Adaptor FYB (Fyn-binding protein) regulates integrin-mediated adhesion and mediator release: Differential involvement of the FYB SH3 domain

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Aggregation of the high-affinity IgE receptor (FcεRI) on mast cells activates a tyrosine phosphorylation cascade that is required for adhesion and degranulation events leading to the release of histamine and other inflammatory mediators. The full range of intracellular mediators that regulate this process is unknown. Recent studies have identified a group of immune cell-specific adaptor proteins that include linker for activation of T-cell (LAT), SH2-domain-containing leukocyte protein (SLP-76), and Fyn-T-binding protein (FYB)/SLP-76-associated protein (SLAP). In this study, we demonstrate that FYB can up-regulate integrin-mediated adhesion to fibronectin and mediator release in RBL-2H3 mast cells. The regulation of these two events could be distinguished from each other by the requirement of the FYB SH3 domain in β-hexosaminidase release, but not adhesion, and the up-regulation of mediator release by FYB in nonadherent cells. FcεRI aggregation increased FYB tyrosine phosphorylation, whereas confocal immunofluorescence microscopy showed that FYB colocalizes with F-actin in membrane ruffles and plaques. Our findings identify FYB as a regulator of integrin-mediated adhesion and degranulation events, which, in the case of mast cells, has potential applications to inflammatory and allergic responses.

Recent studies have identified a spectrum of immune cell-specific adaptors that integrate signals from surface receptors (1–3). These adaptors include linker for activation of T-cell (LAT), Grb2-related adaptor down-stream of Shc (Gads), SH2-domain-containing leukocyte protein of 76 kDa (SLP-76), Fyn-T-binding protein (FYB)/SLP-76-associated protein of 130 kDa (SLAP-130), and *s*rc kinase-associated phosphoprotein of 55 kDa (SKAP55). LAT and SLP-76 are essential for proper thymic differentiation, T cell proliferation, and platelet–mast-cell function (4–8). The major binding partner of SLP-76 is the SH2 domain-binding protein FYB/SLAP (9, 10). FYB also binds to and is preferentially phosphorylated by src kinase FYN-T (9, 11). Two isoforms of FYB at 120 and 130 kDa (FYB-120/130) have been cloned and are restricted to T cells, monocytes, platelets (11, 12), and mast cells (this article). Each isoform has Y^{595}DDV/Y^{651}DDV and Y^{624}DGI motifs for SLP-76 and FYN-T SH2 domain binding, respectively (11, 13), a proline-rich region that binds to the SH3 domain of SKAP-55 (14, 15), two putative nuclear localization sites (9), a C-terminal SH3-like domain that binds to a tyrosine-based RKxxYxxY motif in SKAP55 (16), and an Ena (Enabled)/VASP (vasodilator-stimulated phosphoprotein) homology 1 domain (EVH1) binding site (17).

The role of FYB/SLAP as a positive or negative regulator of immune cell signaling has been the subject of much debate (9–12). FYB overexpression has been reported to either weakly potentiate (9) or to inhibit T cell antigen receptor (TcR)ζ/CD3-driven IL-2 production (10). However, cotransfection of FYB with its binding partners Fyn-T and SLP-76 cooperates in the potent up-regulation of IL-2 transcription (11, 12). TcRζ/CD3-induced IL-2 release depends on intact SLP-76 SH2 binding sites Y^{595}DDV and Y^{651}DDV (13) or an alternate Y^{559}GYI site (18). Further support for a regulatory role for this adaptor is its association with the TcR complex (19) and its localization at the site of TcR ligation (17).

The multimeric Fc ε receptor I (FcεRI) complex on mast cells and basophils forms the high-affinity receptor for the Fc portion of antigen-specific IgE antibodies (20). Multivalent cross-linking of FcεRI activates a tyrosine phosphorylation cascade required for degranulation and the release of histamine and other inflammatory mediators (20–22). The full range of downstream regulators of this process has yet to be established. Although FcεRI lacks intrinsic enzymatic activity, it possesses cytoplasmic immune receptor tyrosine-based motifs that become phosphorylated by src kinase Lyn, thereby leading to recruitment of protein tyrosine kinase Syk (21, 23, 24). Subsequent events include phosphorylation of substrates including phospholipase Cγ1 (PLCγ1), LAT, SLP-76, and proteins in the ≈120-kDa range (21, 24–30). Bone marrow-derived mast cells from LAT- and SLP-76-deficient mice exhibit profound defects in degranulation and cytokine production after FcεRI cross-linking (7, 8). FYB binding to SLP-76 (9, 10) suggested a role for the adaptor in the regulation of mast-cell function.

In this article, we demonstrate that FYB can up-regulate integrin-mediated adhesion to fibronectin (FN) and mediator release in rat basophilic leukemia (RBL)-2H3 mast cells. Furthermore, the regulation of these two events could be distinguished from each other by the requirement of the FYB SH3 domain in β-hexosaminidase release but not in integrin-mediated adhesion. Therefore, different regions of the FYB adaptor regulate different cellular events. These data identify FYB as a previously uncharacterized regulator of FcεRI-mediated mast-cell function having potential applications to inflammation and the maintenance of allergic responses.

