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Co-aggregation of FcγRII with FcεRI on Human Mast Cells Inhibits Antigen-induced Secretion and Involves SHIP-Grb2-Dok Complexes*

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Signaling through the high affinity IgE receptor FcεRI on human basophils and rodent mast cells is decreased by co-aggregating these receptors to the low affinity IgG receptor FcγRII. We used a recently described fusion protein, GE2, which is composed of key portions of the human γ1 and the human ε heavy chains, to dissect the mechanisms that lead to human mast cell and basophil inhibition through co-aggregation of FcγRII and FcεRI. Unstimulated human mast cells derived from umbilical cord blood express the immunoreceptor tyrosine-based inhibitory motif-containing receptor FcγRII but not FcγRI or FcγRIII. Interaction of the mast cells with GE2 alone did not cause degranulation. Co-aggregating FcεRI and FcγRII with GE2 1) significantly inhibited IgE-mediated histamine release, cytokine production, and Ca^{2+} mobilization, 2) reduced the antigen-induced morphological changes associated with mast cell degranulation, 3) reduced the tyrosine phosphorylation of several cellular substrates, and 4) increased the tyrosine phosphorylation of the adapter protein downstream of kinase 1 ($p62^{dok}$; Dok), growth factor receptor-bound protein 2 (Grb2), and SH2 domain containing inositol 5-phosphatase (SHIP). Tyrosine phosphorylation of Dok was associated with increased binding to Grb2. Surprisingly, in non-stimulated cells, there were complexes of phosphorylated SHIP-Grb2-Dok that were lost upon IgE receptor activation but retained under conditions of Fcε-Fcγ co-aggregation. Finally, studies using mast cells from Dok-1 knock-out mice showed that IgE alone triggers degranulation supporting an inhibitory role for Dok degranulation. Our results demonstrate how human FcεRI-mediated responses can be inhibited by co-aggregation with FcγRIIB and implicate Dok, SHIP, and Grb2 as key intermediates in regulating antigen-induced mediator release.

Mast cells generate pro-inflammatory mediators that initiate and propagate allergic inflammation. IgE-mediated mediator release is initiated through the high affinity IgE receptor FcεRI,[1] in which the α subunit has IgE binding activity and the β and γ subunits have signaling activity. Signaling is primarily mediated by immunoreceptor tyrosine-based activation motifs (ITAMs) located in the carboxyl-terminal cytoplasmic tails of each of the β and γ subunits. ITAMs are typically 26- or 27-amino acid stretches including two YXXL motifs separated by 9 or 10 amino acids. Cross-linking IgE-primed receptors with a multivalent antigen results in the activation of Lyn, which in turn phosphorylates ITAMs, creating docking sites for Syk kinase family members and permitting signal propagation (for review, see Ref. 1).

Many immune cells that express ITAM-containing antigen receptors also express receptors with a related immunoreceptor tyrosine-based inhibitory motifs (ITIMs). ITIMs are 13-amino acid sequences containing a single YXXL motif (2). They were first recognized in the cytoplasmic tails of the FcγRIIB isoform (FcγRIIB1) of rodent B cells. Their negative signaling activity was revealed by studies showing that co-aggregating the BCR with FcγRIIB1 inhibits the BCR-mediated activation of rodent B cells (3). Studies in murine bone marrow derived mast cells (BMMCs) showed that co-aggregating murine FcγRIIB to FcεRI also inhibits IgE-induced mast cell degranulation and that this inhibition is dependent on the intact intracytoplasmic tail of the FcγRIIB (4). Further studies using RBL-2H3 cells transfected with cDNA encoding human wild type and mutant FcγRIIB showed that the intracytoplasmic domain responsible for the negative signaling is the ITIM found in FcγRIIB (5). Subsequently, it was shown that the phosphorylation of the mast cell FcγRIIB-ITIM is mediated by Lyn and results in the recruitment of specific tyrosine phosphatases (6).

The negative regulation of signaling through ITAM-containing receptors by signals generated by the co-cross-linked ITIM-

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[1] The abbreviations used are: FcεRI, high affinity receptor for IgE; FcεR, receptor for IgE; FcγR, receptor for IgG; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; BCR, B cell receptor; SHIP, SH2 domain containing inositol 5-phosphatase; Ab, antibody; mAb, monoclonal antibody; NIP, 3-nitro-4-hydroxy-5-iodophenylacetate; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PBS/BSA, phosphate-buffered saline with 0.1% bovine serum albumin; HBSS, Hanks' balanced salt solution; FACS, fluorescence-activated cell sorter; CBMCs, cord blood-derived human mast cells; TNF-α, tumor necrosis factor-α; RasGAP, Ras GTPase-activating protein; BMMCs, bone marrow-derived mast cells; SHP-1, Src homology domain containing protein-tyrosine phosphatase-1.

containing FcγRIIB isoforms has been demonstrated in mouse and human B cells that naturally co-express the BCR and FcγRIIB1, in T cell and rodent mast cell lines that express the T cell receptor or FcεRI plus endogenous or transfected FcγRIIB1 (5), and in human basophils (7). Negative regulation by other ITIM-containing receptors has been demonstrated in several other model systems (8). It is not clear whether human mast cells express ITIM receptors and whether IgE-mediated responses can be down-regulated through their activity. Although there has been a great effort to discover and study ITIM receptors, little progress has been made in harnessing their inherent inhibitory properties for potential therapeutic interventions.

We recently showed that a novel human Fcε-Fcγ receptor-binding fusion protein (GE2) could inhibit human basophil functional and biochemical responses *in vitro* and *in vivo* (9, 64). Subsequently, it has been demonstrated that GE2 can inhibit IgE production by human B cell (10) and cytokine production from FcεRI/FcγRII-expressing, Langerhan cell-like dendritic cells (11).

Here we used umbilical cord blood-derived human mast cells (CBMCs) to test the mechanisms of inhibition by FcεRI-FcγRII co-aggregation on human mast cells. We demonstrated that human mast cells express FcγRII (CD32) but not FcγRI (CD64) or FcγRIII (CD16). We provide the first evidence that the functional responses of human mast cell FcεRI can be down-regulated by FcγRII. The inhibition involves alterations in protein tyrosine phosphorylation, including Syk. We provide evidence that Grb2, Dok, and SHIP phosphorylation is associated with mast cell inactivation and FcγRII-mediated inhibition of FcεRI responses. Although previous rodent studies have implicated SHIP, our findings further implicate Grb2 and Dok as additional "gatekeepers" of human FcεRI signaling.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Anti-FcεRI α subunit mAb 22E7 (IgG1; Ref. 12) was a gift from Dr. J. Kochan. Monoclonal antiphosphotyrosine (Tyr(P); IgG1), anti-Grb2 (rabbit polyclonal), and anti-RasGAP (rabbit polyclonal) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Nonspecific mouse IgG1 (MOPC31C), anti-FcγRIII (CD16), anti-actin, anti-4-hydroxy-3-nitrophenacetyl (NP) IgE (mouse), and CD22 mAbs (IgG1) were from Sigma. The anti-FcγRII AT10 mAbs (IgG1; recognizing all FcγRII isoforms) and anti-FcγRI (anti-CD64; clone 32.2, IgG1) Abs were obtained from Medarex (Annendale, NJ). The chimeric human anti-NIP IgE Abs were from Serotec Ltd. (Oxford, UK). The mAb against signal-regulatory protein-α (SIRP-α) (IgG1) was obtained from Alexis Biochemicals. The anti-Syk Ab (IgG2a) was obtained from Upstate (Waltham, MA). The anti-c-kit (IgG1) and CD81 (IgG1) were from Immunotech (Marseille, France). The mAb anti-CD72 (IgG1) was from Pharmingen. The Ab detecting mast cell-associated antigen (G63; IgG1; MAFA) was a gift from Enrique Ortega, Mexico City, Mexico. Abs recognizing $p62^{dok}$ (Dok) were a gift from Dr. John Cambier, National Jewish Hospital, Denver, CO. The human Fcγ-Fcε Ig chimeric fusion protein (GE2) was produced as described previously (9). All IgE preparations were routinely centrifuged at 60,000 $\times$ *g* to remove aggregates.

