killer cell inhibitory receptors/exocytosis/leukotriene C4)

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ABSTRACT Mouse mast cells express gp49B1, a cell-surface member of the Ig superfamily encoded by the gp9B gene. We now report that by ALIGN comparison of the amino acid sequence of gp49B1 with numerous receptors of the Ig superfamily, a newly recognized family has been established that includes gp49B1, the human myeloid cell Fc receptor for IgA, the bovine myeloid cell Fc receptor for IgG2, and the human killer cell inhibitory receptors expressed on natural killer cells and T lymphocyte subsets. Furthermore, the cytoplasmic domain of gp49B1 contains two immunoreceptor tyrosine-based inhibition motifs that are also present in killer cell inhibitory receptors; these motifs downregulate natural killer cell and T-cell activation signals that lead to cytotoxic activity. As assessed by flow cytometry with transfectants that express either gp49B1 or gp49A, which are 89% identical in the amino acid sequences of their extracellular domains, mAb B23.1 was shown to recognize only gp49B1. Coligation of mAb B23.1 bound to gp49B1 and IgE fixed to the high-affinity Fc receptor for IgE on the surface of mouse bone marrow-derived mast cells inhibited exocytosis in a dose-related manner, as defined by the release of the secretory granule constituent -hexosaminidase, as well as the generation of the membrane-derived lipid mediator, leukotriene C4. Thus, gp49B1 is an immunoreceptor tyrosine-based inhibition motif-containing integral cell-surface protein that downregulates the high-affinity Fc receptor for IgE-mediated release of proinflammatory mediators from mast cells. Our findings establish a novel counterregulatory transmembrane pathway by which mast cell activation can be inhibited.

The rat mAb B23.1 recognizes an epitope expressed on an ~49-kDa glycoprotein on the surface of mouse mast cells, which is synthesized from an intracellular 37-kDa protein precursor (1). Immunofluorescence purification of a cell-surface protein from mast cells with mAb B23.1, determination of its amino terminal amino acid sequence, and screening of a mast cell cDNA library with oligonucleotides encoding the amino terminal sequence provided three cDNAs that encode this amino terminal amino acid sequence (2, 3). One of these (gp49B1) predicts a 35-kDa protein core that contains two C2-type, Ig-like domains in the extracellular region (3). The expression of the gp49B1 cDNA in COS transfectants confers binding of mAb B23.1, indicating that the mAb recognizes an epitope present on gp49B1 (3). One of the other cDNAs (gp49A), isolated in the same screen, predicts a 32-kDa protein core that has two C2-type, Ig-like domains in its extracellular region and is 89% identical at the amino acid level to gp49B1. gp49A also has 100% amino acid identity with gp49B1 in the transmembrane domain and 88% identity in the first 34 amino acids of the cytoplasmic domain. The cytoplasmic region of gp49A then diverges for 8 amino acids because of a frameshift in the nucleotide sequence and terminates so that it is 32 amino acids shorter than gp49B1. Whether the specificity of the epitope recognized by mAb B23.1 is limited to gp49B1 as compared with gp49A had not been previously established.

When B lymphocytes are exposed to F(ab’)2 fragments of anti-membrane Ig antibody, a signal transduction cascade is elicited through the B-lymphocyte antigen receptor that results in B-lymphocyte proliferation and differentiation into anti-body-secreting cells (4, 5). In contrast, stimulation with intact anti-membrane Ig results in attenuated B-lymphocyte signal transduction, as measured by the influx of extracellular calcium (6) and levels of cellular inositol trisphosphates (7). The fact that the suppression of B-cell stimulation can be prevented by blocking the binding of intact anti-membrane Ig to Fc receptors for IgG, type IIIB (FcγRIIb1) on the B lymphocyte (4, 8), indicates that coligation of the B-lymphocyte antigen receptor with FcγRIIb1 inhibits B-lymphocyte stimulation. Amino acid substitution experiments revealed that a 13-amino acid region of the cytoplasmic domain of FcγRIIb1 is sufficient to inhibit B-lymphocyte stimulation (8). Furthermore, a tyrosine in the 13-amino acid sequence is phosphorylated during coligation of the two receptors, and the mutation of this tyrosine to phenylalanine prevents the FcγRIIb1-mediated inhibition of B lymphocyte activation (8). The cytosolic tyrosine phosphatase SHP-1 (formerly termed protein tyrosine phosphatase 1C and hematopoietic cell phosphatase) coinmunoprecipitates with FcγRIIb1 only when the receptor is phosphorylated on tyrosine. As assessed by SDS/PAGE immunoblots, a phosphorylated, but not nonphosphorylated, peptide matching this region of FcγRIIb1 binds SHP-1 from B-lymphocyte lysates in equimolar quantities whether the cells are unstimulated, stimulated, or stimulation-inhibited (9); thus, phosphorylation of the FcγRIIb1 motif is critical to the