Materials and Methods

Reagents and Cells. Monoclonal anti-2,4-dinitrophenyl (DNP) IgE, SPE-7, FN, BSA, ionomycin, and phorbol 12-myristate 13-acetate were obtained from Sigma. Anti-phosphotyrosine mAb, 4G10, and FITC-conjugated anti-mouse IgG1 were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-influenza virus hemagglutinin (HA) mAb, HA.11, was obtained from BabCO (Richmond, CA). Anti-FYB mAb was obtained from Transduction Laboratories (Lexington, KY), whereas the

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Abbreviations: LAT, linker for activation of T cell; SLP-76, SH2-domain-containing leukocyte protein of 76 kDa; FYB, Fyn-T-binding protein; TcR, T cell antigen receptor; FcεRI, high-affinity receptor for Immunoglobulin E; DNP, 2,4-dinitrophenyl; HA, hemagglutinin; KLH, keyhole limpet hemocyanin; FN, fibronectin.

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anti-FYB rabbit antiserum was generated as described (19). DNP-keyhole limpet hemocyanin (KLH) was obtained from Calbiochem. Rhodamine-phalloidin was purchased from Molecular Probes (Eugene, OR). RBL-2H3 cells were obtained from the American Type Culture Collection and maintained in recommended medium.

Generation of Stable Transfectants. Full-length human FYB-120 cDNA and the deletion mutant FYBΔ707 (amino acids 1–707) were cloned into the PEBB mammalian expression vector containing a sequence encoding the influenza HA epitope tag at the N terminus as described. RBL-2H3 cells (10^7) in 0.5 ml of Eagle's minimal essential medium were electroporated with 20 μg of FYB or FYBΔ707 together with 4 μg of pSVneo containing a neomycin resistance gene with an electroporation apparatus from BTX (San Diego) at 960 μF, 310 V, 186 Ω. Stable transfectants were selected in complete medium containing 0.6 mg/ml G418 for 2 weeks. Stable lines overexpressing FYB or FYBΔ707 as determined by Western blotting were subcloned by limited dilution to obtain stable clones.

Cell Activation. RBL-2H3 cell monolayers were washed once with Tyrode's buffer (10 mM Hepes/130 mM NaCl/5 mM KCl/1.4 mM $CaCl_2$/1 mM $MgCl_2$/5.6 mM glucose/0.1% BSA, pH 7.4). Cells were activated with antigen (DNP-conjugated KLH, 100 ng/ml), calcium ionophore ionomycin (0.5 μM), or phobol myristate acetate (40 nM) in Tyrode's buffer. For stimulation with antigen, the cells were cultured for 2 hr with 1 μg/ml DNP-specific IgE before adding the antigen. In experiments to deplete the extracellular calcium, the cells were washed twice with either Ca^{2+}-containing or Ca^{2+}-free (1 mM EGTA) Tyrode's buffer and then stimulated in either Ca^{2+}-containing or Ca^{2+}-free (10 μM EGTA) Tyrode's buffer.

Adhesion Studies. Flat-bottomed nontissue culture-treated 96-well plates were coated with FN (1 μg per well) in PBS at 4°C overnight. Wells were washed once with PBS, then unoccupied sites were blocked by incubating with PBS containing 2% (wt/vol) BSA at 37°C for 1 hr. The wells were washed twice with PBS and once with Eagle's minimal essential medium (EMEM). Adherent transfectants grown overnight were dissociated from flasks by trypsinization and allowed to recover by incubation in EMEM at 37°C for 10 min. To examine cell adhesion to FN-coated surface, 10^5 cells were added to the wells and incubated for various time intervals at 37°C. At the end of the incubation, unbound cells were removed by aspiration, and wells were washed twice with complete EMEM. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to quantify the number of bound cells. Briefly, 25 μl of MTT (2 mg/ml; Sigma) solution was added to each well. The cells were incubated at 37°C for 3 hr, and the plates were centrifuged at 1500 rpm for 10 min (Sorvall RT6000, DuPont). After the supernatant was removed, 100 ml of DMSO (Sigma) was added to each well. Formazan was dissolved by shaking and OD at 595 nm was measured by an ELISA reader (Bio-Rad). The percentage of cells that adhered was obtained by dividing the average OD at a time point by the OD of a control well containing 10^5 cells of the same type.