Umbilical CBMCs—Umbilical cord blood was collected from normal, full-term deliveries as approved by the Human Studies Committees at the Medical College of Virginia. EDTA-treated (0.01% final) cord blood was layered over Ficoll-Hypaque (density 1.077; Sigma) 1:1 (v/v), and the tubes were centrifuged at 350 $\times$ *g* for 20 min at room temperature with no brake. Mononuclear cells recovered at the interphase were washed with phosphate-buffered saline with 0.1% bovine serum albumin (PBS/BSA). If red blood cell contamination was present, the pellet was resuspended in PBS/BSA and was relayered over the Ficoll (1:1, v/v), centrifuged, and washed as above. The CD34+ stem cells were isolated using a negative selection mixture and magnetic separation of non-CD34-positive cells using the technique given by the manufacturer (Miltenyi, Auburn, CA). The cells were suspended at a concentration of $\sim$0.5–1.0 $\times$ 10^6 cells/ml in Iscove's modified Dulbecco's medium (Sigma) supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 100 ng/ml recombinant human stem cell factor and 50 ng/ml recombinant human interleukin-6 (IL-6) (both from BIOSOURCE, Camarillo, CA), 10 μM Hepes, 50 μM 2-mercaptoethanol, 4 mM L-glutamine, 1$\times$ minimum Eagle's medium amino acids, 1$\times$ vitamin solution (Invitrogen), 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin (13). To enhance FcεRI expression, human anti-NIP IgE (1 μg/ml) was added at least 1 week prior to each experiment (13). Cultures were maintained at 5% CO_2 and 37 °C, and the medium was changed every 4–5 days with the cells placed back into the original flask. Cells were used at 6–9 weeks. As reported previously using a similar protocol (13), the purity of the mast cells was always greater than 90% as determined by tryptase immunostaining (14) (data not shown). Aliquots of cells were removed on different days and before use for the analysis of viability (by trypan blue exclusion) and for cytospin preparation.

Flow Cytometry—Cells were recovered by centrifugation at 800 $\times$ *g* at 4 °C, washed with PBS/BSA, and incubated for 30 min at 4 °C with a 1:500 dilution of normal human serum. The cells were washed and incubated with the indicated Abs (10 μg/ml) for 1 h at 4 °C. After antibody labeling, the cells were washed and incubated with a 1:100 dilution of $F(ab')_2$-fluorescein isothiocyanate-goat anti-mouse for 30 min at 4 °C. After three washes, cells were resuspended in 400 μl of PBS/BSA. The mean intensity fluorescence was determined for at least 10,000 cells using a Coulter Epics Elite flow cytometer. MOPC31C nonspecific mouse IgG or non-immune rabbit IgG was used as a negative control. All experiments were performed in duplicate.

Cell Activation—CBMCs were suspended in fresh medium (without cytokines) and sensitized with 10 μg/ml human anti-NIP IgE overnight at 37 °C in a 5% CO_2 incubator. The next morning, GE2 was added for at least 2 h to a final concentration indicated in the "Results" section. Cells were washed and activated, and mediator release was measured as described previously (15).

Intracellular Ca^{2+} Concentration ($[Ca^{2+}]_i$) Measurements—IgE-sensitized mast cells (1 $\times$ 10^5 cells/condition), with or without GE2 challenge, were loaded with 2 μM (final v/v) Indo-1 (Fig. 3*A*) or Fura-2/AM (Fig. 3*B*) for 30 min at room temperature on a rocking platform. Washed cells were resuspended in fresh HBSS and kept at 37 °C. Following acquisition of base-line fluorescence values for $\sim$20–30 s using a FACScaliber flow cytometer (Fig. 3*A*) or laser scanning confocal microscope system Olympus FluoView (Fig. 3*B*), HBSS with or without NIP/BSA or thapsigargin (Calbiochem) was added, and changes in the fluorescent ratio were measured for up to 10 min at 2-s intervals. Each experiment was done in duplicate.

Transmission Electron Microscopy—Suspensions of IgE-sensitized CBMCs were challenged with or without GE2 (10 μg/ml) as above, washed, and incubated at 37 °C in prewarmed $HBSS^+$ (HBSS with 1 mM $CaCl_2$ and 1 mM $MgCl_2$) without or with NIP/BSA (200 ng/ml) for 30 min. Cells were collected by centrifugation, and pellets were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 30 min at room temperature, rinsed with cacodylate, and processed as described previously (16). Thin sections were observed using a Hitachi 600 transmission electron microscope.

Lysis, Immunoprecipitation, and Immunoblotting—IgE-sensitized mast cells, with or without GE2, were activated as above. Preparation of cell lysates, immunoprecipitation, and Western blotting were performed as described previously (7).

RESULTS

Identification of FcγRs on CBMCs—ITIM-containing receptors are an expanding family of immune inhibitory receptors that have the ability to inhibit immune responses (8). We are not aware of studies examining their expression on human mast cells. As seen in Fig. 1, CBMCs express, in addition to FcγRIIB, the ITIM-containing receptor CD22 and the ITIM-like-containing receptor CD81.

Similar to what we have shown previously for human basophil FcγRs, human mast cells exclusively express the low affinity IgG receptor FcγRII. Thus CBMCs react strongly with the pan-anti-FcγRII mAb AT10 (Fig. 1). Confirming earlier studies examining cultured human mast cells, little or no FcγRI or FcγRIII expression was detected (17). Thus, cultured human mast cells express only the low affinity IgG receptor FcγRII. There are multiple FcγRII isoforms, representing the products of three distinct genes (18). Results of reverse transcription-PCR analysis using previously described methods (7, 19) showed that CBMCs contain transcripts for the ITIM-containing FcγRII isoform FcγRIIB and the ITAM-containing

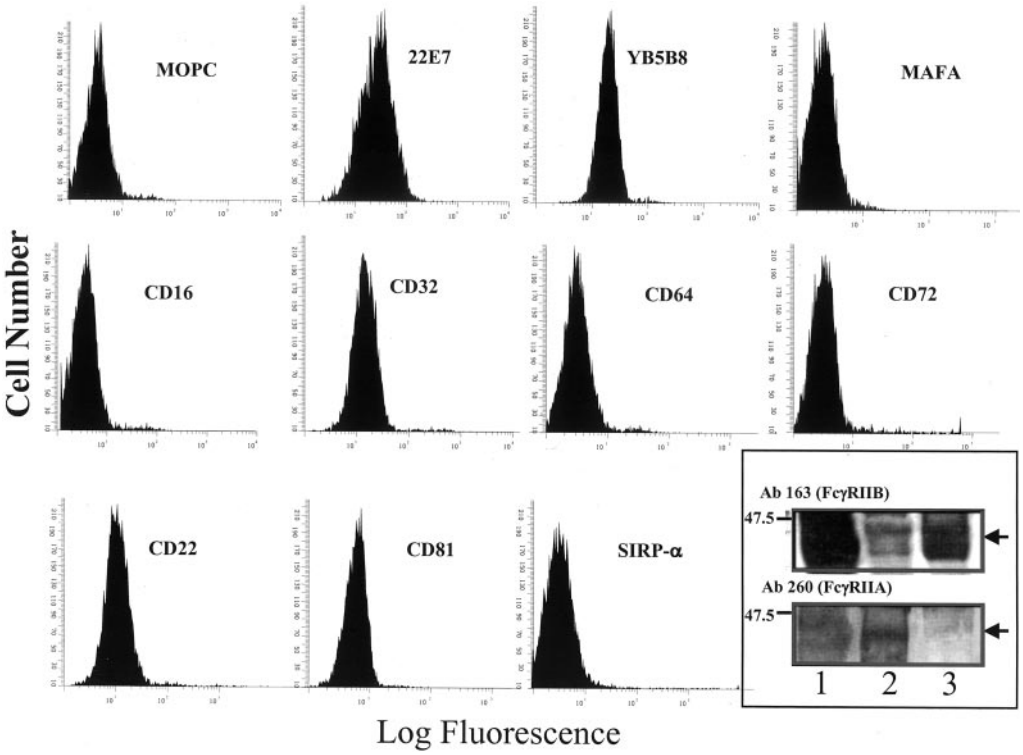

FIG. 1. **ITIM receptor expression on CBMCs.** Mast cells were incubated at 4 °C with Abs raised against a number of cell surface receptors discussed below (10 μg/ml) followed by fluorescein isothiocyanate-labeled goat anti-mouse IgG antibodies. An irrelevant mouse IgG (*MOPC*) was substituted for the receptor Abs as a negative control. mAbs 22E7, which binds the FcεRI α chain, and YB5B8, which binds c-kit, were positive controls. Positive expression was evident for CD22, CD32, and CD81. *MAFA*, mast cell function-associated antigen; *SIRP-α*, signal-regulatory protein-α. *Inset*, CBMCs express FcγRIIB and not FcγRIIA. Lysates of CBMCs (*lane 3*) were probed on two separate blots with the FcγRIIA-specific (Ab260) or FcγRIIB-specific (Ab163.96) Abs. As controls, Raji (*lane 1*, FcγRIIB-positive, FcγRIIA-negative) or U937 (*lane 2*, FcγRIIB-negative, FcγRIIA-positive) cell lines were examined in parallel (20). The *molecular mass marker* is indicated in kilodaltons.