Abbreviations: FceR, Fc receptor for IgA; FcεRI, high-affinity Fc receptor for IgE; FcγR2, Fc receptor for IgG2; FcγRIIb1 and FcγRIIb2, Fc receptors for IgG, type IIb1 and IIb2, respectively; FITC, fluorescein isothiocyanate; H/B/A, HBSS containing 0.1% (wt/vol) BSA and 0.02% (wt/vol) sodium azide; ITIM, immunoreceptor tyrosine-based inhibition motif; KIR, killer cell inhibitory receptor; LT, leukotriene; mMBC, mouse bone marrow-derived mast cells; mMIR, mouse mast cell inhibitory receptor; NK, natural killer; RT, reverse transcriptase; WCM, WEHI-3 cell-conditioned medium.

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binding of SHP-1. Moreover, because binding of the phosphorylated, but not nonphosphorylated, peptide increases the activity of SHP-1 several-fold, phosphorylated FcγRIIB1 may both recruit and regulate the activity of SHP-1. Because the transduction leading to the activation of B lymphocytes requires tyrosine kinase phosphorylation of multiple substrates, coligation of FcγRIIB1 with the B-lymphocyte antigen receptor may promote the intracellular dephosphorylation of one or more substrates critical to activation signaling.

The coligation of FcγRIIB1 or the alternative splicing variant FcγRIIB2 with FceRI (high-affinity Fc receptor for IgE) on mouse bone marrow-derived mast cells (mBMMC) results in the down-regulation of exocytosis elicited through FcεRI (10). In a rat mast cell line transfected with mouse FcγRIIB2, coligation of the mouse receptor with endogenous rat FceRI-inhibited exocytosis and the secretion of tumor necrosis factor-α (10). Simultaneous, independent ligation of FcγRIIB2 and FcεRI did not inhibit exocytosis in the transfectants, nor did coligation when the cytoplasmic domain of the FcγRII species was deleted. Thus, the apposition of cytoplasmic domain sequences in FcγRIIB species with FceRI down-regulates mast cell activation.

Human natural killer (NK) cells and T-lymphocyte subsets express a group of Ig superfamily cell surface receptors, termed “killer cell inhibitory receptors” (KIRs) that recognize allelic groups of the major histocompatibility complex I molecules (11-15). The engagement of KIRs by the appropriate major histocompatibility complex I molecules inhibits the activation pathways of both NK cells and T lymphocytes for cytotoxic effector function (13, 14, 16, 17, 18), and this recognition mechanism protects normal, autologous cells. Both the KIRs and the FcγRIIB species contain a core consensus motif in their cytoplasmic domains, I/VxYxxL/V termed the immunoreceptor tyrosine-based inhibition motif (ITIM) (19, 20); the ITIM core is within the 13-amino acid region of the FcγRIIB species that mediates inhibition of B-lymphocyte stimulation. Phosphorylated, but not nonphosphorylated, peptides containing ITIMs from KIR cytoplasmic domains interact with and stimulate SHP-1 activity (19, 20). Moreover, the transfection of cytolytic, KIR-deficient NK cells exhibiting low levels of SHP-1 with constructs encoding a KIR and active SHP-1 inhibits their cytotoxicity for an appropriate target cell, whereas transfected constructs encoding the KIR and enzymatically inactive SHP-1 does not. Thus, the recruitment of SHP-1 activity is directly involved in KIR-mediated inhibition of NK cell cytotoxicity (19). The identity of the activating receptor(s) on NK cells whose cytotoxic function signals are inhibited by SHP-1 is unknown.

