Targeting of MIST to Src-family kinases via SKAP55–SLAP-130 adaptor complex in mast cells[1]

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Abstract MIST (mast cell immunoreceptor signal transducer; also termed Clnk) is an adaptor protein structurally related to SLP-76-family hematopoietic cell-specific adaptor proteins. We demonstrate here that two major MIST-associated phosphoproteins expressed in mast cell lines are SLAP-130 and SKAP55, adaptors known to interact with the Src-homology (SH) 2 domain of Src-family protein tyrosine kinases (PTKs). MIST directly associated with SLAP-130 via its SH2 domain, and collaboration of SLAP-130 with SKAP55 was required for the recruitment of MIST to Lyn. Furthermore, MIST was preferentially recruited to Fyn rather than Lyn, which is regulated by higher affinity binding of SLAP-130 and SKAP55 with the Fyn-SH2 domain than the Lyn-SH2 domain. Our results suggest that the MIST–SLAP-130–SKAP55 adaptor complex functions downstream of high-affinity IgE receptor-associated Src-PTKs in mast cells.

Key words: Signal transduction; Protein tyrosine kinase; Mast cell; IgE receptor; Adaptor

1. Introduction

Engagement of the high-affinity IgE receptor (type I, FcεRI) by IgE and multivalent antigens induces the release of various preformed inflammatory mediators and also production of cytokines from mast cells and basophils, which leads to the development of an array of acute and chronic allergic reactions including asthma and atopic dermatitis (reviewed in [1,2]).

The FcεRI is composed of four polypeptide chains, the α-subunit that binds to IgE, the β-subunit for signal-amplification, and a disulfide-bonded pair of γ-subunits that lack intrinsic protein tyrosine kinase (PTK) activity but possess immunoreceptor tyrosine-based activation motifs (ITAMs) for initiating the phosphorylation-mediated signaling cascade (reviewed in [3]). In this signaling cascade, the initial step is considered to be activation of FcεRI-associated Src-family PTKs such as Lyn and Fyn, and phosphorylation of the ITAMs in the γ-subunit by these PTKs. Another PTK Syk is then recruited through its Src homology (SH) 2 domain to the phosphorylated ITAMs. These activated PTKs in turn phosphorylate various cellular enzymes such as phospholipase C (PLC)γ and Vav, as well as adaptor or linker proteins lacking catalytic activities.

Recently, it has become clear that adaptor proteins play a key regulatory role in immunoreceptor signaling by linking receptor-proximal PTKs with downstream effectors (reviewed in [4,5]). Adaptor molecules contain multiple modules for protein–protein interactions, by which they recruit PTKs, effectors and other adaptors, and facilitate signal transduction. With regard to the FcεRI-signaling in mast cells, two adaptor proteins, SLP-76 and LAT, have been demonstrated to be essential for the signal transduction from the analyses of mice carrying null-mutation alleles of these genes [6,7]. In both SLP-76- and LAT-deficient mast cells, FcεRI-induced PTK activation is not affected but subsequent downstream effector functions including phosphorylation of PLCγ and activation of mitogen-activated protein kinases (MAPKs) are impaired, resulting in the defective degranulation response and cytokine production.

We and others have identified the third member of SLP-76/BLNK adaptor family, termed either MIST (mast cell immunoreceptor signal transducer) or Clnk (cytokine-dependent hematopoietic cell linker) which is expressed in mast cell lines and IL-3-induced bone marrow-derived mast cells [8,9]. We have demonstrated that a mutated form of MIST expressed in a rat mast cell line RBL-2H3 inhibits FcεRI-mediated degranulation [8], suggesting that MIST, in addition to SLP-76, is

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[1] The rat SKAP55 cDNA nucleotide sequence has been deposited in DDBJ database under accession number AB092812.