FcγRII isoforms FcγRIIA and FcγRIIC (data not shown). However, we did not detect surface expression of FcγRIIA by FACS analysis using the FcγRIIA-specific Ab IV.3 (data not shown). In addition, no FcγRIIA was detected by Western blot analysis in mast cell lysates using an Ab raised against the cytoplasmic tail of FcγRIIA (Ab260), whereas strong signals were detected when using an FcγRIIB-specific Ab (Ab163; a gift of Dr. Susheela Tridandapani, Ohio State University, Columbus, OH) (Fig. 1, *inset*) (20). Thus, although CBMCs express mRNA for FcγRIIA, it appears that only FcγRIIB is expressed on the cellular membrane in unstimulated, human CBMCs.

FcγRII-FcεRI Co-aggregation Inhibits FcγRI-dependent Human Mast Cell Degranulation—Previous investigators have established that FcγRII-FcεRI co-aggregation can inhibit FcεRI responses in a variety of model systems. We designed and described an FcγR-FcεR-binding chimeric protein that can directly induce this interaction (GE2; Ref. 9). Fig. 2*A* shows the secretory responses induced by adding optimal concentrations of NIP/BSA (200 ng/ml) to CBMCs that had been sensitized with human anti-NIP IgE and treated with the GE2 fusion protein. GE2 incubation for 2 h prior to antigen stimulation inhibited IgE-mediated histamine release in a dose-dependent fashion. This inhibition could not be accounted for by competition between IgE and GE2 for FcεRI binding as substitution of nonspecific purified IgE myeloma protein (PS-IgE) for the GE2 resulted in IgE-mediated release comparable with non-GE2-challenged control cells. Similar to what we reported previously using human basophils (9), no secretion is induced by adding chimeric protein alone to IgE-sensitized and unsensitized cells (data not shown). In four separate experiments, co-aggregating FcεRI and FcγRII with GE2 (10 μg/ml) reduced FcεRI-mediated histamine secretion by an average of 68%.

FcγRII-FcεRI Co-aggregation Inhibits CBMCs FcεRI-mediated TNF-α Production—Human mast cells produce TNF-α following IgE-mediated stimulation (21). The results in Fig. 2*B* show that anti-NIP IgE-sensitized CBMCs that were treated with GE2 released significantly less TNF-α in response to 12-h stimulation with NIP/BSA compared with sensitized but GE2-untreated cells. The average inhibition of FcεRI-mediated TNF-α production by FcγRII-FcεRI co-aggregation was 60 and 64% using 5 and 10 μg/ml, respectively.

FcγRII-FcεRI Co-aggregation Inhibits CBMCs FcεRI-mediated Ca^{2+} Mobilization—Previous studies have indicated that human mast cell FcεRI-mediated degranulation and cytokine production both depend on the release of Ca^{2+} from intracellular stores and on the subsequent sustained influx of extracellular Ca^{2+} (22, 23). We used the Ca^{2+} indicator dye Indo-1 and FACS analysis to examine the pattern of FcεRI-mediated Ca^{2+} mobilization in human mast cells following GE2 treatment (Fig. 3).

Cross-linking anti-NIP IgE-primed FcεRI with NIP/BSA leads, after a lag phase, to a rapid increase in intracellular Ca^{2+} levels (Fig. 3*A*). This Ca^{2+} spike is followed by a sustained elevation in Ca^{2+} levels, the Ca^{2+} plateau, which persists for up to 3 min. In the experiment shown in Fig. 3*A*, the lag time from antigen addition to FcεRI-mediated Ca^{2+} influx was ~24 s. When cells were treated with GE2 prior to antigen challenge, the lag time from antigen addition to the initial rise in $[Ca^{2+}]$ more than doubled to 60 s. Thus, co-aggregating FcγRII and FcεRI with GE2 decreases the initial Ca^{2+} mobilization in human mast cells.

To further investigate whether co-aggregation inhibits the release of intracellular calcium stores, we utilized thapsigargin, which induces an increase in $[Ca^{2+}]_i$ because of store leakage from the endoplasmic reticulum. As seen in Fig. 3*B*, thapsigargin induced a sustained increase in $[Ca^{2+}]_i$. However,

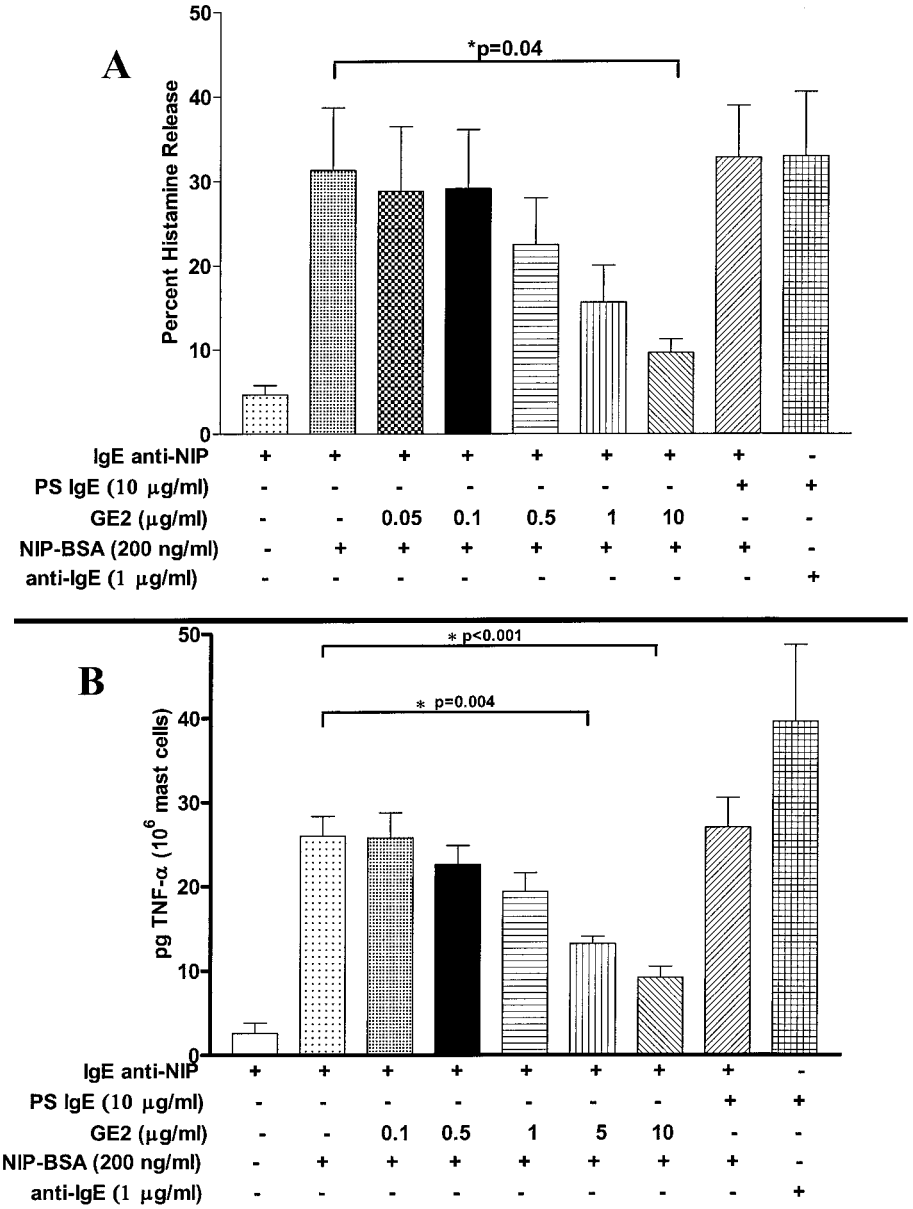

FIG. 2. **FcγRII-FcεRI co-aggregation inhibits FcεRI-mediated mast cell degranulation and TNF-α production.** CBMCs were incubated with anti-NIP IgE (10 μg/ml) with or without a 2-h incubation with GE2 or nonspecific IgE (*PS*) in Iscove's medium at 37 °C in a 5% CO_2 incubator. The cells were washed and incubated with or without 200 ng/ml NIP/BSA or anti-IgE for 45 min (histamine release) or 12 h (cytokine production), and mediator release was measured in the supernatants. Results are represented as the mean ± S.E. of four (*A*) or two (*B*) separate experiments. * designates values significantly reduced ($p < 0.01$) when comparing cells challenged with or without GE2.

when GE2 (10 μg/ml) and antigen were added prior to challenge with thapsigargin, the increase observed in $[Ca^{2+}]_i$ was smaller, and a sustained phase of Ca^{2+} was lacking. These findings indicate that the effect of GE2 appeared to be mainly due to an inhibition of the endoplasmic reticulum Ca^{2+} store as well as sustained, store-dependent $[Ca^{2+}]_i$ influx.

FcγRII-FcεRI Co-aggregation Inhibits CBMCs FcεRI-mediated Morphological Changes—Under transmission electron microscopy, unstimulated cultured human mast cells (Fig. 4*A*) show large unsegmented nuclei and relatively sparse endoplasmic reticulum and mitochondria. Granules are numerous and contain loosely packed matrix material plus occasional dense core material, multilamellar membranes, and internal vesicles. Fig. 4*B* shows the intracellular fusion of granules (*arrows*) and the extracellular release of granule contents associated with normal IgE-mediated mast cell activation (24–26). In contrast, the granules in cells treated with GE2 remain individual and intact following antigen stimulation (Fig. 4*C*).