We now report that the amino acid sequence of gp49B1 bears statistically significant homology to human KLRs human Fc receptors for IgA (FcεR) and bovine Fc receptors for IgG2 (Fcγ2R), which together form a newly recognized family within the Ig superfamily. This family does not include FcγRIIB or the other FcγR of mouse or human origin. We also show that the cytoplasmic domain of gp49B1 contains two ITIMs, implying functional homology with members of the Ig superfamily that have amino acid sequences that are not homologous to the amino acid sequence of gp49B1. Using stable transfectants that express either gp49B1 or gp49A, we demonstrate that mAb B23.1 specifically binds gp49B1 on the surface of cells. Most importantly, we establish that the coligation of FcεRI and gp49B1 on the surface of mast cells suppresses FcεRI-mediated exocytosis, defined by the release of the secretory granule mediator β-hexosaminidase, and the generation of the membrane derived pro-inflammatory lipid mediator leukotriene (LT) C₄. Thus, the apposition of gp49B1 with FcεRI sends a negative regulatory signal that inhibits the signal transduction cascade emanating from FcεRI, thereby attenuating mast cell activation.
P815 clones expressed high steady-state levels of gp49A mRNA, and one (clone 9) was selected for study.

**Preparation of Antibodies.** Monoclonal rat IgE anti-dinitrophenyl hapten (clone LO-DNP-30) was obtained from Zymed. Rat IgM mAb B23.1 anti-mouse gp49B1 and rat IgM mAb RATNP 17.3 were purified by affinity chromatography from ascites produced in BALB/c nu/nu mice as described (1). For production of antibody to gp49A, a synthetic peptide (CEVPLENRR7KFKIRF; prepared at the Brigham and Women's Hospital Protein Microsequencing Laboratory) was synthesized corresponding to amino acids 72–87 of the extracellular domain of gp49A; this peptide differs from the sequence of gp49B1 at the italicized amino acids (3). The amino terminal cysteine is not found in the gp49A amino acid sequence but was added to facilitate coupling to carrier protein. The peptide (2 mg) was coupled to 2 mg of maleimide-activated keyhole limpet hemocyanin (Imject Kit, Pierce). The conjugate (125 μg in 250 μl PBS, pH 7.2) was mixed with an equal volume of Hunter's Titermax adjuvant emulsion (CytRx, Norcross, GA) and injected intramuscularly at four sites in each rabbit. A booster immunization consisting of 100 μg of each peptide conjugate and the same adjuvant was administered 3 weeks later, and 2 weeks after the booster immunization blood was collected.

The titer of the antiserum was determined by ELISA. Peptides (10 μg in PBS, pH 7.5) were incubated for 1 h at room temperature in 96-well Immulon II plates (Dynatech). The wells were washed three times with PBS, blocked by incubation with 1% BSA in PBS (BSA/PBS) for 1 h at room temperature, washed three times with 1% Tween 20 in PBS (TPBS), and incubated with serial dilutions of rabbit serum in 1% BSA/PBS for 1 h at room temperature. The wells were washed five times with TPBS, incubated for 1 h at room temperature with a 1:2000 dilution of horseradish peroxidase-labeled goat-anti-rabbit IgG (Bio-Rad) in 1% BSA/PBS, washed six times with TPBS, and incubated for 20 min at room temperature with 100 μl of a 200 μg/ml solution of 2,2'-azino-di-[3-ethylbenzthiazolinesulfonate] (Boehringer Mannheim) containing 0.015% hydrogen peroxide in citrate buffer (pH 4.25). Absorbance at 405 nm was measured on a Titertek Multiscan MCC/340 plate reader (Labsystems, Finland). Half-maximal binding of anti-gp49A72-87 Serum to the immobilizing peptide occurred at an approximate 1000-fold dilution of serum. The reactivity of anti-gp49A72-87 Serum with a peptide consisting of a cysteine plus gp49B1 amino acids 72–87 (CQVPLETRNAKFNPS) was ≈10% of its activity with the immobilizing peptide, as defined by reactivity with dilutions of the serum.