Abbreviations: Ab, antibody; FcεRI, Fc receptor type I for IgE; GST, glutathione *S*-transferase; ITAM, immunoreceptor tyrosine-based activation motif; MAPK, mitogen-activated protein kinase; PLC, phospholipase C; PR, proline-rich; PTK, protein tyrosine kinase; SH, Src homology

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an important component in the signaling pathways downstream of FcεRI on mast cells. MIST contains similar structural modules for molecular interaction with SLP-76, including N-terminal tyrosine phosphorylation sites, a central proline-rich (PR) region and a C-terminal SH2 domain, and through these domains MIST can associate with PLCγ and Grb2 in mast cells [10]. However, the precise role of MIST in the FcεRI-signal transduction remains unclear.

In the present study, we identified two adaptor proteins, SKAP55 and SLAP-130/Fyb, as the major binding partners of MIST in mast cells, and also demonstrated that these proteins are required for recruiting MIST in the vicinity of FcεRI-associated PTKs.

2. Materials and methods

2.1. Cell lines and reagents

COS-7 cells, rat mast cell line RBL-2H3 cells and their stable transfectants were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS and antibiotics. Mouse IL-3-dependent mast cell line MC/9 was also maintained in RPMI1640 medium supplemented with 10% FCS in the presence of 5 ng/ml of recombinant mouse IL-3 (Peprotech). The anti-MIST peptide antibody (Ab) was produced by immunizing rabbits with a peptide encompassing amino acids 15–39 of the mouse MIST sequence that was coupled to keyhole limpet hemocyanin. The resultant antiserum was affinity-purified by peptide-bound Sepharose column and its reactivity to mouse MIST was confirmed. Other Abs used were anti-Lyn, anti-SKAP55, anti-SLAP-130 and anti-phosphotyrosine (RC20) Abs (Transduction Laboratories), anti-T7 Ab (Novagen, Madison, WI, USA), anti-Flag and anti-Myc Abs (Upstate Biotechnology, Lake Placid, NY, USA), anti-glutathione *S*-transferase (GST) Ab (Amersham Bioscience) and polyclonal anti-Lyn and anti-Fyn Abs (Santa Cruz Biotechnology, Santa Cruz, CA, USA). GST proteins fused with the SH2 domain of mouse Lyn, Fyn and Lck were kindly provided by Dr. Y. Takemoto (Nippon Glaxo Ltd.) [11].

2.2. Purification and identification of MIST-binding phosphoproteins

RBL-2H3 cells stably expressing T7 epitope-tagged wild-type mouse MIST (2×10^9 cells) were lysed with NP-40 buffer (1% NP-40, 150 mM NaCl, 50 mM Tris–HCl [pH 8.0], and 2 mM EDTA) containing 10 mM NaF, 2 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml aprotinin. After centrifugation at $17\,000\times g$, the supernatant was precleared with a protein-G agarose (Roche Molecular Biochemicals, NJ, USA), followed by incubation with anti-T7 Ab conjugated with agarose beads (Novagen). After washing with 10-bed volumes of 0.5% NP-40 buffer, the immunoprecipitated complexes eluted with citrate buffer (pH 4.0) were resolved by SDS–PAGE, transferred to PVDF membranes, and visualized with Coomassie staining. The 55- and 116-kDa protein bands were excised, digested with Lysyl endopeptidase, chromatographed on the reverse-phase HPLC column, and then subjected to protein microsequencing.

2.3. cDNA cloning and expression constructs

The entire coding regions of rat SKAP55 and mouse SLAP-130 cDNAs were amplified by PCR using a single-stranded cDNA synthesized from RBL-2H3 cells and mouse spleen, respectively. The oligonucleotide primer pairs used for PCR amplification were as follows: 5′-GCTGGATCCCATGCAGGCTGTCGCCCTCCCGGAGGAGAT-3′ and 5′-CCCGAATTCTCATCTTCCTTCCATTTCAAAGGCGGT-3′ for rat SKAP55, and 5′-ATCAAGCTTAATGGCGAAGTTCAACACGGGGAGTAAC-3′ and 5′-AAACTCGAGCTAGTCATTGTCATAGATGCAACCATCA-3′ for mouse SLAP-130. The amplified PCR products were subcloned into pBluescript plasmids and subjected to sequencing by the dideoxy-chain termination method with an automatic DNA sequencer (Applied Biosystems, Foster City, CA, USA). After verifying their sequences, SLAP-130 and SKAP55 cDNAs were either subcloned into pCMV-tag2 (Stratagene, La Jolla, CA, USA) in flame with the Flag epitope or pCMV-tag3 (Stratagene) with the c-Myc epitope. The expression plasmids containing T7 epitope-tagged wild-type and mutant forms of mouse MIST cDNAs were generated as described previously [10]. The pME18-Lyn plasmid was kindly provided by Dr. T. Yamamoto (University of Tokyo).