FcγRII-FcεRI Co-aggregation Affects FcεRI-mediated Tyrosine Phosphorylation of Multiple Substrates—Protein tyrosine phosphorylation is a key event linking FcεRI cross-linking to downstream signaling in human mast cells and basophils (15, 16, 23). To investigate the effects of co-aggregating FcγRII-FcεRI on IgE-mediated protein tyrosine phosphorylation, whole cell lysates of IgE-activated cells, with or without GE2 preincubation, were resolved by SDS-PAGE and probed with anti-Tyr(P) antibodies. As shown in Fig. 5*A*, NIP/BSA cross-linking of anti-NIP IgE-sensitized FcεRI induced the tyrosine phosphorylation of several different substrates at 2, 5, and 15 min (*lanes 2*, *4*, and *6*) compared with non-activated cells (*lane 1*). The most prominent signals were species appearing at ~110, 85, 76, 72, 66, 44, 42, 38, and 28 kDa. When cells were treated with GE2, tyrosine phosphorylation of several of these substrates was reduced. These included proteins with molecular masses of ~85, 72, 66, 44, and 38 kDa (indicated with *arrows*). Thus, the down-regulation of IgE-mediated degranulation by co-aggregation of FcγRII and FcεRI by GE2 appears to involve the reduction of tyrosine phosphorylation of several different proteins.

Previous investigations have shown that the tyrosine kinase Syk is quickly phosphorylated in IgE-stimulated human FcεRI-positive cells (15, 23, 27). In human basophils, co-aggregation of FcεRI and FcγRII with antigen-specific IgE and IgG (7) or GE2 (9) inhibits the tyrosine phosphorylation of Syk. As seen in

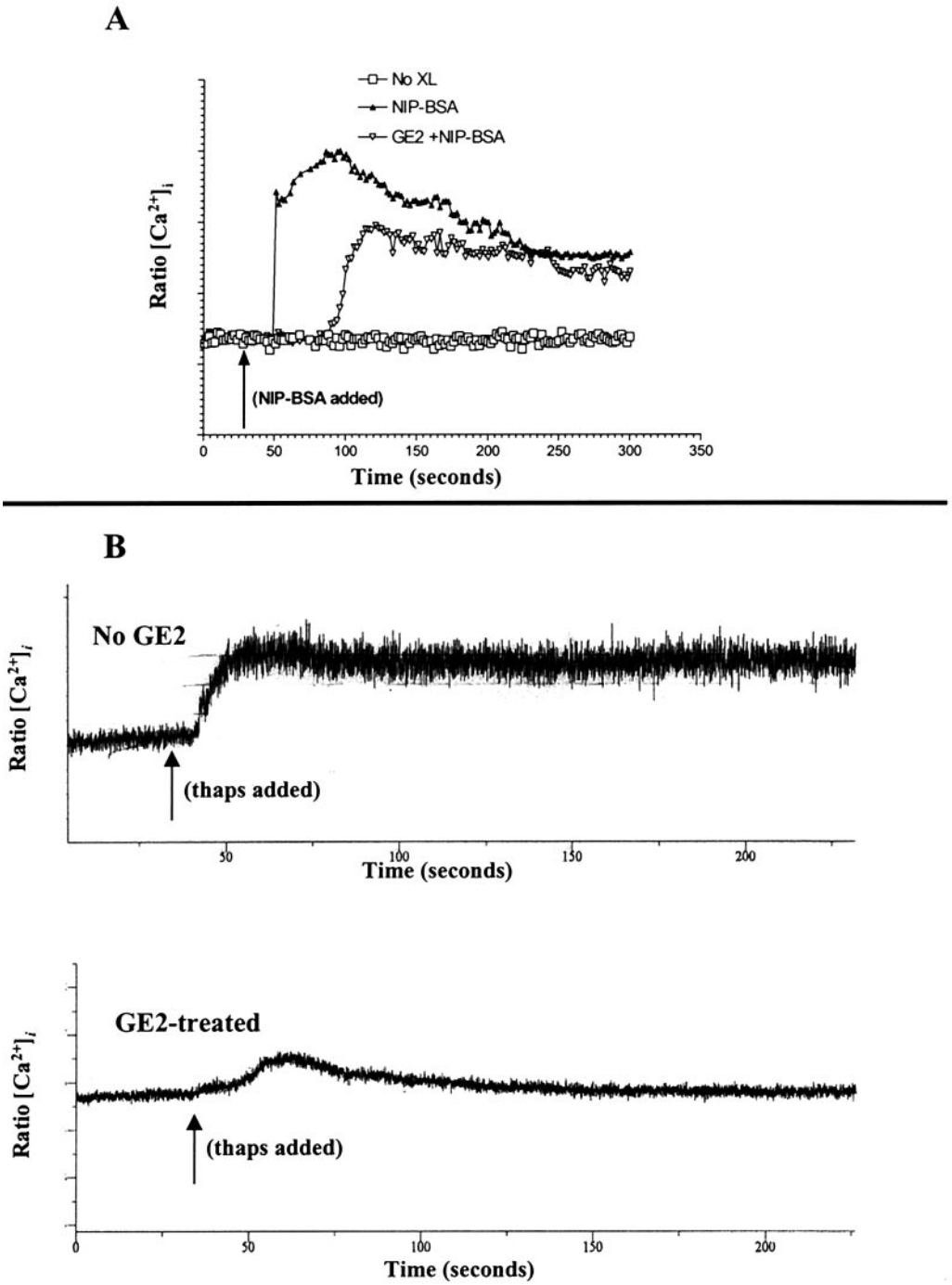

FIG. 3. *A*, FcγRII-FcεRI co-aggregation inhibits FcεRI-mediated Ca^{2+} mobilization in CBMCs. Cells were sensitized with 10 μg/ml of anti-NIP IgE with or without GE2, loaded with Indo-1, and challenged with 200 ng/ml NIP/BSA for 4 min. Change in absorbance was monitored using FACS analysis as described under "Experimental Procedures." Each *plot* represents the Ca^{2+} response of whole cell populations. The data are representative of two separate experiments. *B*, GE2 inhibits the release of intracellular calcium stores. Fura-2/AM-loaded cells were challenged with thapsigargin (*thaps*) with or without preincubation with GE2 (10 μg/ml). Change in absorbance was monitored as described under "Experimental Procedures." *XL*, cross-linker.

Fig. 5*B* using whole cell lysates of CBMCs, co-aggregation by GE2 reduced the tyrosine phosphorylation of a protein with the approximate molecular mass of Syk (~72 kDa). Thus, we wanted to examine specifically the phosphorylation of Syk in GE2-treated CBMCs after IgE stimulation. Cells with or without GE2 preincubation were stimulated for the indicated periods and lysed, and anti-Syk immunoprecipitates were analyzed by immunoblotting with anti-Tyr(P) Ab. As seen in Fig. 5*B* Syk was tyrosine-phosphorylated within 2 min after addition of antigen. The amount of Syk tyrosine phosphorylation was markedly reduced in IgE-activated cells that had been treated with GE2 compared with non-treated IgE-activated cells.

Grb2 Phosphorylation Is Increased in Resting and FcγRII-FcεRI-co-aggregated Cells—Grb2 is an adapter protein associated with FcεRI and FcγRIIB signaling (28, 29). Initial experiments indicated Grb2 was differentially phosphorylated and associated with other signaling molecules under FcεRI-FcγRIIB-co-aggregating conditions (not shown). Grb2 was immunoprecipitated from lysates of variously activated cells, and immune complexes were resolved by SDS-PAGE and probed

