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Identification of Fyn-binding proteins in MC/9 mast cells using mass spectrometry☆

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Abstract

Fyn is a Src kinase known to have an essential role in mast cell degranulation induced following aggregation of the high affinity IgE-receptor. Although Fyn possesses SH2 and SH3 protein binding domains, the molecules that interact with Fyn have not been characterized in mast cells. We thus analyzed Fyn-binding proteins in MC/9 mast cells to explore the Fyn-mediated signaling pathway. On mass spectrometric analysis of proteins binding to the SH2 and SH3 domains of Fyn, we identified six proteins that bind to Fyn including vimentin, pyruvate kinase, p62 ras-GAP associated phosphoprotein, SLP-76, HS-1, and FYB. Among these proteins, vimentin and pyruvate kinase have not been shown to bind to Fyn. After IgE-receptor mediated stimulation, binding of vimentin to Fyn was increased; and this interaction was via binding to the SH2, but not the SH3, domain of Fyn. Mast cells from vimentin-deficient mice showed enhanced mediator release and tyrosine phosphorylation of intracellular proteins including NTAL and LAT. The observation that vimentin and pyruvate kinase bind to Fyn provides additional insight into Fyn-mediated signaling pathways, and suggests a critical role for Fyn in mast cell degranulation in interacting with both cytosolic and structural proteins. Published by Elsevier Inc.

Keywords: Fyn; Mast cell; Signaling; Protein; Mass spectrometry

Mast cells are secretory cells that play an important role in the genesis of allergic inflammation [1]. In this type of inflammation, mast cell activation follows antigen-mediated aggregation of cell surface high-affinity IgE receptor (FcεRI) molecules via receptor-bound antigen-specific IgE [2]. This initiates a tyrosine-phosphorylation cascade that leads to degranulation with generation and/or release of inflammatory mediators [2,3]. Although the complete sequence of this signaling process has yet to be established, the tyrosine phosphorylation of the cytoplasmic immune receptor tyrosine-based activation motifs (ITAMs) of FcεRI by the Src kinase Lyn, and the subsequent recruitment of the protein tyrosine-kinase Syk has been suggested as one of the first events in IgE-receptor mediated signaling [2]. However, mast cells from Lyn-deficient mice exhibit limited impairment in FcεRI-dependent degranulation [4].

Recently, the Src kinase Fyn was recognized as being important in FcεRI-dependent degranulation of mast cells [5]. Fyn is activated following FcεRI aggregation in bone marrow-derived mast cells (BMMCs); and FcεRI-dependent degranulation is impaired in BMMC from Fyn-deficient mice [5]. On the basis of these observations, the existence of at least two different pathways (Lyn and Fyn-mediated) of FcεRI-dependent activation of mast cells has been proposed [5]. However, the molecules involved in the Fyn-mediated signaling pathway in mast cells are incompletely characterized [5,6].

☆ *Abbreviations:* SH, Src homology; FcεRI, high affinity receptor for IgE; LAT, linker for activation of T cells; NTAL, non-T cell activation linker; BMMC, bone marrow-derived mast cell; DNP,l dinitrophenyl; LDS, lithium dodecyl sulfate; DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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As with other members of the Src family of tyrosine kinases, Fyn possesses a single Src homology (SH)2 and SH3 domain. SH2 domains bind in an inducible manner to phosphorylated tyrosine residues whereas SH3 domains bind to proline-rich regions of associating proteins. Thus, protein–protein interactions regulated by both SH2 and SH3 domains play a central role in signaling processes within cells and most signaling proteins act as components of protein complexes [7,8]. Traditionally, identifying these proteins has been technically challenging, employing labor- and time-intensive biochemical techniques including protein purification and gene cloning. However, in concert with the human genome project, mass spectrometry-based proteomic analysis has become a powerful tool to identify proteins, including those in complex mixtures [7,8] and this approach has provided the opportunity to study protein–protein interactions involved in signaling pathways [7,8]. Glutathione *S*-transferase (GST) fusion proteins containing SH2 and SH3 domains have thus been successfully used to isolate and enrich the specific binding proteins for mass spectrometry analysis. This approach has resulted in the identification of signaling molecules interacting with Src kinases [9,10]. In the present study we analyzed Fyn-binding proteins in mast cells using GST fusion proteins and mass spectrometry to explore the Fyn-mediated signaling pathway involved in mast cell activation.