2.4. Transfection, generation of stable cell lines and stimulation

COS-7 cells were transfected with 0.5 μg of plasmids encoding wild-type or mutant forms of MIST, in various combinations with 0.5 μg of mock plasmid or plasmids encoding SKAP55 and/or SLAP-130 by using an Efectene reagent (Qiagen, Germany). The plasmid encoding mouse Lyn (0.2 μg) was also co-transfected for tyrosine-phosphorylation of transfected MIST, SKAP55 and SLAP-130. RBL-2H3 cells stably expressing a SH2 mutant form of MIST were generated, as described previously [8]. FcεRI cross-linking was carried out by incubating RBL-2H3 cells with 10 μg/ml of anti-DNP mouse IgE (Sigma) for 30 min on ice and then stimulated with 100 ng/ml of DNP-HSA at 37°C for the indicated period of time. MC/9 cells were sensitized with 1 μg/ml of anti-DNP-IgE overnight at 37°C, and stimulated with 500 ng/ml of DNP-HSA.

2.5. Immunoprecipitation, GST pulldown and immunoblotting

Cells were lysed with 1% NP-40 lysis buffer containing protease and phosphatase inhibitors, and precipitated with an indicated Ab or GST-fusion proteins bound to glutathione–Sepharose (Amersham Biosciences). The precipitates and aliquots of total cell lysates were resolved on SDS–PAGE, and transferred to PVDF membranes. The membranes were immunoblotted with Abs described above and the secondary Ab conjugated with horseradish peroxidase, and then developed with the Enhanced Chemiluminescence system (Amersham Biosciences).

3. Results and discussion

In RBL-2H3 cells stably expressing wild-type mouse MIST, constitutively tyrosine-phosphorylated MIST was associated with two prominent proteins of about 55- and 116-kDa, both of which were tyrosine-phosphorylated (Fig. 1A). The association of the 116-kDa phosphoprotein with endogenous MIST was also detected in MC/9 mast cells, whereas the presence of the 55-kDa phosphoprotein was not evident in the anti-MIST precipitate, because of its similar molecular weight with endogenous MIST (Fig. 1B). These phosphoproteins were purified from lysates of RBL-2H3 cells expressing MIST, digested by Lysyl endopeptidase, chromatographed on a reverse-phase HPLC column, and then subjected to protein microsequencing. The amino acid sequences of five peptides from the 116-kDa protein (GTTKPPLAAK,

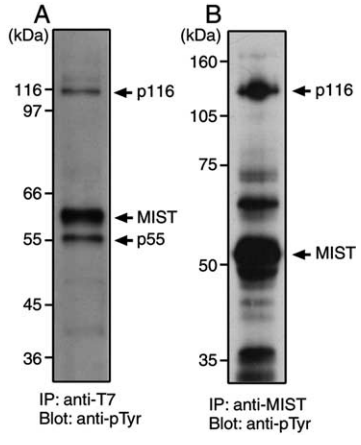

Fig. 1. Phosphoproteins of 55- and 116-kDa are associated with MIST in mast cells. MIST was immunoprecipitated from cell lysates of RBL-2H3 cells expressing the T7-tagged mouse MIST by anti-T7 Ab (A) or from lysates of MC/9 mast cells by anti-MIST Ab (B), and an aliquot of immunoprecipitates was immunoblotted with anti-phosphotyrosine (anti-pTyr) Ab. MIST-associated phosphoproteins, p116 and p55, are indicated by arrowheads.