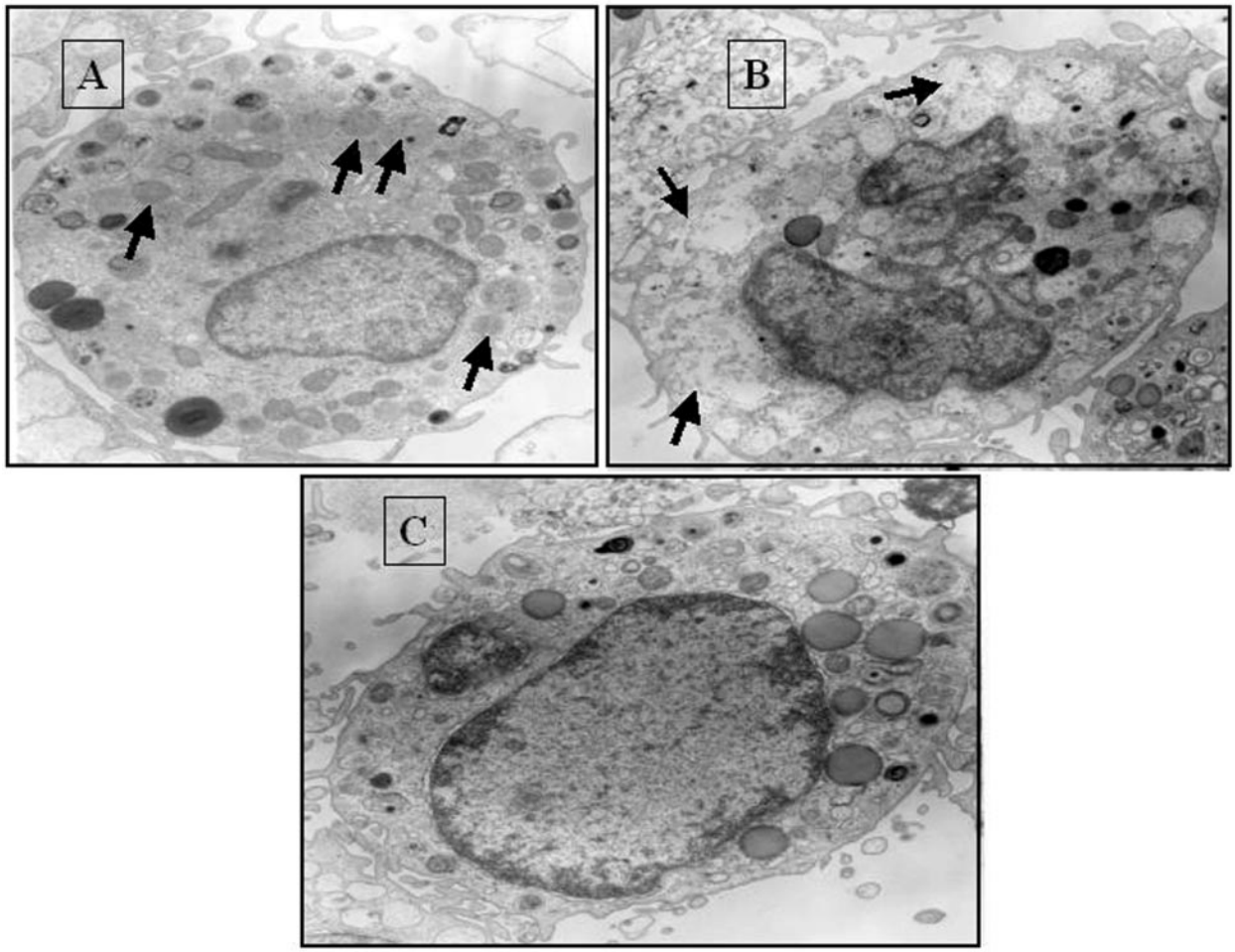

FIG. 4. **FcγRII-FcεRI co-aggregation inhibits FcεRI-mediated morphological changes in CBMCs.** Unstimulated (*A*) CBMCs show characteristic morphological features of mast cells with numerous intact cytoplasmic granules (*arrows*). FcεRI cross-linking induces granule-granule fusion in non-GE2-challenged (*arrows* in *B*) but not in GE2-challenged (*C*) CBMCs. Magnification is ×8,000.

with anti-Tyr(P) Abs. As seen in Fig. 6, in unstimulated cells Grb2 immunoprecipitated tyrosine-phosphorylated proteins with apparent molecular masses of 60–65, 54–60, 35–37, and 20–25 kDa. Upon antigen-driven FcεRI cross-linking, the interaction of Grb2 with the phosphorylated species at 20–25 and 60–65 kDa was significantly reduced. However, these reductions were not observed when antigen-treated cells had been treated with GE2 so as to drive Fcε-Fcγ co-aggregation. Stripping and reprobing with anti-Grb2 antibodies determined that the molecular mass species at about 25 kDa was Grb2 (Fig. 6, *bottom blot*). The increased phosphorylation of Grb2 in resting *versus* IgE-stimulated cells suggests that Grb2 phosphorylation is important for preventing FcεRI activation and for inhibitory signaling.

Phosphorylated Grb2 and Dok Complexes Are Increased in Resting and FcγRII-FcεRI-co-aggregated Cells—We hypothesized that the 60–65-kDa species complexed to Grb2 was Dok. Grb2 was immunoprecipitated, and the blots were probed with anti-Tyr(P) Abs. Confirming the results seen in Fig. 6, *top blot*, the phosphorylation of the 60–65-kDa species was reduced under IgE-cross-linking conditions. However, in resting and co-aggregated cells a stronger phosphorylation signal was observed (Fig. 7, *top*). Probing with anti-Dok Abs revealed that the Dok-Grb2 interaction was incrementally lost upon IgE activation but was maintained and slightly increased in resting and Fcε-Fcγ-co-aggregated cells (Fig. 7, *middle*). This difference was not because of unequal protein loading as shown in the Grb2 blot (Fig. 7, *bottom*). Studies are underway to determine the identity of the 35–40- and 50–55-kDa proteins, but linker for activation of T cells (LAT) and Shc are likely candidates, respectively. Nonetheless, Grb2 and Dok appear to form a phosphorylation-dependent complex in resting mast cells that is lost upon activation but is maintained and slightly increased under Fcε-Fcγ inhibitory signaling.

Phosphorylated SHIP and Dok Complexes Are Increased in Resting and FcγRII-FcεRI-co-aggregated Cells—Rodent studies suggest that SHIP may mediate FcγRII inhibitory function in mast cells through the recruitment of Dok (30). Dok was immunoprecipitated from cells as described above, and blots were probed with anti-Tyr(P) Abs. As seen in Fig. 8*A*, a phosphorylated protein shown to be SHIP (*middle*) at an apparent molecular mass of 145 kDa was differentially tyrosine-phosphorylated in resting, Fcε-, and Fcε-Fcγ-stimulated cells (*top*). Specifically, the strong basal tyrosine phosphorylation was decreased in IgE-stimulated cells at 2, 5, and 15 min. However, the tyrosine phosphorylation was increased in Fcε-Fcγ-stimulated cells at 5 and 15 min. In addition, immunoblot analysis of Dok immunoprecipitations using antiphosphotyrosine Abs demonstrated that Dok is constitutively phosphorylated on tyrosine at a low level in unstimulated cells, and phosphorylation is enhanced following aggregation of FcεRI alone. RasGAP was found to associate with Dok in unstimulated and GE2-co-aggregated cells (Fig. 8*B*). Thus, in human mast cells, tyrosine-phosphorylated SHIP may exert its gatekeeper role (31) through its interaction with Dok. By binding to Dok, SHIP