β-Hexosaminidase Release Assay. The secretory response was monitored by measuring the activity of β-hexosaminidase in the cell supernatants after treatments indicated for each particular experiment (31). Cells (10^5 per well) grown overnight on 96-well plates were loaded with IgE for 2 hr and stimulated for 30 min with DNP-KLH (250 ng/ml) or ionomycin (2 μM) in 100 μl of Tyrode's buffer. For each sample assayed, supernatant aliquots (20 μl) were transferred to microtiter plates. Fifty microliters of substrate solution (1.3 μg/ml P-nitrophenyl-*N*-acetyl-β-D-glucosamine in 0.1 M sodium citrate, pH 4.5) was then added and plates were incubated for 1 hr at 37°C. The reaction was then stopped, and the color was developed by the addition of 150 μl of 0.2 M glycine solution, pH 10.7. The light absorption by the substrate hydrolysis product was measured at 405 nm in an ELISA plate reader. To determine the total cell content of this enzyme, an equivalent number of cells were lysed with 1% Triton X-100, and a lysate aliquot was assayed in parallel to the other experimental samples. The extent of secretion is expressed as the net fraction of the total β-hexosaminidase activity present in the cells.

Immunofluoresence and Confocal Microscopy. RBL-2H3 cells were cultured on cover slips and then stimulated as described above. Cells were fixed with 2% (wt/vol) paraformaldehyde in PBS for 10 min and permeabilized with 0.1% Triton X-100/PBS for 10 min. After rinsing with PBS and blocking with 1% BSA/PBS, the cells were incubated with anti-FYB mAb for 1 hr at 37°C. FYB was detected by incubation with FITC-labeled secondary antibody for 1 hr at 37°C. Rhodamine-labeled phalloidin was added together with the secondary antibody to detect F-actin. Coverslips were mounted in glycerol–gelatin (Sigma) onto microscope slides. Immunofluorescence was analyzed with the confocal laser scanning microscope LSM 410 (Zeiss, Germany) equipped with an external argon–krypton laser (568 nm).

Results

FcεRI Aggregation Induces FYB Tyrosine Phosphorylation. Previous studies have reported that ≈120-kDa proteins are tyrosine phosphorylated upon FcεRI stimulation of RBL-2H3 cells, one of which coprecipitates with SLP-76 (27–29). Given that FYB/SLAP is a 120- to 130-kDa phosphoprotein that interacts with the SH2 domain of SLP-76 (9, 10), we initially examined whether FYB was expressed in mast cells and whether FcεRI ligation could induce FYB tyrosine phosphorylation. As shown in Fig. 1*A Upper*, FcεRI cross-linking induced rapid tyrosine phosphorylation of FYB that peaked at 1 min and remained high after 30 min of stimulation. The rapidity and intensity of this phosphorylation was considerably more pronounced than that observed in T cells (9–11). Equivalent levels of FYB were expressed among samples, as verified by anti-FYB blotting (Fig. 1*A Lower*). FcεRI-mediated substrate phosphorylation can occur both prior to and after the rise of intracellular calcium and protein kinase C activation (32–34). To address whether FYB phosphorylation could be regulated by either of these events, calcium ionophore (i.e., ionomycin) and phorbol 12-myristate 13-acetate also were used to activate RBL-2H3 cells. Both treatments induced tyrosine phosphorylation of FYB (Fig. 1*B Upper*). To assess whether phosphorylation required extracellular Ca^{2+}, the divalent ion was chelated with EGTA before cross-linking (Fig. 1*C*). The removal of Ca^{2+} had little apparent effect on FcεRI-mediated FYB phosphorylation (Fig. 1*C*). Blotting confirmed the expression of equivalent amounts of FYB (Fig. 1 *B* and *C*, *Lower*). Low levels of tyrosine phosphorylation of FYB were evident in unstimulated cells (Fig. 1). Overall, our findings demonstrate that FcεRI stimulation can induce rapid phosphorylation of FYB adaptor independently of extracellular Ca^{2+}.

FYB Colocalizes with Actin in Membrane Ruffles. FcεRI cross-linking is known to induce increased membrane ruffling and adhesion of RBL-2H3 cells (35). F-actin localizes to the lamellae at the apical surface and in foot-like plaques (35). Given that FYB possesses putative EVH1 and actin binding sites (17), we next investigated by confocal microscopy whether FYB might localize to actin-rich ruffles. As shown in Fig. 2 *a–c*, resting RBL-2H3 cells have an elongated shape with few extensions or adhesion plaques. A ventral view of anti-FYB-stained cells showed mostly perinuclear/cytoplasmic and limited peripheral membrane