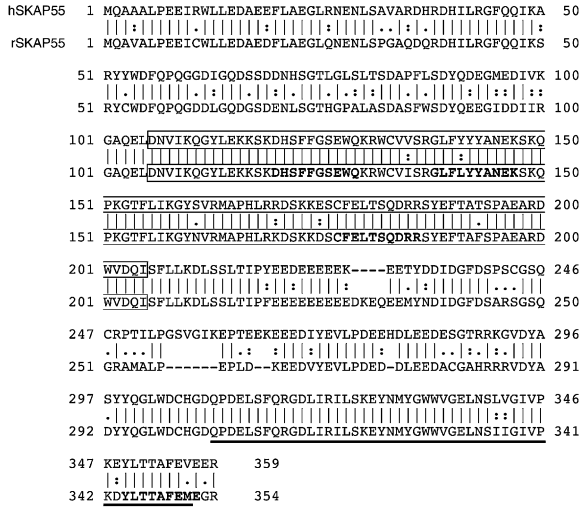

Fig. 2. Comparison of rat and human SKAP55 amino acid sequences. The deduced amino acid sequence of rat SKAP55 contains a highly conserved pleckstrin-homology domain (boxed) and a SH3 domain (underlined) with those of human SKAP55. The sequence of the peptide obtained by microsequencing is shown in bold. The sequence data of rat SKAP55 has been deposited in DDBJ database (accession number: AB092812).

FNTGSNPTEE, WGTRDLQIKV, DGEIRVLYST and PKPPFLKPTG) completely matched those of reported mouse SLAP-130 (also termed Fyb) sequences [12,13]. Furthermore, two out of four amino acid sequences derived from the 55-kDa protein (DHSFFGSEWQ and CFELTSQDRR) were identical with those found in human SKAP55 sequence [14]. The remaining peptide sequences (YLTTAFEME and GLFLYYANEK) were also confirmed to match those in SKAP55 by comparison with the rat counterpart of SKAP55 cDNA cloned from RBL-2H3 cDNA library (Fig. 2). These results demonstrate that MIST-associated phosphoproteins of 55- and 116-kDa in mast cells are the rat counterparts of SKAP55 and SLAP-130, respectively.

SLAP-130 was originally identified as a protein that interacts with the SH2 domain of SLP-76 [12], a member of the same family as MIST, and also independently isolated as a Fyn SH2 domain-binding protein, thus called Fyb (Fyn-binding protein) [13]. SKAP55 was also isolated as a binding protein to the SH2 domain of Fyn [14], and SKAP55 and SLAP-130 have been reported to bind to each other via their SH3 domains [15–17]. We therefore examined the mode of interaction of MIST with SKAP55 and SLAP-130 by expressing T7-tagged mouse MIST, Flag-tagged SKAP55 and Myc-tagged SLAP-130 in COS-7 cells. Since MIST, SKAP55 and SLAP-130 are substrates for Src-family PTKs, Lyn was also co-expressed for their phosphorylation. In the presence of Lyn, Flag-tagged SKAP55 was co-precipitated with MIST only when SLAP-130 was co-expressed in COS-7 cells, whereas SLAP-130 was co-precipitated with MIST even in the absence of SKAP55, indicating that the association of MIST with SKAP55 is indirect and mediated by SLAP-130 (Fig. 3A, compare lane 1 with lanes 2 and 3). In addition, we also found that Lyn was associated with MIST only in the presence of both SKAP55 and SLAP-130, and that the tyrosine phosphorylation of MIST was significantly increased under these conditions (Fig. 3A, compare lanes 1 and 4 in the third panel from the bottom). Interestingly, even in the absence of Lyn, the association of SLAP-130 with MIST was detectable when SKAP55 was co-expressed (Fig. 3A, lane 5). This interaction of MIST with SLAP-130 was probably mediated by residual phosphorylation of SKAP55 in COS-7 cells (data not shown and Fig. 3A, lane 5). It is likely that SKAP55 phosphorylated by endogenous Src-PTK in COS-7 cells [18] recruits MIST as well as SLAP-130 in the vicinity of the PTK, leading to the phosphorylation of associated partners,