Materials and methods

Antibodies and reagents. Rabbit polyclonal antibody to Fyn and agarose beads conjugated to GST fusion proteins containing the SH2 (amino acids 145–247), SH3 (amino acid 85–139), and SH2–SH3 (85–247) domains of recombinant mouse Fyn were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody to Linker for Activation of T-cell (LAT) was a kind gift of Dr. Petr Draber (Prague, Czech Republic) and monoclonal antibody to Non-T cell Activation Linker (NTAL) was a kind gift of Dr. Vaclav Horejsi (Prague, Czech Republic). Sheep polyclonal antibody to SLP-76, rabbit polyclonal antibodies to tyrosine-phosphorylated LAT (Tyr191), which recognized both phosphorylated LAT and NTAL (unpublished data), and mouse monoclonal antibody to phosphotyrosine (clone 4G10) were obtained from Upstate Biotechnologies (Lake Placid, NY). Goat polyclonal antibody to pyruvate kinase was obtained from Research Diagnostics (Flanders, NJ). Sheep polyclonal antibody to vimentin was obtained from Enzyme Research Laboratories (South Bend, IN). Fluorescein isothiocyanate (FITC)-conjugated monoclonal rat anti-mouse IgE and FITC-conjugated rat IgG1κ monoclonal immunoglobulin isotype control were obtained from BD PharMingen (San Diego, CA).

Cells, mice, and stimulation. The mouse mast cell line MC/9 and primary BMMC from mice were cultured at 37 °C, in 5% CO_2 in RPMI 1640, supplemented with 10% fetal calf serum, 2 mM L-glutamine, nonessential amino acids, 20 mM Hepes buffer, 1 mM pyruvate, penicillin/streptomycin (all from Biofluids, Rockville, MD), 50 μM of 2-mercaptoethanol (Sigma, St. Louis, MO), and 30 ng/ml of recombinant murine IL-3 (Peprotec, Rocky Hill, NJ). The vimentin knockout (KO) mouse (Vim1 mutation) has been described [11]. Control 129/Sv mice were purchased from the Jackson Laboratories (Bar Harbor, ME). All animals were treated according to National Institutes of Health animal protocol guidelines.

Expression of FcεRI was determined by flow cytometric analysis as described [5] with FITC-conjugated anti-mouse IgE and FITC-conjugated isotype control. BMMCs were cultured for 4–5 weeks for all studies. Before stimulation, cells were sensitized overnight with 100 ng/ml of anti-DNP mouse IgE (monoclonal antibody clone SPE-7, Sigma) and then washed twice with Tyrode–BSA buffer (37 °C, 20 mM Hepes buffer at pH 7.4, 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, and 0.05% bovine serum albumin). Mast cells (10^7 cells/ml) were stimulated with 100 ng/ml of dinitrophenyl (DNP)–human serum albumin (Sigma) for 2 min. For mass spectrometry analysis of Fyn binding proteins, cells were stimulated with 125 μM of pervanadate prepared as described for 15 min to induce a maximal tyrosine phosphorylation of mast cell signaling proteins and thereby increase the binding to the SH2-domain of Fyn [12].

GST fusion protein binding assay, immunoprecipitation and immunoblotting. Unstimulated and stimulated cells were lysed in buffer containing 1% Triton X-100, 0.04% LDS, 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 2 mM PMSF, 10 μg/ml aprotinin, 2 μg/ml leupeptin and pepstatin, 5 mM sodium pyrophosphate, 50 mM NaF, and 1 mM sodium orthovanadate. For the GST fusion protein binding assay, cell lysates were incubated overnight with 50 μg GST fusion protein coupled to agarose beads. Immunoprecipitation was performed using antibodies prebound to protein G–Sepharose beads (Sigma). After capture, the beads were washed five times. The proteins were then eluted by boiling with an equal volume of SDS sample buffer and resolved by SDS–PAGE (Invitrogen, Carlsbad, CA). Proteins were then transferred to a polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ). The membranes were blocked with either 4% BSA or 5% dried milk (depending on the primary antibody) in Tris-buffered saline with 0.1% Tween 20, probed with the desired primary antibody and an appropriate secondary HRP-conjugated antibody, and visualized by enhanced chemiluminescence. For protein normalization, membranes were stripped and re-probed or gels were loaded with identical amounts of samples in parallel.