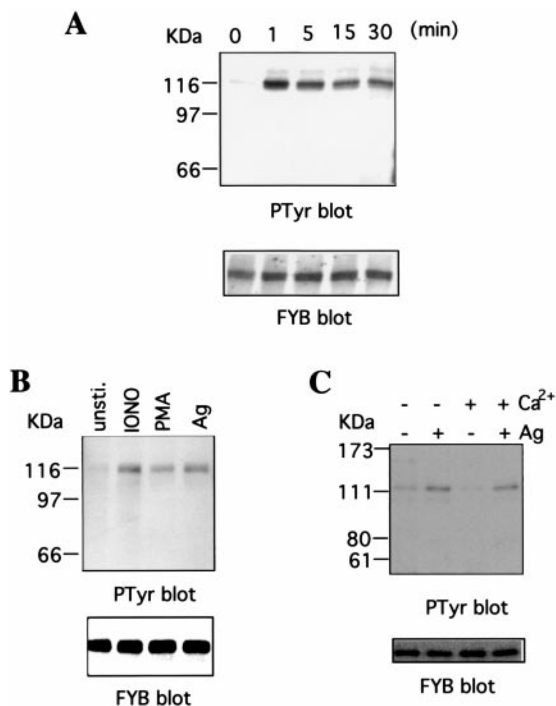

Fig. 1. FcεRI-induced tyrosine phosphorylation of FYB. (*A*) Time course of the FcεRI-induced tyrosine phosphorylation of FYB. RBL-2H3 cells preincubated with DNP-specific IgE were washed and stimulated with DNP-KLH antigen at 100 ng/ml for the indicated times. Cell lysates were subjected to immunoprecipitation with anti-FYB (PTyr) antisera and analyzed by blotting with anti-phosphotyrosine 4G10 (*Upper*) or anti-FYB mAb (*Lower*). (*B*) Cells were either unstimulated (unsti.) or stimulated with 0.5 mM calcium ionophore ionomycin (IONO), 40 nM phorbol 12-myristate 13-acetate (PMA), or antigen (Ag) for 10 min. (*C*) FcεRI-induced tyrosine phosphorylation of FYB is independent of Ca^{2+} influx. Cells were washed and stimulated for 10 min with antigen (Ag) in Tyrode's buffer containing either 1.4 mM $CaCl_2$ (Ca^{2+}, +) or 10 μM EGTA (Ca^{2+}, −).

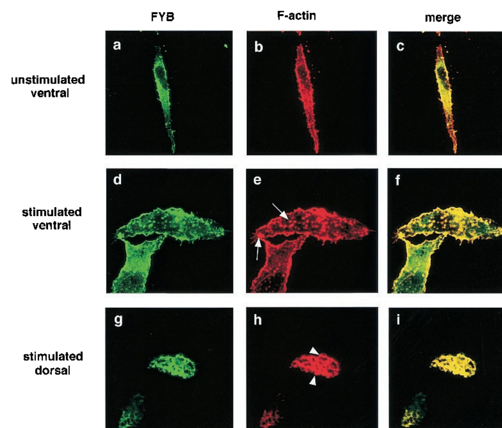

Fig. 2. Colocalization of FYB and F-actin in adhesion plaques and membrane ruffles after FcεRI stimulation. Immunofluorescent localization of F-actin and FYB visualized by confocal microscopy. FITC-labeled FYB (green) and/or rhodamine-phalloidin-labeled F-actin (red) is illustrated for RBL-2H3 cells that are either unstimulated (*a–c*) or stimulated with antigen (*d–i*). The focal cut was made either along the ventral (i.e., bottom) cell surface (*a–f*) or the dorsal cell surface (*g–i*). Anti-FYB-FITC staining observed in *a*, *d*, and *g*, whereas phalloidin staining with rhodamine is seen in *b*, *e*, and *h*. Overlapping images can be seen in *c*, *f*, and *i*. Antigen-induced actin plaques or podosomes (arrows) and membrane ruffles (arrowheads) are indicated.

staining (Fig. 2*a*). Rhodamine-tagged phalloidin showed more extensive membrane staining (Fig. 2*b*), whereas an overlay of images showed colocalized cytoplasmic staining with a limited degree of membrane colocalization (Fig. 2*c*). By comparison, FcεRI activation caused a significant increase in size, spreading, and the number of membrane ruffles and points of attachment with the substratum (Fig. 2 *d–i*). In these activated cells, a ventral (i.e., bottom) cut of cells showed extensive actin staining at the membrane periphery as well as in the characteristic dot-like F-actin assembly points (adhesion plaques or podosomes) where cells attach to the substratum (see arrows, Fig. 2*e*). This characteristic pattern of staining of RBL-2H3 cells has been well documented in other reports (35) and was particularly evident at the extended poles of cells (see left arrow, Fig. 2*e*). Importantly, anti-FYB staining also showed intense staining at the membrane periphery and adhesion plaques (Fig. 2*d*). An overlay of the images showed a striking degree of colocalized FYB and F-actin (Fig. 2*f*). In terms of membrane ruffles, the FYB and F-actin colocalization was particularly evident with a dorsal cut along the surface of the activated mast cell (Fig. 2 *g–i*). In this case, almost complete colocalized staining was evident in the multiple membrane ruffles on the surface of cells (Fig. 2 *g–i*, see arrowheads in *h*). These confocal images demonstrate that FYB and F-actin can colocalize in the FcεRI-induced adhesion plaques and surface membrane ruffles.