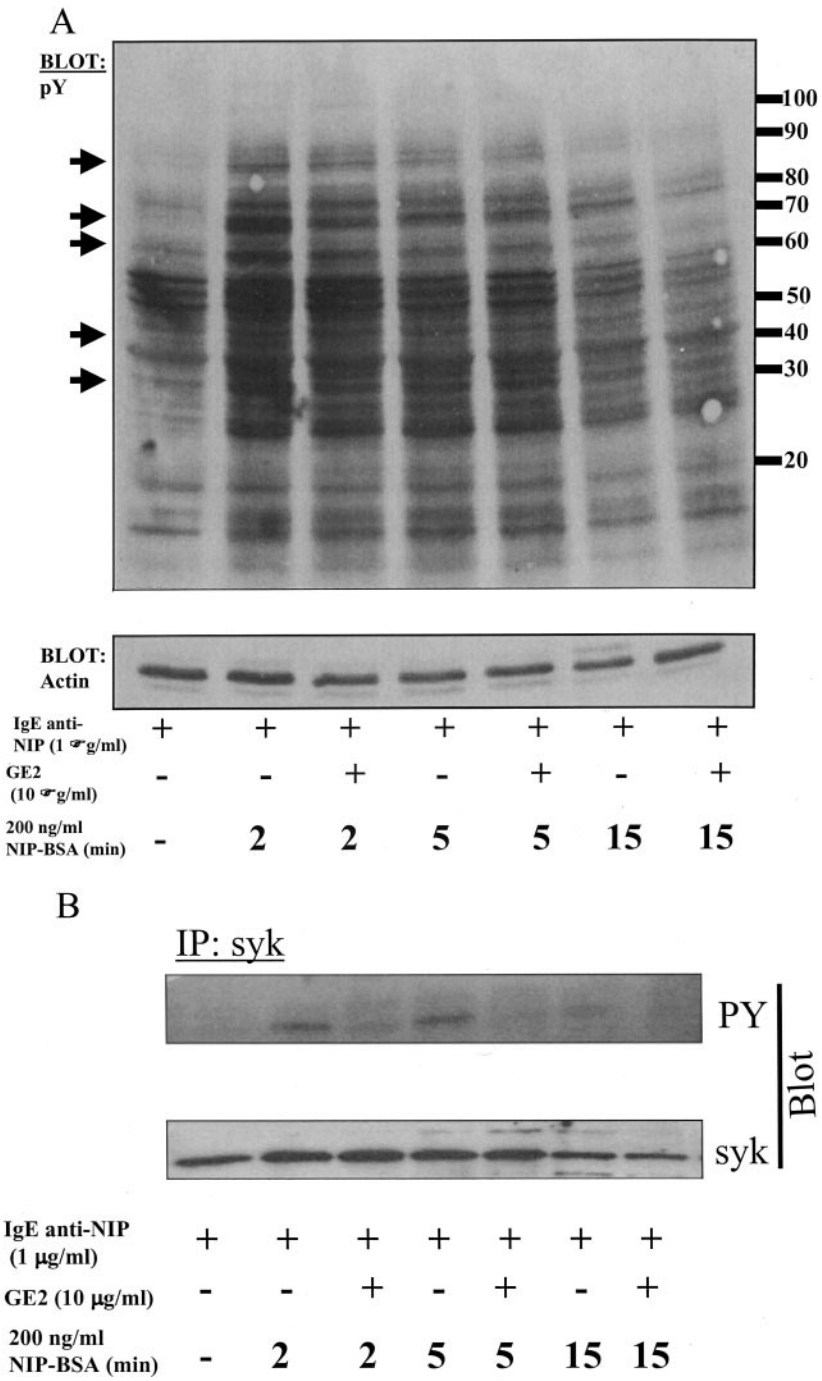

FIG. 5. *A*, differential cellular phosphorylation induced by FcγRII-FcεRI co-aggregation. IgE-sensitized CBMCs were incubated with NIP/BSA (200 ng/ml) for the indicated time with or without GE2 preincubation. Cells were lysed as described under "Experimental Procedures," and proteins in the cleared cell lysates were analyzed by SDS-PAGE (1.3×10^6 cell equivalents/lane). Proteins were transferred onto nitrocellulose membranes and probed with antiphosphotyrosine (*pY*) Ab. Immunoreactivity was detected using ECL. The *arrows* on the *left* indicate tyrosine-phosphorylated proteins differentially phosphorylated in GE2- and non-GE2-challenged cells (*top blot*). To ensure equal protein loading, blots were stripped and reprobed with 1 μg/ml anti-actin Ab as described above (*bottom blot*). Similar results were obtained with two other separate experiments. *B*, FcγRII-FcεRI co-aggregation inhibits FcγRI-mediated Syk kinase phosphorylation. Cells were lysed, and the clarified supernatants were immunoprecipitated (*IP*) with anti-Syk antibodies. Immunoprecipitates were analyzed by Western blotting with antiphosphotyrosine Abs (*top*), stripped, and reprobed with anti-Syk Ab. Each *lane* shows Syk immunoprecipitated from 2.8×10^5 cells. The results are representative of two separate experiments.

may negatively regulate Ras function by enhancing its intrinsic GTPase activity through RasGAP.

IgE Sensitization Alone Induces Degranulation in Dok Knock-out Bone Marrow Mast Cells—The data presented above suggest that Dok may play a role in setting the threshold for degranulation in mast cells. To determine whether Dok was involved in maintaining FcεRI in a quiescent state, we investigated the effects that IgE binding alone had on BMMCs from Dok knock-out mice that have been described previously (32, 33). As seen in Fig. 9, degranulation of Dok knock-out BMMCs was observed simply with the addition of IgE clones from either of two different murine clones. Degranulation from these cells did not follow exposure to mouse IgG, nor did wild type BMMCs undergo degranulation following exposure to the same IgE. Taken together, these studies demonstrate a vital role for Dok in setting the threshold for degranulation in mast cells.

DISCUSSION

The ability of FcγRII to down-regulate human basophil FcεRI-induced degranulation was first reported by Daeron and co-workers (4) and confirmed in later studies (7). Based on our recent studies using human basophils and a novel chimeric human Fcγ-Fcε Ig protein (9), we chose to focus on the use of ITIM/ITAM-receptor cross-linking as a way to block the initiation of the FcεRI response in mast cells. Because mast cells are clearly a distinct lineage from basophils in humans and have a number of distinctive functional properties in mediating human disease (34), it is very important to demonstrate their specific response to FcγRII-FcεRI co-aggregation. We report here that co-aggregating FcγRII and FcεRI on human mast cells inhibits FcεRI-induced mediator release. These results provide the first demonstration of antigen-specific down-regu-

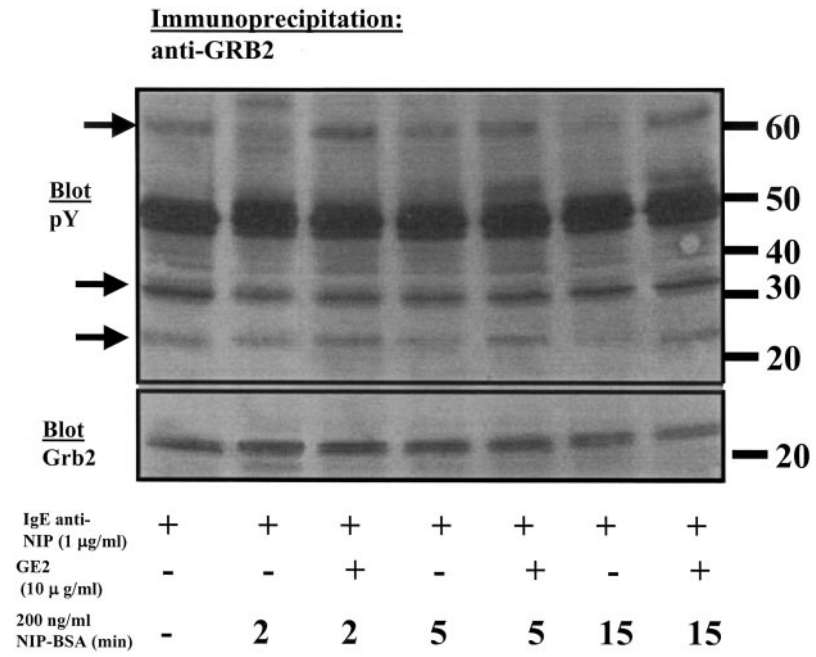

FIG. 6. **FcγRII-FcεRI co-aggregation induces differential Grb2 phosphorylation and association of several signaling proteins.** CBMCs were lysed, and the clarified supernatants were immunoprecipitated with anti-Grb2 antibodies. Immunoprecipitates were analyzed by Western blotting with anti-Tyr(P) (*pY*) Abs (*top blot*) and were stripped and reprobed with anti-Grb2 Abs (*bottom blot*). The *arrows* on the *left* indicate tyrosine-phosphorylated proteins differentially phosphorylated in GE2- and non-GE2-challenged cells. Each *lane* shows Grb2 immunoprecipitated from 8.0×10^5 cells. The results are representative of five separate experiments.

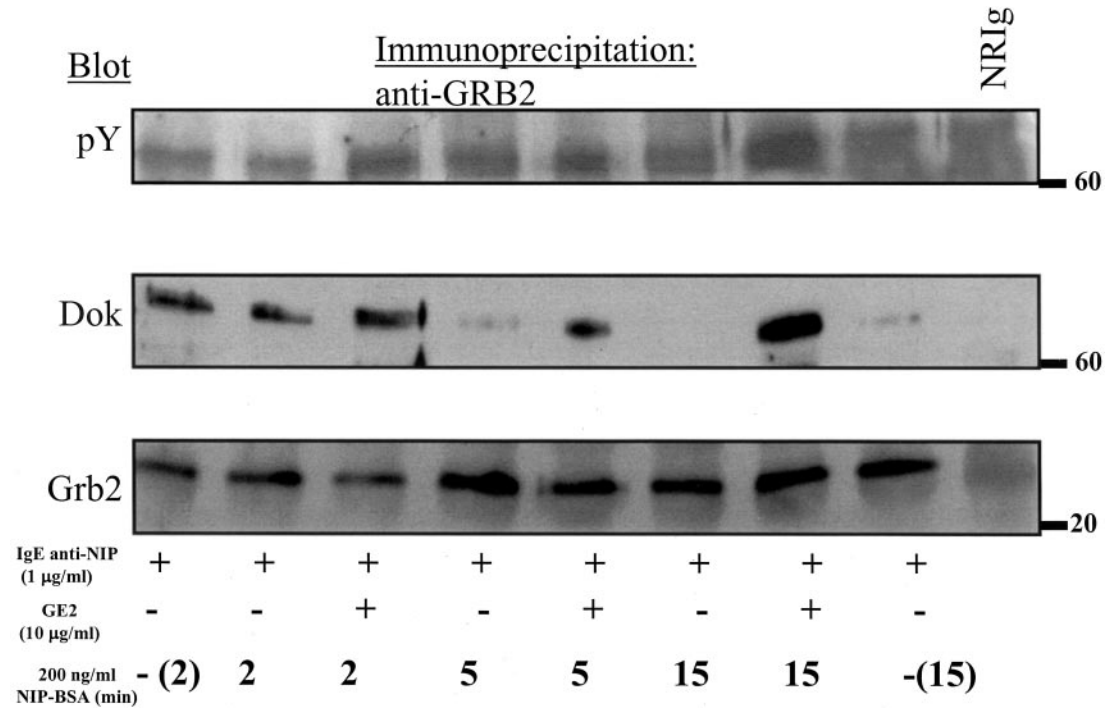

FIG. 7. **FcγRII-FcεRI co-aggregation induces Dok phosphorylation and Grb2 interaction.** CBMCs were lysed, and the clarified supernatants were immunoprecipitated with anti-Grb2 Abs. Immunoprecipitates were analyzed by Western blotting with anti-Tyr(P) (*pY*) Abs (*top*), stripped, and reprobed with anti-Dok (*middle*) and anti-Grb2 (*bottom*) Abs. Each *lane* shows Grb2 immunoprecipitated from 3.6×10^5 cells. The results are representative of two separate experiments. *NRIg*, normal rabbit IgG.

lation of FcεRI-mediated signaling though FcγRII-FcεRI co-aggregation on human mast cells.