Anti-gp4972-87 IgG was purified on peptide-affinity columns. Each peptide (10 mg) was coupled to 1 mg of cyanogen bromide-activated Sepharose 4B (Pharmacia LKB), according to the manufacturer's protocol. An 8-ml sample of anti-gp4972-87 serum was applied to a column containing 2 ml of resin coupled with the heterologous gp49B172-87 peptide, and the flow-through was applied to a column containing the homologous peptide. The second column was washed sequentially with 20 column vol of 10 mM Tris-HCl (pH 7.5), 20 column vol of 500 mM NaCl in 10 mM Tris-HCl (pH 7.5), and 20 column vol of 10 mM Tris-HCl (pH 7.5). Ig was then eluted with 2 column vol of 100 mM glycine (pH 2.5) into 0.2-column vol of 1 M Tris-HCl (pH 8.0) and was dialyzed twice against PBS (pH 7.2). Nonimmune Ig was purified on a protein A-Sepharose column with the same steps described above.

**Flow Cytometric Analyses.** Samples containing 2–2.5 × 10^5 cells were added into tubes, and the cells were resuspended in 25 μl of either 0.20 μg/ml mAb B23.1 or an equal concentration of rat myeloma IgM in calcium- and magnesium-free Hanks' Balanced Salt Solution (HBSS) containing 0.1% (wt/vol) BSA and 0.02% (wt/vol) sodium azide (H/B/A). The cells were then incubated for 30 min at 4°C, washed by centrifugation in cold H/B/A, and incubated for 30 min at 4°C in a saturating concentration of FITC-labeled F(ab')2 fragments of goat anti-rabbit IgM (μ chain specific). To stain cells with anti-gp49A72-87 IgG, an analogous procedure was used with 90 μg/ml anti-gp49A72-87 IgG or an equal concentration of nonimmune rabbit Ig in the first step. A saturating concentration of FITC-labeled F(ab')2 fragments of goat anti-rabbit IgG heavy and light chains was used as the second antibody. All second antibodies were obtained from Jackson ImmunoResearch. After incubation with second antibodies, the cells were washed by centrifugation, fixed in 2% paraformaldehyde, and analyzed with a Becton Dickinson FACSort with linear fluorescence amplification. The mean fluorescence channel number was calculated as the mean fluorescence channel number of cells incubated with immune primary antibody minus that of cells incubated with the nonimmune primary reagent.

**Activation of Mast Cells.** mBMCC (1 × 10^7/ml) were incubated for 1 h at 4°C in 50% WCM containing mAb B23.1 or RATNP 17.3 (20 μg/ml each), rat IgE (3 μg/ml), rat IgE + RATNP, or rat IgE + dilutions of mAb B23.1. After being washed once by centrifugation at 4°C, replicate cell pellets were resuspended on ice at their original volume with 50% WCM either alone or containing 25 μg/ml F(ab')2 mouse IgG anti-rat IgG (heavy and light chain reactive) (Jackson ImmunoResearch). The cells were then incubated for 15 min at 37°C with agitation, and 50-μl samples were removed from each tube in duplicate and diluted with an equal volume of 0.15 M EDTA in HBSS. The cells were sedimented by centrifugation at 250 × g for 5 min at 4°C, the supernatants were decanted and retained, and the pellets were resuspended to their original volumes with a 1:1 (vol/vol) mixture of 50% WCM/0.15 M EDTA and sonicated on ice.

β-hexosaminidase was quantitated in a 30-μl sample of each supernatant and pellet in duplicate by spectrophotometric analysis of the hydrolysis of p-nitrophenyl-β-D-2-acetamido-2-deoxyglucopyranoside (27). The percent release values for each experimental condition were calculated by the formula [S/(S + P)] × 100, where S and P are the mediator contents of the samples of each supernatant and cell pellet, respectively. The net percent release values were obtained by subtracting the percent release of sensitized cells incubated in 50% WCM alone from that of replicate cells challenged with F(ab')2 mouse IgG anti-rat IgG heavy and light chains. LTC4 release was measured by radioimmunoassay (PerSeptive Diagnostics, Cambridge, MA) as described (28).