FYB Overexpression Enhances Adhesion to Fibronectin. The colocalization of FYB with F-actin in adhesion plaques and ruffles suggested FYB might play a role in cell–substrate adhesion. To address this question further, stable transfectants were generated that overexpress HA-tagged wild-type FYB or an FYB mutant (FYBΔ707) that lacks the C-terminal SH3 domain (Fig. 3*A*). Immunoblotting with anti-HA confirmed the presence of HA-tagged FYB in FYB-transfected cells (*Left*), but not in the vector (PEBB)-transfected cells (*Right*). Anti-FYB blotting showed that transfected FYB (FYB) was moderately expressed at a 2- to 3-fold higher level than endogenous FYB protein. In FYBΔ707, the expression of endogenous FYB was unaffected relative to vector-transfected cells (data not shown). As seen in Fig. 3*B*, vector-transfected control cells showed a time-dependent increase in the percentage of cells that bound to FN over a time course of 0–30 min. Whereas 5% of cells remained bound at 10 min, more than 35% were found attached at 30 min. It is interesting, however, that transfectants expressing FYB-HA showed a 2-fold increase in the percentage of cells relative to the vector-transfected control. This increase was observed over the entire time course of binding and between different transfectants. As seen with the two clones FYB34 and -35, 15% of cells were bound by 10 min and 50–55% were bound by 30 min. Surprisingly, FYBΔ707 transfectants (i.e., Δ707.4) also showed an increase in adhesion that was similar to that observed for wild-type transfectants. Wild-type FYB and FYBΔ707 were expressed at similar levels as assessed by blotting and showed the expected size difference (Fig. 3*A*, lanes 3 and 2, respectively). A comparison of three independently derived clones of each type (wild-type FYB, FYB Δ707, and vector PEBB) yielded similar conclusions. Results at 30 min from five separate experiments are presented in Fig. 3*B Inset*. A Student's *t* test confirmed that the responses of wild-type FYB are significantly higher than the vector clones ($P < 0.02$) and not significantly different from the FYB Δ707 clones ($P > 0.1$). This increase in adhesion also could be visualized by viewing plates under the light microscope (data not shown). Last, to control for possible differences in the level of FN receptors on the surface of the various transfectants—β1 (CD29) and β3 (CD61)—expression was assessed by fluorescence-activated cell sorting analysis (Fig. 3*C*). β1 and β3 are components in the full range of potential receptors ($\alpha4\beta1$, $\alpha5\beta1$, and $\alpha v\beta3$) for FN on the surface of cells. β1 and β3 expression

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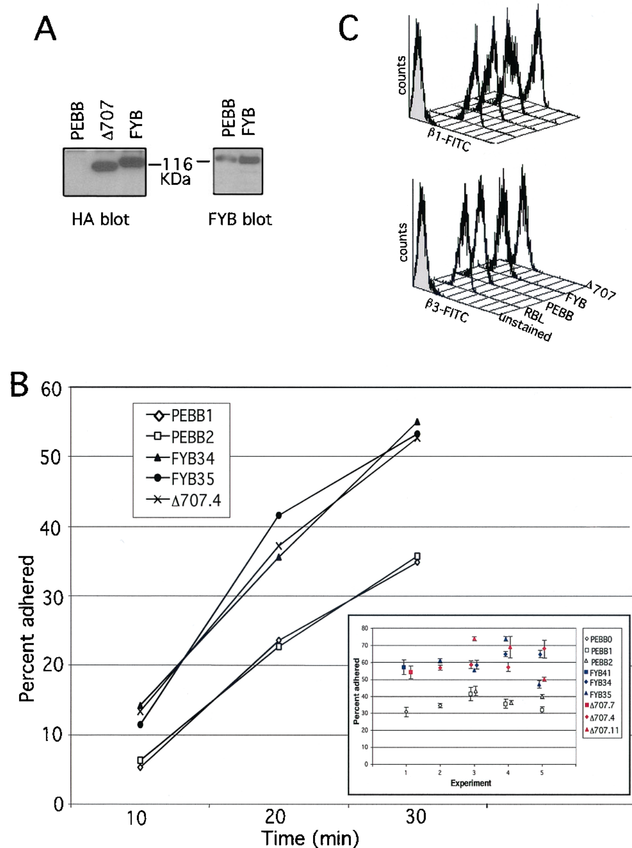

Fig. 3. FYB overexpression enhances integrin-mediated adhesion to FN. (*A*) Expression of FYB and Δ707 in RBL-2H3 stable transfectants. Cell lysates from HA-tagged FYB, or HA-tagged Δ707 transfectants and the PEBB vector transfectants were analyzed for expression by immunoblotting with anti-HA (lanes 1–3) or anti-FYB mAb (lanes 4, 5). (*B*) FYB and Δ707 transfectants show enhanced binding to FN. Transfectants or vector control (PEBB) transfectants were allowed to adhere to FN-coated plates for various time periods, and the extent of cell adhesion was examined by quantifying the adherent cells with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The mean of triplicate wells is presented. (*Inset*) The mean percentages of adhered cells at 30 min along with the SDs derived from five separate experiments are presented for three of each FYB- (blue), FYB Δ707- (red), and PEBB-transfected (white) clones. (*C*) Various transfectants show equivalent levels of β1 (CD29) and β3 (CD61) expression. β1 (CD29) and β3 (CD61) expression was assessed by fluorescence-activated cell sorting analysis. β1 and β3 are components in FN receptors (α4β1, α5β1, and αvβ3). Unstained RBL cells were used as negative control (filled). (*Upper*) β1 staining. (*Lower*) β3 staining.