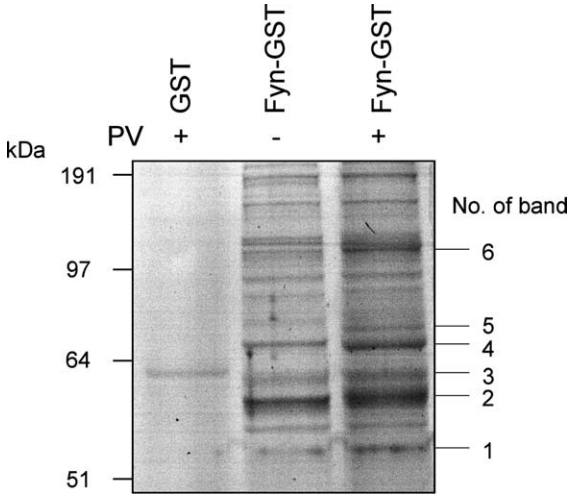

Fig. 1. Identification of Fyn-binding proteins in MC/9 mast cell lysates. Cell lysates from pervanadate (PV)-treated or untreated MC/9 cells were added to glutathione–agarose beads coupled to mouse recombinant Fyn–SH2 and SH3 domain-GST fusion proteins. Bound proteins were eluted by boiling with SDS sample buffer, separated by SDS–PAGE, and stained by colloidal Coomassie blue. Numbered protein bands were excised for mass spectrometry analysis.

Mass spectrometry analysis. For mass spectrometry analysis, the Fyn-binding proteins were eluted from the GST–Fyn SH2–SH3 domain fusion proteins coupled to agarose beads, by boiling with SDS sample buffer. Eluted proteins were then again separated by SDS–PAGE. The gel was stained by colloidal Coomassie blue (Invitrogen, Carlsbad, CA), the protein bands were excised from the gel (Fig. 1), and the excised bands were digested with trypsin (Boehringer–Mannheim). After in-gel digestion, liquid chromatography–electrospray tandem mass spectrometry (LC–MS/MS) analysis was performed on a quadrupole time-of-flight mass spectrometer (QTOF2, Micro mass, Beverly, MA). Protein identification was done with the Global Server software package (Micro mass, Beverly, MA) using the National Center for Biotechnology Information (Bethesda, MD) non-redundant protein database.

Mast cell degranulation assay. Mast cell degranulation was determined by β-hexosaminidase release [13]. Cells (10^6) sensitized with anti-DNP IgE were stimulated with various concentrations of DNP–human serum albumin antigen for 30 min at 37 °C in 0.5 ml of Tyrode–BSA and placed on ice. As a control, mast cells were also stimulated by 20 μM calcium ionophore A23187 (Sigma). Cells were centrifuged at 300*g* for 10 min and the supernatant and pellet were collected. The β-hexosaminidase content of these fractions was determined as described [13].

Results

Identification of Fyn-binding proteins in MC/9 mast cell lysates

To identify Fyn-binding proteins in MC/9 cells, we used glutathione–agarose beads coupled to mouse recombinant Fyn–SH2–SH3–GST fusion proteins, similar to the approach used to isolate proteins binding to other Src family kinases [10]. Thus, MC/9 cells were treated