**RESULTS**

**Analysis of the Relationship Between the gp49 Family and Other Members of the Ig Superfamily.** A homology-based search of the amino acid sequences in the Brookhaven National Laboratory, SWISS-PROT, Protein Identificaton Resource, translated GenBank, and Kabat data bases revealed that both gp49A and gp49B1 possess greatest homology with the human myeloid FcR, the bovine Fcy2R, and members of the human KIR family that are expressed on NK cells and certain cytotoxic T lymphocytes. To determine formally whether a relationship exists between gp49B1 and the other receptors, gp49B1 was compared by ALIGN analysis, which was used previously to define gp49A and gp49B1 as members of the Ig superfamily (2, 3), with receptors of the Ig superfamily that, like gp49B1, possess two C2-type, Ig-like domains. Analysis of the complete amino acid sequences revealed statistically significant homology (ALIGN scores ≥3) with human FcR, bovine Fcy2R, and eight members of the KIR family, but not with mouse and human FcyR species or FceRIα (Fig. 1). Delineation of the two groups of proteins was also observed when each Ig-like domain of gp49, including 20 amino acids flanking each end (23), was compared by ALIGN analysis with molecules representative of the set shown in Fig. 1 (data not
shown). To assess the relationship of these proteins by another means, evolutionary tree analysis of the full-length amino acid sequences was conducted with the CLUSTAL W program (31) to perform multiple sequence alignment and PHYLIP TREE (32) with default settings to generate the tree. The segregation of the mouse gp49, bovine FcγR2, and human FcαR and KIR proteins into a family distinct from the other receptors was confirmed in the analysis because their branches localized as a group that radiated ~180° away from the branches that contained the other receptors analyzed (data not shown).

Analysis of the Binding Specificity of mAb B23.1 for gp49B1 and gp49A. Stable transfectants that express gp49A or gp49B1 were generated in the mouse P815 mastocytoma cell line, which binds little or no mAb B23.1 (33). As determined by flow cytometry, mAb B23.1 bound to gp49B1, but not gp49A, as transfectants as compared with background binding to P815 cells transfected with the pMH-NEO expression vector alone (Fig. 2). In contrast, anti-gp49A72-87 IgG bound to gp49A, but not gp49B1 transfectants, thereby demonstrating that gp49A was expressed on the surface of the appropriate transfectants. Thus, it is reasonable to assume that cell-surface gp49B1 can be selectively bound by mAb B23.1 on nontransfected cells, such as mBMMC.

Effects of Ligation of gp49B1 and FcεRI on Mast Cell Activation. The cytoplasmic domain of gp49B1 contains two sequences that conform to the ITIM consensus (20), namely, 1VYAQV and VYQAQL (3). To determine whether coligation of gp49B1 with FcεRI altered activation–secretion, mBMMC were incubated at 4°C with rat IgE (3 μg/ml) alone or together with the RATNP 17.3 isotype control (20 μg/ml) or incremental concentrations of mAb B23.1 (0.625–20 μg/ml). Samples of cells were also incubated with RATNP 17.3 or mAb B23.1 alone. The cells were washed once and incubated for 15 min at 37°C in medium either alone or with the coligating antibody, a F(ab')2 mouse IgG that is reactive with the light chains of rat IgE and IgM. No release of the secretory granule mediator β-hexosaminidase occurred unless mBMMC were primed with rat IgE in the first step and then stimulated with the second antibody (Fig. 3A). The release of β-hexosaminidase was inhibited in a dose-dependent fashion when mBMMC were concomitantly incubated with the fixed concentration of rat IgE and incremental concentrations of mAb

![Fig. 2. Flow cytometric analysis of the binding of rat IgM mAb B23.1 (Upper) and rabbit anti-gp49A72-87 IgG (Lower) to pMH-NEO vector control, gp49A, and gp49B1 transfectants. Cells were incubated with primary antibodies for 30 min at 4°C, washed, and incubated with F(ab')2 fragments of appropriate, FITC-labeled secondary antibody for 30 min at 4°C before flow cytometry. Data are expressed as net mean fluorescence channel numbers ± SEM, n = 5 (anti-gp49A72-87 IgG) and n = 3 (mAb B23.1).](image-url)

B23.1 before coligation of their respective FcεRI and gp49B1 molecules. The release of LTC4 was inhibited in a similar manner (Fig. 3B).