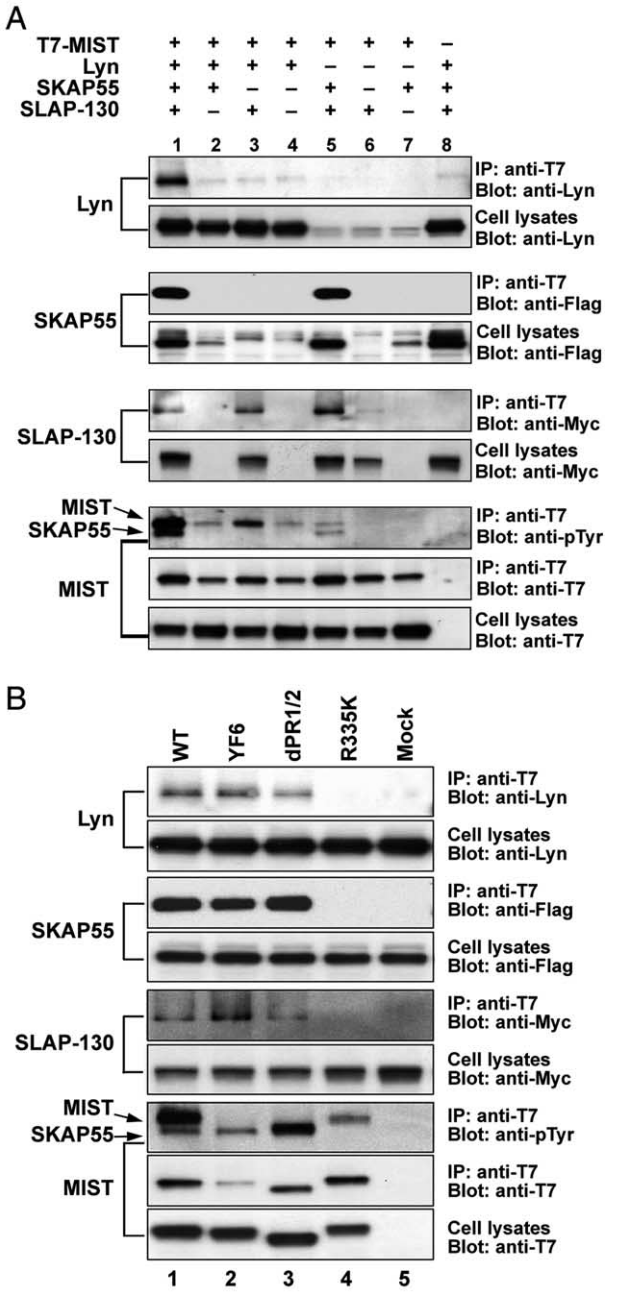

Fig. 3. Association of MIST with Lyn is mediated by SKAP55 and SLAP-130. A: COS-7 cells were transfected with T7-tagged wild-type mouse MIST cDNA, with various combination of Flag-tagged SKAP55, Myc-tagged SLAP-130 or Lyn cDNAs as indicated. Cell lysates were immunoprecipitated with anti-T7 Ab, and the precipitates and an aliquot of whole cell lysates were immunoblotted with indicated Abs. B: COS-7 cells were transfected with T7-tagged various forms of MIST cDNAs (YF6, dPR1/2 and R335K), together with SLAP-130, SKAP55 and Lyn cDNAs. Cell lysates were immunoprecipitated and immunoblotted as in (A).

and thus MIST–SLAP-130–SKAP55 trimolecular complex may be formed without Lyn. Although both SLAP-130 and SKAP55 were shown to associate directly with the SH2 domain of Fyn [13,14], SLAP-130 alone may not be sufficient to induce a tight association of MIST with Lyn, but SKAP55 and SLAP-130 may cooperate in this process. This notion was supported by a substantial increase in the level of tyrosine phosphorylation of MIST by the presence of SKAP55 in addition to SLAP-130 (Fig. 3A, compare lanes 1 and 3 in the third panel from the bottom). Taken together, these results demonstrate that SLAP-130 mediates interaction of MIST with SKAP55 and then SKAP55, in cooperation with SLAP-130, enhances the association of MIST–SLAP-130 complex with Lyn. The results also indicate that the trimolecular complex formed by MIST–SLAP-130–SKAP55 is essential for the full tyrosine phosphorylation of MIST by Lyn.