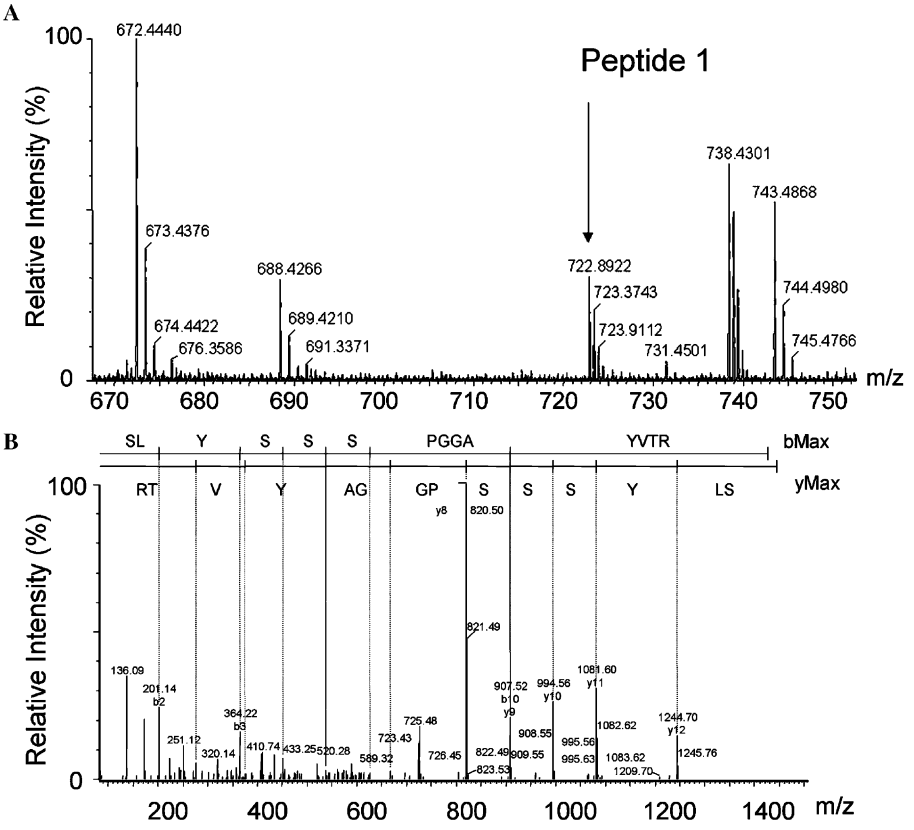

Fig. 2. Mass spectrometric identification of Fyn-binding proteins. Protein band (number 1 from Fig. 1) was excised from the gel and digested with trypsin, and the resulting peptide fragments were separated and analyzed by liquid chromatography–electrospray tandem mass spectrometry (LC–MS/MS). (A) Mass spectrum of the tryptic peptides. The marked tryptic peptide peak (peptide 1) corresponds to a peptide from mouse vimentin. (B) Tandem mass spectrum of doubly charged peptide ions from peptide 1. The sequence of SLYSSSPGGAYVTR was derived from the mass difference of the nested set of peptide fragments. The positions of the assigned series of COOH-terminal (Y ions) and NH_2-terminal ions (B ions) are marked. (C) Sequence and mass data from four peptides matched with mouse vimentin.

with pervanadate and lysates from these cells were incubated with the GST-fusion protein-bound beads. After extensive washing, bound proteins were eluted by boiling in SDS sample buffer and then subjected to SDS–PAGE and proteins were visualized by colloidal Coomassie stain (Fig. 1). With this approach, we observed six principle proteins that bound to Fyn in pervanadate-treated mast cell lysates. These proteins were excised from the gel, digested with trypsin, and analyzed by mass spectrometry (Fig. 1). The resulting peptide fragments then were separated and analyzed by LC–MS/MS as exemplified in Fig. 2A. In this instance (band 1 from Fig. 1), the peptide corresponding to an *m*/*z* value of 722.89 was subsequently fragmented, the tandem mass spectrum of doubly charged peptide ions from peptide 1 was obtained, and the sequence of SLY-SSSPGGAYVTR was derived from the mass difference of the nested set of peptide fragments (Fig. 2B). Fig. 2C shows four peptides matching the mass and sequence of mouse vimentin. These data confirm the presence of vimentin in the protein band 1.

In a similar manner, bands 2–6 in Fig. 1 were characterized. Thus, six Fyn-binding proteins were identified: vimentin, pyruvate kinase, p62 ras-GAP associated phosphoprotein, SLP-76, HS-1, and FYB (Table 1). Of these interactions, the binding of vimentin and pyruvate kinase to Fyn has not been reported. We therefore selected these protein interactions for further study.

In vitro and in vivo binding of Fyn with vimentin and pyruvate kinase

To determine whether the interactions between Fyn and vimentin or pyruvate kinase are mediated by the SH2 domains or SH3 domains of Fyn, immunoblot analysis was done using cell lysates from pervanadate-treated MC/9 cells and agarose beads coupled to Fyn–SH2–GST and Fyn–SH3–GST fusion proteins. SLP-76, known to an essential for mast cell function [14], was employed as a control. Vimentin, pyruvate kinase, and SLP-76 were thus shown to preferentially bind to the Fyn–SH2 domain (Fig. 3).