Here, we confirm that human mast cells express CD32 (FcγRII) but not CD16 (FcγRIII) or CD64 (FcγRI). Although CBMCs contained RNA transcripts for all three FcγRII isoforms, we were not able to detect FcγRIIA protein expression by FACS analysis with Ab IV.3 or by Western blotting with an Ab raised against the cytoplasmic tail of FcγRIIA. It is still possible that there is donor variability in FcγRII expression in human mast cells as has been shown in human monocytes (35), platelets (36), and NK cells (19). Human monocytes can also alter FcγRII isoform expression in response to certain cytokines and culture conditions (37). Indeed, we have encountered periodically human FcεRI-positive cell preparations that did not respond to negative signaling, and we are investigating the relationship between FcγRII isoform expression and the ability of CBMCs to undergo co-aggregation and inhibition of FcεRI signaling.

Granule secretion and cytokine production are both Ca^{2+}-dependent processes in human mast cells. We found the lag time between antigen addition and initial Ca^{2+} mobilization very similar to those reported previously using cultured human mast cells (23, 38). However, as with human basophils (7), GE2-driven FcγRII-FcεRI co-aggregation extended the lag time

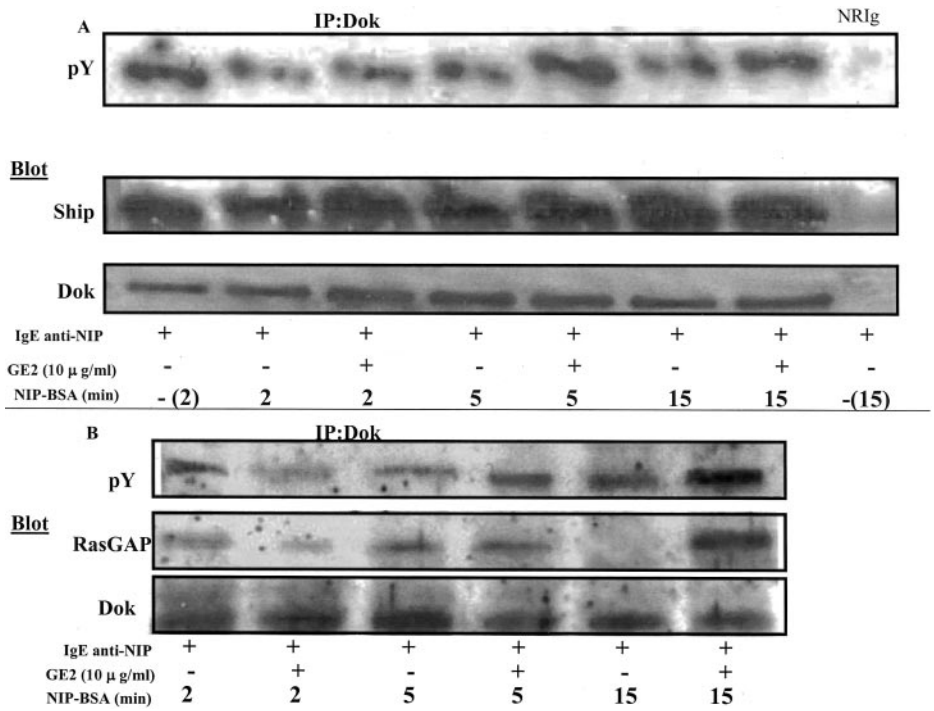

FIG. 8. *A*, FcγRII-FcεRI co-aggregation induces Dok-associated SHIP phosphorylation. CBMCs were antigen-activated for the indicated times with or without preincubation with GE2. Cells were lysed, and the clarified supernatants were immunoprecipitated (*IP*) with anti-Dok Abs. Immunoprecipitates were analyzed by Western blotting with anti-Tyr(P) (*pY*) Abs (*A*, *top*), stripped, and reprobed with anti-SHIP (*A*, *middle*) and anti-Dok (*A*, *bottom*) Abs. Each *lane* shows Dok immunoprecipitated from 4.9×10^5 cells. *B*, FcγRII-FcεRI co-aggregation induces Dok-associated RasGAP phosphorylation. CBMCs were treated as above and probed with anti-Tyr(P) followed by anti-RasGAP Abs. Each lane represents 5.6×10^5 cells. *NRIg*, normal rabbit IgG. The results are representative of two separate experiments.

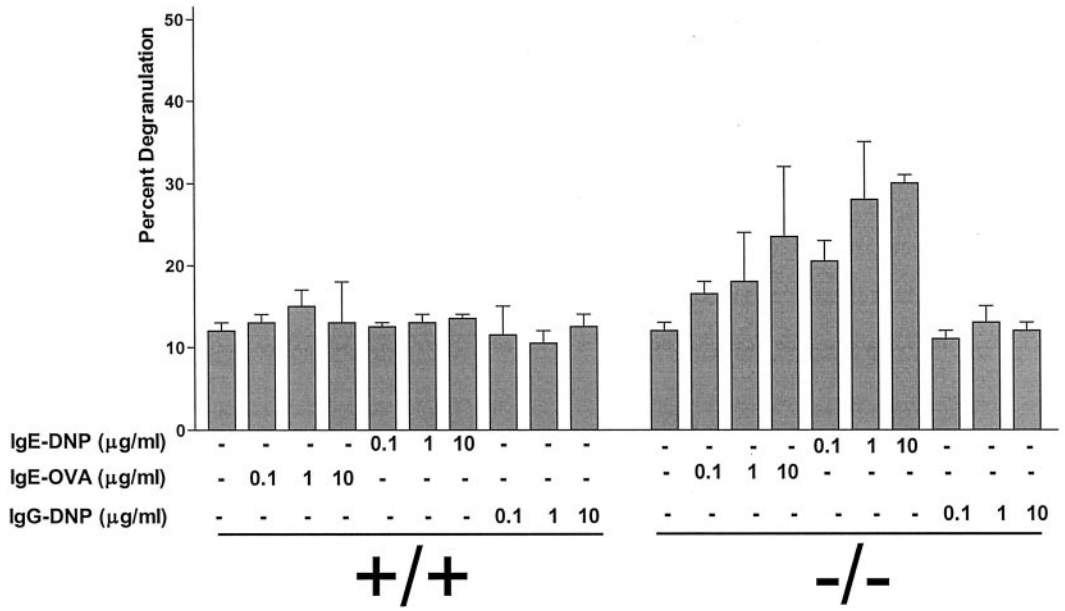

FIG. 9. **IgE alone induces degranulation in Dok knock-out BMMCs.** BMMCs from Dok+/+ or Dok−/− were challenged with varying concentrations of mouse IgE or IgG and centrifuged, and the supernatant was removed for the analysis of β-hexosaminidase as described previously (61). The results are representative of three separate experiments.

from FcεRI cross-linking to the Ca^{2+} spike response and reduced the magnitude of the Ca^{2+} plateau. In addition, GE2 appears to inhibit the release of intracellular calcium stores as thapsigargin-induced release was inhibited by GE2. Co-aggregation of FcγRII-BCR on B cells under a variety of co-cross-linking conditions results in premature termination of Ca^{2+} influx, sometimes associated with a reduced Ca^{2+} spike response but not an increased delay to the spike response (39–42). This difference between mast cells/basophils and B cells is unexplained but likely reflects distinct signaling properties of B cells and human FcεRI-positive cells and particularly the presence of FcγRIIA on resting and activated B cells.