To determine the region of MIST responsible for the interaction with SLAP-130–SKAP55–Lyn complexes, we expressed mutant forms of MIST as well as wild-type MIST together with Lyn, SKAP55 and SLAP-130 in COS-7 cells. The mutant forms of MIST includes a YF6 mutant containing six tyrosine-to-phenylalanine substitutions at amino acid positions 69, 96, 110, 125, 137 and 145, a dPR1/2 mutant lacking two PR motifs corresponding to amino acid positions at 234–239 and 245–256, and the non-functional SH2 mutant (R335K) harboring an arginine-to-lysine amino acid substitution at position 335. As shown in Fig. 3B, the Lyn-SKAP55–SLAP-130 complex was co-precipitated with wild-type MIST, YF6 or dPR1/2, but not with the R335K mutant. Because the direct binding partner of MIST is SLAP-130 in this signaling complex (Fig. 3A), the inability of R335K MIST mutant to interact with SKAP55 as well as Lyn reflects its failure to interact with SLAP-130. The SH2 domain in MIST is therefore essential for the binding with SLAP-130 that links MIST to SKAP55 and Lyn. In addition, the tyrosine-phosphorylation status of the SH2 domain mutant was markedly reduced compared with that of wild-type MIST (Fig. 3B, compare lanes 1 and 4 in the third panel from the bottom). These results indicate that MIST interacts with SLAP-130 via its SH2 domain, leading to the formation of signaling complex with SKAP55 and Lyn, whereby MIST is efficiently phosphorylated by Lyn.

We next examined whether the MIST–SLAP-130–SKAP55 adaptor complex is recruited to Lyn upon FcεRI cross-linking in RBL-2H3 mast cells. As shown in Fig. 4A, MIST constitutively associated with SLAP-130 and SKAP55 and the amount of SLAP-130 and SKAP55 proteins associated with MIST was not altered after FcεRI stimulation. In sharp contrast, Lyn did not associate with MIST before FcεRI stimulation, but the association of Lyn with MIST, perhaps through SLAP-130 and SKAP55, become detectable as early as 30 s upon stimulation, reached maximum at 1 min and thereafter gradually disappeared within 10 min. The FcεRI-induced association of Lyn with the trimolecular complex consisting of MIST, SLAP-130 and SKAP55 was not detectable in RBL-2H3 cells expressing the MIST mutant with the non-functional SH2 domain (Fig. 4B), confirming that the SH2 domain-mediated interaction of MIST with SLAP-130 is the critical process for the recruitment of MIST to Lyn.

Since Fyn is also expressed in mast cells and functions in FcεRI-mediated mast cell activation [19], we further examined whether the MIST–SLAP-130–SKAP55 adaptor complex is