To determine whether Fyn interacts with these proteins in vivo and whether these interactions are FcεRI-dependent, we immunoprecipitated Fyn-binding proteins from MC/9 mast cell lysates with an anti-Fyn antibody before and after FcεRI aggregation. On immunoblot analysis, vimentin, pyruvate kinase, and SLP-76 were observed to co-immunoprecipitate with Fyn in MC/9 mast cell lysates. The amount of vimentin associated with Fyn in mast cells increased after FcεRI-dependent stimulation (Fig. 3). These results confirm the in vivo and in vitro binding of Fyn with vimentin, pyruvate kinase, and SLP-76.

Tyrosine phosphorylation of vimentin and pyruvate kinase

In order to test whether vimentin and pyruvate kinase are tyrosine-phosphorylated after FcεRI-dependent stimulation, these proteins were immunoprecipitated and then the immunoprecipitates were probed with an anti-phosphotyrosine antibody. Vimentin and pyruvate kinase were observed to be constitutively tyrosine-phosphorylated in MC/9 mast cells (Fig. 4). No significant

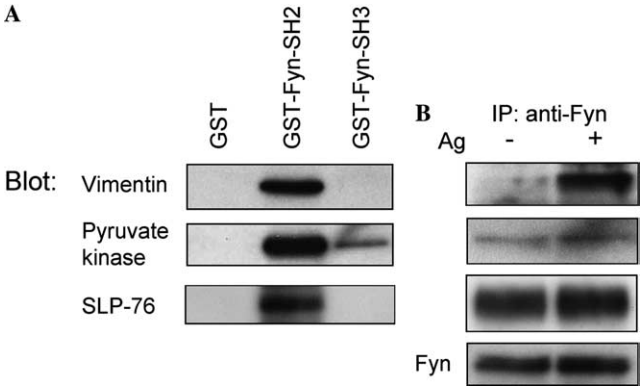

Fig. 3. In vitro and in vivo binding of Fyn with vimentin, pyruvate kinase, and SLP-76. (A) Vimentin, pyruvate kinase, and SLP-76 in pervanadate-treated MC/9 mast cells preferentially bound to the SH2 domain of Fyn. (B) Vimentin, pyruvate kinase, and SLP-76 were immunoprecipitated from MC/9 mast cell lysates by anti-Fyn antibody. Ag is antigen.

Table 1
Identification of Fyn binding proteins in MC/9 mast cells by mass spectrometry (LC–MS/MS)

Band No.	Protein name	Gi No.*	No. of peptides sequenced	Previous report on Fyn binding in mast cells	Previous report on Fyn binding in other cells
1	Vimentin	202370	4	No	No
2	Pyruvate kinase	1405933	1	No	No
	P62 ras-GAP associated phosphoprotein	608528	3	No	Yes [17]
3	P62 ras-GAP associated phosphoprotein		2		
4	SLP-76	806768	6	No	Yes [18]
5	HS1	806522	2	No	Yes [19]
6	FYB	4107509	1	Yes [20]	Yes [27]

* NCBI GenBank Accession No..

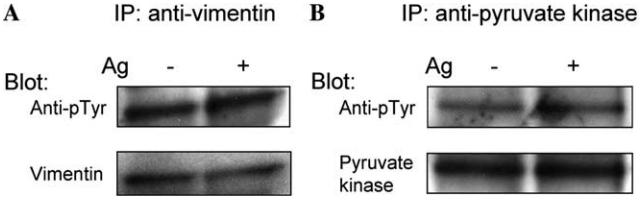

Fig. 4. Tyrosine phosphorylation of vimentin and pyruvate kinase in MC/9 mast cells following FcεRI-stimulation. Cell lysates were subjected to immunoprecipitation with specific antibodies to vimentin and pyruvate kinase and analyzed by immunoblotting with anti-phosphotyrosine antibodies or specific antibodies to vimentin (A) and pyruvate kinase (B).

change in tyrosine phosphorylation was observed after FcεRI-dependent stimulation, suggesting that constitutive tyrosine phosphorylation of these molecules was sufficient for their function.