**DISCUSSION**

We have established that the coligation of gp49B1 with FcεRI inhibits mast cell activation–responses. The recognition that gp49B1 might be an inhibitory protein initially arose from ALIGN analysis of the amino acid sequences of gp49B1 versus other members of the Ig superfamily containing two C2-type, Ig-like domains. This analysis revealed statistically significant homology between gp49B1 and human KIRs. There was also homology between gp49B1, human FcαR, and bovine FcγR2, but not with other FcγR species or FcεRI of the human and mouse (Fig. 1). The segregation of the homologous proteins into a distinct family was supported by separate evolutionary tree analysis (data not shown) (31, 32). A second finding suggested a basis for a function for gp49B1, namely, suppression of an activation event in mast cells, because the cytoplasmic domain possessed two ITIMs, a motif that is also recognized in FcγRIIb species and in KIRs of NK cells and T-lymphocyte subsets. Moreover, although the cytoplasmic domain of mouse FcγRIIib contains only one ITIM, coligation of this Fe receptor with the B-lymphocyte antigen receptor or with FcεRI inhibits B lymphocyte and mast cell activation, respectively (8–10). Of note is the finding that mouse gp49B1 does not exhibit statistically significant amino acid sequence homology with mouse FcγRIIib (Fig. 1). Thus, our findings establish that separate homology-defined families within the Ig superfamily, with possibly distinct counterligands, inhibit mast cell activation.

We previously demonstrated that COS cells transfected with a cDNA encoding gp49B1 are immunoreactive with mAb B23.1 (3). However, the specificity of the mAb with regard to gp49B1 was unknown because of the 89% identity between the extracellular domains of gp49B1 and gp49A. To address this issue, stable transfectants expressing either the gp49A or the gp49B1 cDNA were created in the P815 mastocytoma cell line, which binds little or no mAb B23.1 (Fig. 2). In addition, to measure specifically the expression of gp49A in transfectants, rabbit anti-gp49A72-87 IgG was prepared to a peptide in the extracellular domain that differs in gp49A and gp49B1 (3). mAb B23.1 specifically bound to transfectants that expressed...
were reminiscent of the finding that gp49B1 contains two core ITIM motifs and suppresses secretory granule and lipid-derived mediator release when coligated with FcεRI. It was proposed that gp49B1 be designated "mouse mast cell inhibitory receptor" (mMIR).

The mechanism by which ITIMs inhibit cellular activation events appears to be through the downstream recruitment of one or more tyrosine phosphatases that reverse a tyrosine phosphorylation step(s) critical to progressive signal transduction. Coligation of the B-lymphocyte antigen receptor with FcγRIIB1 causes tyrosine phosphorylation of each receptor with different resultant intracellular protein associations. Whereas the B-lymphocyte antigen receptor associates with syk kinase via the receptor's immunoreceptor tyrosine-containing activation motif in the Ig-α and Ig-β subunits (8), phosphopeptides encompassing the FcγRIIB1 ITIM bind SHP-1 (9). Furthermore, tyrosine-phosphorylated KIR peptides from the two ITIMs of certain human KIRs bind SHP-1 and SHP-2 [formerly PTP1D] (19, 20). Thus, ITIMs may mediate inhibitory effects by recruiting tyrosine phosphatases that could reverse and/or suppress tyrosine phosphorylation events at one or more steps of signal transduction cascades that activate cellular functions.

The finding that mMIR is homologous to members of the Ig superfamily that bind other members of the superfamily (e.g., Fc receptors/Ig, KIRs/major histocompatibility complex class I molecules) suggests that its counterligand may also be a member of the Ig superfamily. Although mouse NK cells express a family of KIRs (Ly49) that contain the ITIM motif (20) and function like human NK cell KIRs in interacting with major histocompatibility complex class I molecules, the Ly49 family consists of type II transmembrane proteins with an extracellular C-type lectin domain, rather than an Ig-like structure (14). It is possible therefore, based on the structural and functional considerations presented here, that mMIR may belong to a family of molecules that are the mouse equivalents of human NK and T-lymphocyte KIRs.

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