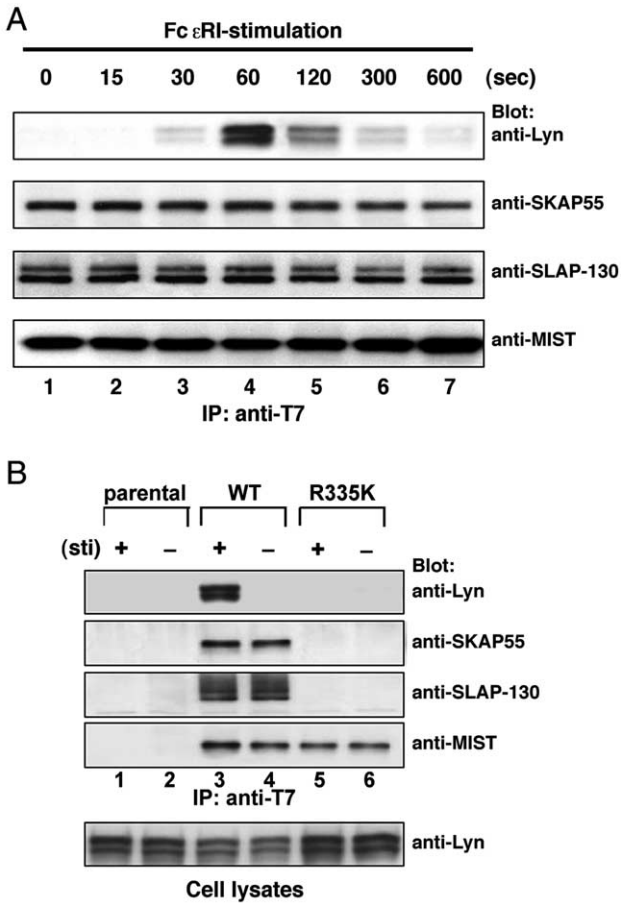

Fig. 4. Engagement of FcεRI induces the association of MIST with Lyn in RBL-2H3 mast cells. A: RBL-2H3 cells stably expressing T7-tagged mouse MIST sensitized with anti-DNP IgE were either unstimulated or stimulated with 100 ng/ml DNP-HSA for the indicated time periods before cell lysis. Cell lysates were then immunoprecipitated with anti-MIST Ab and immunoprecipitates were immunoblotted with the indicated Abs. B: Parental RBL-2H3 cells and stable transfectants expressing T7-tagged wild-type MIST (WT) or its SH2-mutant (R335K) were sensitized with anti-DNP IgE, and then unstimulated or stimulated with 100 ng/ml DNP-HSA for 1 min at 37°C. Immunoprecipitation and immunoblotting were carried out as described in (A).

associated with Fyn using MC/9 mast cells. MC/9 cells express equivalent levels of Fyn and Lyn, whereas RBL-2H3 cells predominantly express Lyn and the expression level of Fyn protein is below the detection limit by Western blotting [20]. In MC/9 cells, MIST constitutively associated with SLAP-130 and SKAP55 (Fig. 5A, lanes 1 and 2), as in RBL-2H3 cells. However, to our surprise, Fyn, but not Lyn, was co-precipitated with MIST irrespective of FcεRI cross-linking (Fig. 5A, lanes 1 and 2). Immunoprecipitation of Fyn and Lyn also revealed the binding of MIST with Fyn but not with Lyn (Fig. 5A, lanes 3–6 in the third panel from the bottom). Consistent with this, SLAP-130 and SKAP55 were co-precipitated only with Fyn but not Lyn (Fig. 5A, lane 3–6 in second and third panels from the top). To understand the reason why the Lyn-SKAP55–SLAP-130–MIST complex was undetectable in the presence of Fyn, we compared the affinities of the SH2 domains of Fyn and Lyn to bind SLAP-130 and SKAP55. Precipitation of SLAP-130 and SKAP55 from cell lysates of COS-7 cells transiently expressing these proteins with or with-