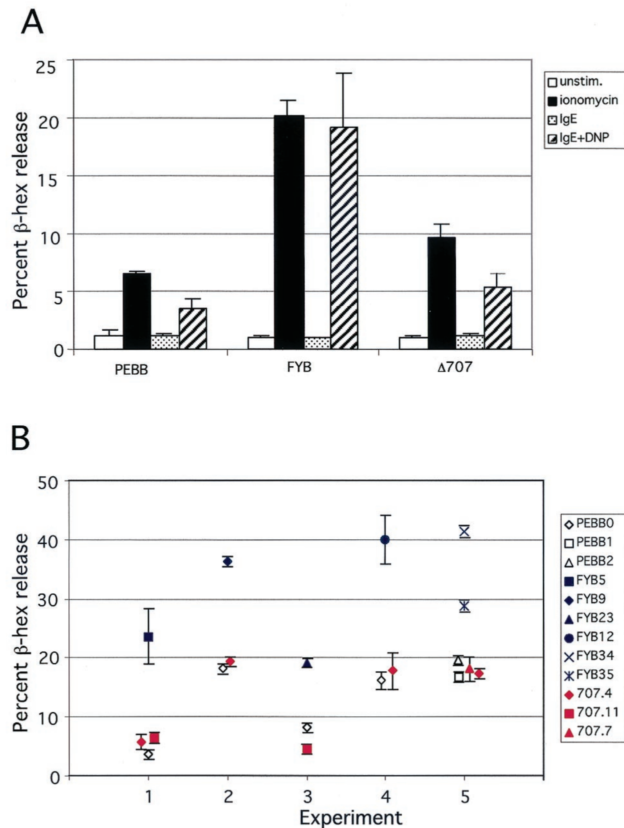

Fig. 4. FYB overexpression enhances β-hexosaminidase release after stimulation. (*A*) FYB-, Δ707-, or vector-transfected cells were either unstimulated or stimulated with ionomycin, IgE, or IgE plus antigen for 30 min. The amount of β-hexosaminidase in the supernatant as well as in the cell lysates was assayed. The mean of triplicate wells is presented. (*B*) The mean percentages β-hexosaminidase release after IgE plus DNP stimulation for 30 min along with the SDs derived from five separate experiments are presented for six FYB- (blue), three FYB Δ707- (red) and three PEBB-transfected (white) clones.

was found to be comparable on the various transfectants. Therefore, consistent with the observation that FYB localizes at adhesion plaques, moderate FYB overexpression enhanced adhesion to FN of RBL-2H3 cells. Further, unlike with β-hexosaminidase release (see ahead), this enhancing effect on adhesion occurred without the need for the C-terminal SH3 domain of FYB.

FYB Enhances β-Hexosaminidase Release in Response to FcεRI Ligation. FcεRI signaling induces the release of inflammatory mediators such as histamine (36). To examine whether FYB might also influence this event, the vector- and FYB-transfected cells were stimulated for 30 min and assessed for the release of β-hexosaminidase. IgE and antigen were used at slightly suboptimal concentrations to enhance our ability to detect alterations caused by FYB overexpression. In this case, IgE plus antigen caused a shift in β-hexosaminidase release from 1% to 3–5% in vector-transfected cells (Fig. 4*A*, hatched bars). By contrast, as shown with a representative clone FYB34, FcεRI cross-linking of FYB transfectants induced a higher level of β-hexosaminidase to 18%. Importantly, FYB expression had no effect on non-cross-linked cells (dotted columns). In a comparison of multiple experiments, FYB overexpression was found to increase β-hexosaminidase by 2- to 6-fold. FYB also enhanced β-hexosaminidase release induced by ionomycin from 6–8% in vector-transfected cells to 20–22% in FYB transfectants. Consistent with this result, ionophore and FcεRI cross-linking both induce FYB phosphorylation (Fig. 1). Overall, our findings demonstrate that FYB overexpression enhances β-hexosaminidase release in mast cells (and, by inference, histamine release) in response to antigen and ionomycin.