Mast cell degranulation and protein-tyrosine phosphorylation in mast cells from vimentin-deficient mice

SLP-76 deficient mast cells have been documented to be defective in mast cell activation [14]. As we observed that vimentin bound to Fyn in a similar manner to SLP-76, we wished to investigate whether the vimentin-deficient BMMCs were also defective in FcεRI-mediated activation. We thus evaluated mast cell degranulation and protein-tyrosine phosphorylation in BMMC from vimentin-deficient mice compared to wild type mice. The FcεRI-dependent release of β-hexosaminidase was significantly increased in BMMC from vimentin-deficient mice compared to wild type mice (Fig. 5A, $p < 0.05$). To determine if the difference in degranulation between BMMC from vimentin-deficient mice and from wild type mice is due to a difference in surface expression of FcεRI, we employed flow cytometry using FITC-conjugated anti-mouse IgE and FITC-conjugated isotype control. We found no meaningful differences in FcεRI

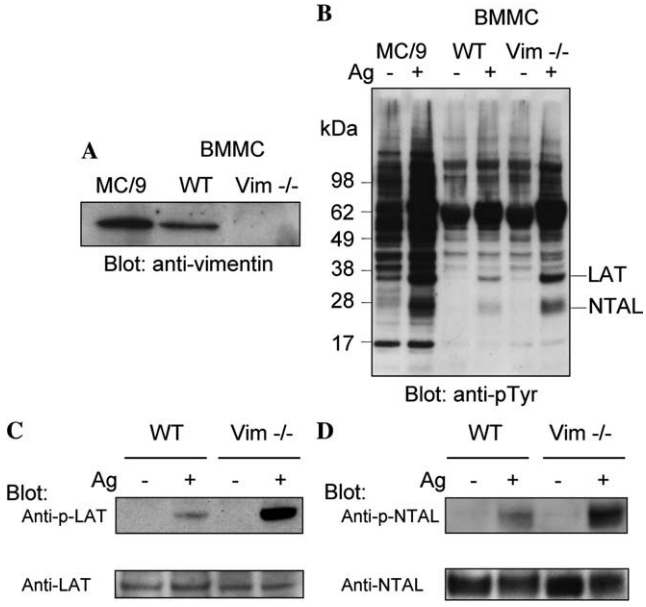

Fig. 6. Protein-tyrosine phosphorylation in BMMC from vimentin-deficient and wild type mice following FcεRI-mediated activation. (A) Immunoblot analysis of vimentin in cell lysates from MC/9 cells and from BMMC from wild type and vimentin deficient mice. (B) Immunoblot analysis of protein-tyrosine phosphorylation following FcεRI-stimulation in MC/9 mast cells and BMMC from wild type and vimentin-deficient mice using anti-phosphotyrosine antibody. (C) Tyrosine phosphorylation of LAT (C) and NTAL (D) in BMMC from wild type and vimentin-deficient mice. Results shown are representative of four experiments.

surface expression between BMMC from vimentin-deficient mice and from wild type mice (Fig. 5B).

The protein-tyrosine phosphorylation pattern was then compared in total cell lysates of BMMC from vimentin-deficient mice and wild type mice before and after FcεRI-dependent mast cell stimulation. The lack of expression of vimentin in vimentin-deficient mice was first confirmed (Fig. 6A). From Fig. 6B, it can be seen