Tyrosine phosphorylation of cellular substrates is an integral part of IgE-mediated signaling in human mast cells (38) and basophils (7). Several substrates were differentially phosphorylated upon antigen activation in CBMCs (Fig. 5*A*). We reported previously that the tyrosine kinase Syk is critical in human basophil IgE-mediated signal transduction (15, 16) and that co-aggregation inhibits Syk phosphorylation (7, 9). Here we show little or no tyrosine-phosphorylated Syk present in resting mast cells, whereas FcεRI cross-linking induced a large increase in Syk phosphorylation (Fig. 5*B*). The kinetics of phosphorylation observed upon IgE activation were similar to previous reports using human mast cells (23). As with human basophils, Syk phosphorylation was markedly decreased by co-aggregating FcγRII and FcεRI.

Surprisingly, we observed increased Grb2 phosphorylation in resting and FcγRII-FcεRI-co-aggregated cells with reduced Grb2 phosphorylation seen in mast cells activated through FcεRI alone. We are not aware of any studies that associate

Grb2 tyrosine phosphorylation with inhibitory FcεRI signaling. Grb2 functions as an adaptor protein associated with protein-tyrosine kinase signaling (43). Published studies, mostly non-physiological, focus on the phosphorylation of other signaling intermediates, which then leads to the binding of Grb2 SH2 and SH3 domains. The tyrosine phosphorylation of Grb2 itself and the effect on signaling responses have not been characterized, especially in human mast cells. However, Grb2 protein has several tyrosines capable of being phosphorylated (44), and Grb2 tyrosine phosphorylation has been detected under physiological conditions in several cell types (45–47). Analogous to our findings, Grb2 tyrosine phosphorylation was clearly shown to negatively regulate the downstream activation of tyrosine kinase signaling pathways in other cell types (48). Our data support these previous studies and suggest that tyrosine phosphorylation of Grb2 may be an important step in a novel mechanism of down-regulation of FcεRI activation of human mast cells.

We also found that Dok is involved in IgE receptor signaling in human mast cells. Similar to Grb2, Dok phosphorylation was reduced in FcεRI-stimulated cells, whereas it was maintained in co-aggregated "inhibited" cells. The increase in Dok tyrosine phosphorylation was also associated with increased interaction with Grb2. Previous studies have shown that Grb2 SH2 domains can bind to phosphorylated Dok (and a phosphorylated 30–40-kDa protein, as we also found) (Fig. 6) (49). Reports with transfected RBL-2H3 cells demonstrated that co-aggregation of FcγRII with FcεRI stimulates enhanced SHIP tyrosine phosphorylation leading to association with Shc and Dok. An increase in Dok phosphorylation was observed under co-aggregation conditions, with subsequent increased association with RasGAP (33). Similarly, co-aggregating FcγRII with FcεRI on B cells leads to increased tyrosine phosphorylation of Dok associated with negative signaling (50, 51). Dok also plays a negative role at multiple steps in T cell signaling (37) and through the ITIM-containing mast cell function-associated antigen (MAFA) receptor on RBL-2H3 cells (52). Overall, our data also support a role for Dok in inhibitory signaling through FcγRIIB via its increased association with Grb2 and SHIP.

We observed Dok-Grb2 interaction in non-activated cells, interaction that was decreased at 2 and 5 min in IgE-activated cells and completely absent at 15 min. Thus tyrosine-phosphorylated Dok-Grb2 appears to be important for maintaining FcεRI in its unactivated state and suggests a previously unrecognized gatekeeper role for Dok and Grb2 in human mast cells. This contrasts with a rodent study that found that Dok is not needed for FcγRIIB-mediated inhibitory signaling in Dok-deficient mast cells (33), suggesting that another molecule can compensate for loss of Dok or that, as in other signaling systems, there are important differences between rodents and humans.

We have confirmed rodent studies in which Dok was found to interact with SHIP and in which co-aggregation of Fcε with Fcγ led to increased SHIP phosphorylation (33, 53). We observed a high basal tyrosine phosphorylation of Dok-associated SHIP in resting cells that was slightly decreased in IgE-activated (FcεRI-cross-linked) cells. RBL-2H3 cells have also been shown to exhibit high basal tyrosine phosphorylation of SHIP (33). Others have reported an increase in tyrosine phosphorylation of SHIP following FcεRI cross-linking (33). It is possible under these conditions that SHIP is recruited away from Dok and to the FcεRI β chain (33, 54, 55) to regulate FcεRI signaling through an FcγRII-independent mechanism. We are currently examining this hypothesis.

Our data using Dok knock-out BMMCs further point to a role for Dok in the FcγRIIB-mediated inhibition of IgE-FcεRI degranulation. We found that monomeric IgE alone induced degranulation in these cells. Previous studies found similar results in SHIP knock-out mast cells, although the maximal degranulation we observed (≈30%) was lower than that in the SHIP knockouts (≈80%) (31). This suggests that Dok contributes to the process of BMMCs IgE receptor quiescence, but other factors are also needed, probably SHIP. To this end, Ott *et al.* (33) found Dok was sufficient, but not necessary, for FcγRIIB inhibitory signaling in BMMCs from Dok knock-out mice. Taken together, these results suggest that Dok, through its interaction with Grb2 and SHIP, keeps FcεRI in a quiescent state and that this inhibitory complex is maintained under co-aggregating conditions.

We confirm previous studies that implicate SHIP in FcγRII-induced inhibition of FcεRI signaling. The absence of SHIP in mice leads to increased releasability of BMMCs, even during IgE sensitization (31). In human FcεRI studies, Vonakis *et al.* (56) found a strong negative correlation between the amount of SHIP protein and maximal degranulation in human basophils. Although our results showing reduced Syk phosphorylation support a role for inhibitory signaling by SHP-1 (57), SHIP activation also appears to be important in our human mast cell system. Given that FcγRIIB can interact with both SHP-1 and SHIP (58, 59), it is still unclear which phosphatase plays the more dominant role in down-regulating FcεRI in human mast cells. It is quite possible that multiple phosphorylation sites in FcγRIIB, where phosphatase binding occurs, contribute uniquely to transduction of FcγRIIB-mediated inhibitory signals.

Tyrosine phosphorylation of Grb2 and the differential binding of Dok and SHIP may regulate inhibitory function through several different mechanisms. Grb2 may help to localize Dok and/or SHIP to the cell membrane, regulating their inhibitory function. It is well established that SHIP can inhibit FcεRI functional and biochemical responses (60), but it is not as clear how SHIP exerts such effects. Dok tyrosine phosphorylation (or enhanced SHIP tyrosine phosphorylation) may facilitate the recruitment of Dok (through Grb2 as seen in Fig. 7) to the FcεRI-FcγRII receptor complex. Indeed, Dok recruitment to FcεRI is enough to inhibit FcεRI-induced signal transduction (30). Thus, because SHIP and Dok can associate with RasGAP under co-aggregating conditions (33, 50), FcεRI may be "turned off" by up-regulating Ras GTPase activity. Our findings suggest that the mechanisms that keep FcεRI in its non-activated state are similar to the mechanisms of inhibition through FcεRI-FcγRIIB co-aggregation.

Many investigators have sought inhibitors of human FcεRI signaling to block IgE-mediated allergic inflammation. One approach is to block FcεRI signaling by altering the propagation of signals generated from the cross-linked receptors. Strategies based on the specific inhibition of the earliest events in the FcεRI signaling cascade, such as we have shown here using GE2 to co-aggregate FcεRI with FcγRII, may ultimately generate new therapies for allergic inflammation. In addition to FcγRII, we also demonstrate that CBMCs also express the ITIM-containing receptors CD22 and CD81. We did not detect other ITIM-receptor expression on mast cells but were limited to those which had Abs detecting human isoforms. CD22 is widely known for its ability to down-regulate B cell receptor-mediated B cell activation (62), whereas CD81 negatively regulates FcεRI responses in rodent mast cells (63). These and other ITIM-containing receptors on human mast cells are also potential novel targets for chimeric bifunctional proteins to "down-regulate" FcεRI function as described here.

In summary, we have demonstrated that IgE activation of cultured human mast cells can be inhibited by co-aggregating

FcεRI with FcγRII through a novel mechanism involving Grb2, Dok, and SHIP. By achieving this with a human chimeric immunoglobulin, we have sought to turn off allergic responses utilizing the powerful negative regulatory ability of ITIM receptor motifs. The highly specific and avid association of Fc and FcεRI provides a highly specific target by which to deliver the off switch to effector cells of allergy and asthma. This approach can be extended in several ways. Firstly, chimeric proteins capable of binding more that one ITIM receptor (*e.g.* G-G-E2 or CD81L-G-E2) may be more potent inhibitors. Secondly, it is possible to make chimeric allergen-γ proteins that will bind to FcγRII and indirectly bind to FcεRI via their high affinity interaction with the antigen-specific IgE on the mast cells. Not only would this latter approach serve to inhibit antigen-specific immediate reactivity, but also it should be able to provide a platform for safer and more effective allergen immunotherapy. As such, these improved versions of the chimeric protein described may prove to be promising agents for the treatment of human allergic disorders.

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