Because we previously found that the SH3 domain was not needed for FYB-mediated increases in adhesion (Fig. 3), we were interested in whether the SH3 domain was needed for the enhancement of β-hexosaminidase release (Fig. 4). Interestingly, FYBΔ707 transfectants showed a markedly reduced level of β-hexosaminidase release relative to wild-type FYB transfectants. This reduced potentiation was observed in response to IgE plus antigen or ionomycin. β-Hexosaminidase release in the FYBΔ707 transfectants was roughly equivalent to that observed in vector-transfected controls, and about a third to half that observed in the FYB wild-type transfectants. FYB and Δ707 transfectants were selected with similar levels of expression (Fig. 3*A*). A comparison of the responses to IgE plus antigen with

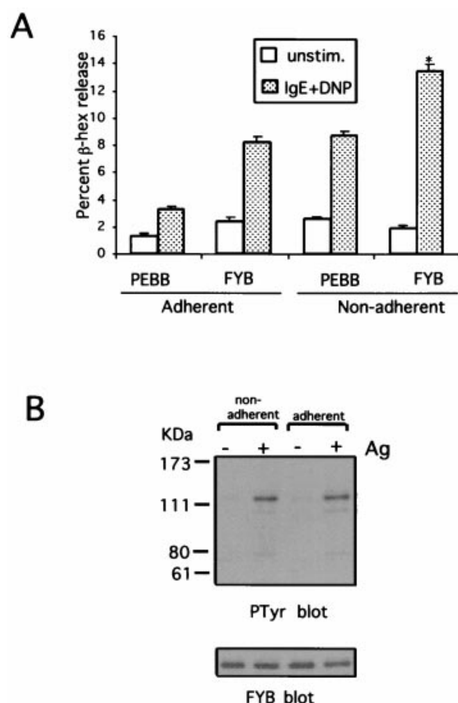

Fig. 5. (*A*) FYB enhances β-hexosaminidase release in adherent and nonadherent cells. FYB or PEBB (vector control) transfectants grown overnight on 96-well plates were preincubated with DNP-specific IgE. The cells were then either left attached to the plate or dissociated from the well by trypsinization and placed in Eppendorf tubes. Cells were stimulated with 100 ng/ml DNP-KLH either on the plate (adherent) or in the tubes (nonadherent). Data are representative of two experiments with two FYB clones and indicate the mean of triplicate wells. (*B*) FcεRI induces tyrosine phosphorylation of FYB in adherent and nonadherent cells. RBL-2H3 cells preincubated with DNP-specific IgE were dissociated from the tissue-culture flask and seeded for 20 min on non-tissue-culture-treated plates that had been coated with either BSA (nonadherent) or FN (adherent). Cells were either unstimulated (Ag, −) or stimulated with DNP-KLH (Ag, +) for 10 min. Lysates were immunoprecipitated with anti-FYB antisera and analyzed by immunoblotting with anti-phosphotyrosine or anti-FYB mAb.

multiple independently derived clones of each type (six wild-type FYB, three FYBΔ707, and three vector-PEBB-transfected clones) yielded similar conclusions. Results from five separate experiments are presented in Fig. 4*B*. A Student's *t* test confirmed that the responses of wild-type FYB are significantly higher than those of the vector clones ($P < 0.01$), and the responses of FYBΔ707 clones are not significantly different from those of the vector clones ($P > 0.2$). These results also were confirmed in a time course analysis (10–40 min of stimulation) with the above clones (data not shown). Our findings demonstrate that FYB potentiates β-hexosaminidase release in response to FcεRI or ionomycin stimulation. In contrast to adhesion, this potentiation depends on the presence of the SH3 domain.

FYB Enhances FcεRI-Induced β-Hexosaminidase Release in Adherent and Nonadherent Cells. The data indicate that the FYB SH3 domain differentially regulates adhesion and β-hexosaminidase release. This result suggested that FYB enhancement of β-hexosaminidase occurs in a manner distinct from its effect on adhesion (Fig. 5*A*). To assess this question further, we compared the effect of FYB on β-hexosaminidase release in response to IgE and DNP in adherent and nonadherent cells. As seen in Fig. 5*A*, both adherent and nonadherent FYB transfectants showed enhanced β-hexosaminidase release. FYB enhanced β-hexosaminidase release by some 2.5-fold in adherent cells and some 1.5-fold in suspended cells. The increase in nonadherent cells was statistically significant at $P < 0.02$ by a Student's *t* test. These observations show that FYB can regulate mediator release in nonadherent cells, albeit to a lesser degree than that observed in adherent cells. To pursue this issue further, we also assessed whether FcεRI could induce equivalent levels of tyrosine phosphorylation of FYB in adherent and nonadherent cells (Fig. 5*B Upper*). Equivalent levels of FYB were found in the different cells (Fig. 5*B Lower*). These observations demonstrate that FcεRI induced FYB phosphorylation, and FYB enhancement of FcεRI-induced β-hexosaminidase release can occur independently of adhesion.