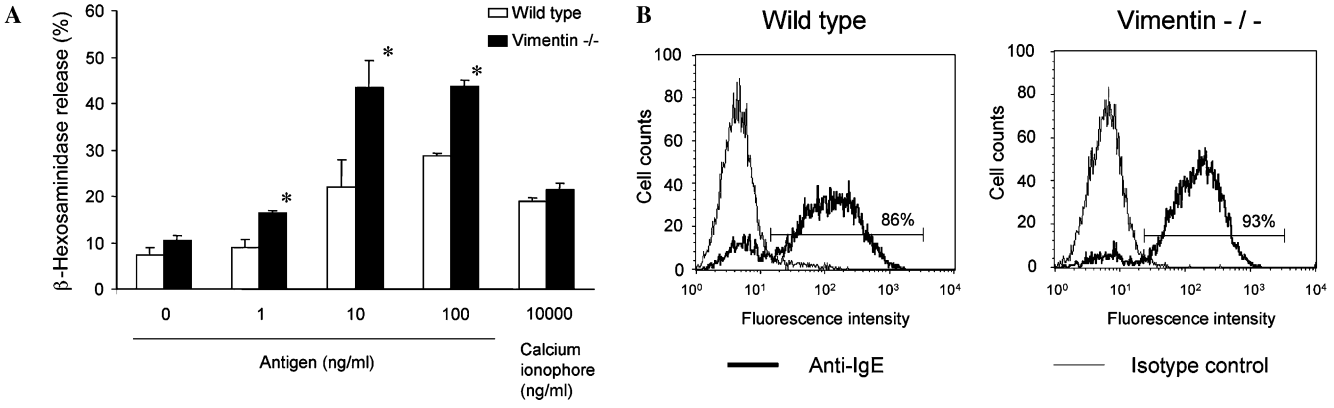

Fig. 5. Mast cell degranulation following FcεRI aggregation in BMMC from vimentin-deficient and wild type mice. (A) β-Hexosaminidase release in BMMC from vimentin-deficient mice compared to wild type mice. Data are presented as means and standard error, $N = 4$ (*$p < 0.05$ by unpaired two-tailed t test). (B) FcεRI expression on BMMC from wild type and vimentin-deficient mice analyzed by flow cytometry.

that following receptor aggregation, there was enhancement of phosphorylation of several proteins in the vimentin-deficient BMMC, particularly those of approximately 36–38 and 28 kDa, believed to be the adaptor molecules LAT and NTAL [15,16]. Data presented in Figs. 6C and D confirm this conclusion. These results suggest that vimentin association with Fyn may have a negative regulatory role in mast cell degranulation and the tyrosine phosphorylation of signaling molecules induced by FcεRI-stimulation.

Discussion

In this study, we identified six Fyn-binding mast cell proteins: vimentin, pyruvate kinase, p62 ras-GAP associated phosphoprotein, SLP-76, HS-1, and FYB using mass spectrometry and GST fusion proteins. Among these six proteins, only FYB was known to bind to Fyn in mast cells, although p62 ras-GAP associated phosphoprotein, SLP-76, and HS-1 were known to bind Fyn in other cell types [17–20]. This is the first documentation that vimentin and pyruvate kinase bind to Fyn. These novel protein–protein interactions were confirmed by immunoprecipitation and immunoblot analysis. These observations confirm the utility of combining GST fusion protein binding assays and mass spectrometry for the identification of interactions between components of signaling cascades [8,10].

SLP-76 is a major cytosolic adaptor molecule, which has been demonstrated to be essential for mast cell degranulation [14]. As both SLP-76 and Fyn-deficient BMMCs display defective FcεRI-mediated degranulation [5,14], the evidence in this paper provides additional support for a pathway involving both molecules. In this study, the adaptor molecule FYB (also known as ADAP or SLAP-130) also bound to Fyn in mast cells, as reported [20]. It is possible that the binding between Fyn and SLP-76 in mast cells may also involve a series of Fyn–FYB–SLP-76 interactions as demonstrated in T cells [18].

The p62 ras-GAP associated phosphoprotein and HS-1 have been reported to bind to Fyn in other cell types [17,19] and are known to be tyrosine-phosphorylated in mast cells after FcεRI-stimulation [21,22]. Although HS-1 is known to be a major substrate for tyrosine kinases [19], its function is unknown. The p62 ras-GAP associated phosphoprotein has a role in the regulation of the ras–raf MAP kinase pathway [23].

We identified vimentin and pyruvate kinase as novel Fyn-binding proteins. Both vimentin and pyruvate kinase bound to the SH2 domain of Fyn, similar to other known studies on the Fyn-binding proteins [17,19]. Pyruvate kinase aids the conversion of glucose to energy when oxygen is not adequate. It also reportedly binds to FcεRI in mast cells [24]. The Fyn–pyruvate kinase interaction may have a role in the high-energy requiring process of structural changes induced by FcεRI-stimulation.

Vimentin is a structural protein that has been suggested to be involved in mast cell degranulation, but its function in mast cell activation is unknown [25]. In mast cells, vimentin is associated with secretory granules and structural changes during degranulation are induced by compound 40/80 [25]. Lymphocytes from vimentin-deficient mice are known to be more deformable compared to lymphocytes from wild type mice and vimentin was suggested as a primary source of lymphocyte rigidity [26]. We found that mast cells from vimentin-deficient mice exhibited increased mediator release, which was associated with enhanced FcεRI-dependent tyrosine phosphorylation of LAT and NTAL. It may be that more deformable mast cells degranulate more easily following FcεRI aggregation. Thus, the data present in this paper suggest a critical role for Fyn in mast cell degranulation, which may involve both cytosolic and structural proteins.

Acknowledgments

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