Discussion

FYB is an immune cell-specific adaptor that has been shown to up-regulate IL-2 transcription in T cells (9, 11–13). Given its expression in other immune cells, a major issue has been to define the full range of functions regulated by the adaptor, and to determine whether FYB operates in other cells of the immune system. Previous studies have implicated SYK, LAT, and SLP-76 in FcεRI-mediated degranulation and in the release of leukotrienes and cytokines in mast cells (7, 8, 24, 36, 37). In this study, we demonstrate that FYB can up-regulate two functions: integrin-mediated adhesion to FN and mediator release in immune cells. Furthermore, we show that the regulation of these two events can be distinguished from one another such that enhanced β-hexosaminidase release, but not adhesion, depends on the presence of an intact FYB SH3 domain. These observations make the important point that a region in FYB is differentially involved in the regulation of two functions. The ability to distinguish FYB involvement in these two events was confirmed further by the ability of FYB overexpression to potentiate mediator release in nonadherent cells. Therefore, whereas we previously showed that FYB can enhance TcR/CD3-induced cytokine transcription in T cells (11, 12), our present findings demonstrate that FYB also can potentiate integrin-mediated adhesion and β-hexosaminidase release of mast cells. In this context, the requirement for FYB in optimal TcR regulation of IL-2 transcription may be related indirectly to an effect on integrin binding and adhesion. The independent regulation of mediator release eventually may be found to regulate other secretory functions such as in cytotoxic T cells. In terms of mast-cell function, the function of the FYB adaptor in regulating mediator release points to a role for the adaptor in the control of allergic responses in a manner analogous to that recently suggested for SLP-76 (7).

FYB also colocalized with F-actin in adhesion plaques or podosomes and at the poles of extended FcεRI-activated cells (Fig. 2). Concordant with this result, multiple individually derived FYB transfectants showed enhanced adherence to FN-coated plates (Fig. 3). Importantly, FYB expression did not alter integrin (i.e., β1 or β3 subunits) expression on the surface of cells, which strongly suggests that FYB can increase the affinity/avidity of integrin binding for FN. Other processes, such as increased spreading, also could contribute to this event. Podosomes were described originally in the v-Src-transformed cell and have been thought to regulate movement of hematopoietic cells (38). Src kinase Lyn also has been reported to localize to adhesion plaques (39), whereas Csk overexpressing cells have shown markedly reduced podosome formation and cell locomotion (40). Because we showed that FYB is the preferred target of Fyn-T in T cells (11), an alternate src kinase (i.e., such as Lyn) is likely to substitute for this kinase in mast cells. In this context, Lyn and FYB could cooperate in the regulation of adhesion. Colocalization with F-actin is also consistent with a recent report that FYB can coprecipitate Ena/VASP and WASP proteins, components involved in actin assembly (17). Interestingly, SLP-

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$76^{-/-}$ mast cells are functionally defective (4–8). Given that FYB is the principal binding partner of SLP-76 (9, 11), these observations support a model in which FYB-SLP-76 cooperates in the regulation of adhesion and mast-cell function. Indeed, recently SLP-76 overexpression also has been reported to induce increased lamellipodia formation in Chinese hamster ovary cells (41). Further studies will be needed to clearly establish the roles of FYB in the regulation of the actin cytoskeleton.

In terms of β-hexosaminidase release, FYB transfectants showed a consistent 2- to 6-fold higher level of β-hexosaminidase release than that observed in vector-transfected cells (Fig. 4). Specificity was shown by the fact that the amplification was evident only in response to FcεRI ligation, and by the fact that the potential was reduced with the loss of the SH3 domain (i.e., Δ707 mutant). Therefore, β-hexosaminidase depends more on the presence of the SH3 domain than does adhesion (Fig. 4). This observation, combined with the finding that FYB can enhance release in nonadherent cells, indicates that FYB can modulate β-hexosaminidase release independently of adhesion.

SH3 domains are involved in diverse functions linked to the cytoskeleton (43). We have shown that the SH3 domain in FYB can recognize a tyrosine-based RKxxYxxY motif in SKAP55 (16). NMR studies confirmed that the RKxxYxxY motif binds to a more restricted charged region of the SH3 domain that overlaps with classic R/KxxPxxP binding (16). The differential involvement of the SH3 domain in β-hexosaminidase release relative to adhesion indicates that distinct modules in FYB regulate different functions. From this result, one might predict an involvement of the FYB SH3 binding protein SKAP-55 or SKAP55R Hom in mast-cell function. A similar distinction in the role of protein modules has been made with adaptor SH2-B, in which the SH2 domain is needed for Janus kinase activation but not for cytoskeletal reorganization (44). Further studies will be required to assess the role of FYB binding to partners SLP-76 and SKAP55 in the regulation of mast-cell function.

Note Added in Proof. The finding of FYB regulation of β-1/3 integrin-mediated adhesion has been extended to β-2 mediated adhesion with the loss of LFA-1 adhesion and capping in FYB-deficient T cells (45, 46). Based on these combined studies, we propose that FYB/SLAP-130 be redesignated "adhesion and degranulation-promoting adaptor protein" (ADAP).

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