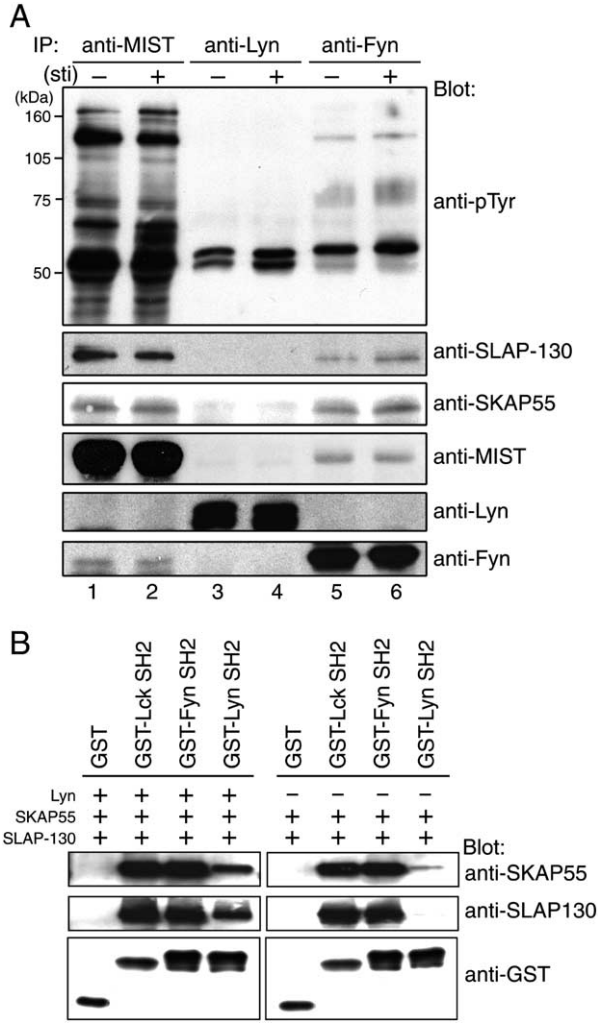

Fig. 5. MIST–SLAP-130–SKAP55 adaptor complex favors an association with Fyn rather than Lyn. A: MC/9 mast cells sensitized with IgE were stimulated with 500 ng/ml of DNP-HSA for 1 min. Cell lysates were then immunoprecipitated with anti-MIST, Lyn or Fyn Ab, and immunoblotted with the indicated Abs. B: COS-7 cells were transfected with SLAP-130 and SKAP55 cDNAs with or without Lyn cDNA. Each cell lysate was divided equally into four aliquots, and was precipitated with glutathione–Sepharose beads precoated with GST or GST fusion proteins containing the SH2 domain of Fyn, Lyn or Lck. The precipitates were immunoblotted with anti-SLAP-130, anti-SKAP55 and anti-GST Abs.

out Lyn by GST fusion proteins containing the SH2 domain of Lyn or Fyn showed that the amounts of both SLAP-130 and SKAP55 interacting with the SH2 domain of Lyn were considerably lower than those interacting with the SH2 domain of Fyn or Lck (Fig. 5B). These findings indicate that MIST–SLAP-130–SKAP55 adaptor complex favors an association with Fyn rather than Lyn when both Fyn and Lyn are present in mast cells.

Studies using Fyn- and Lyn-deficient mast cells have recently revealed that Fyn and Lyn function independently in FcεRI-proximal signal transduction [19,21]. Lyn is required for the phosphorylation of Syk, LAT and PLCγ and the calcium response, although FcεRI-mediated degranulation is intact in Lyn-deficient mast cells [19,21]. In contrast, Fyn deficiency does not affect the phosphorylation of Syk and LAT or the calcium response, however, it causes severe defects in mast cell degranulation [19]. Since not only Fyn but also Syk [22] and LAT [7] are essential for mast cell activation, both Lyn-initiated and Fyn-initiated pathways, though they appear to have non-redundant functions in FcεRI-proximal signaling, are probably mutually integrated into the activation of common downstream effectors that are essential for mast cell activation. Based on our finding that the MIST–SLAP-130–SKAP55 adaptor complex associates strongly with Fyn and weakly with Lyn, this adaptor complex appears to function mainly downstream of Fyn. However, the balance of Fyn and Lyn expressed in different mast cell populations might influence the potential involvement of the MIST-nucleated adaptor complex in Lyn- or Fyn-mediated signaling. Geng et al. [23] have recently demonstrated that over-expression of SLAP-130 in mast cells can up-regulate the FcεRI-mediated degranulation response. Furthermore, similar to the Fyn-deficient phenotype in FcεRI-proximal signaling, the loss of SLAP-130 did not affect T cell antigen receptor (TCR)-proximal signaling pathways such as calcium flux and MAPK activation. However, TCR-induced integrin-mediated adhesion was impaired in SLAP-130-deficient T cells [24,25], suggestive of a Fyn–SLAP-130 axis of immunoreceptor signaling. Therefore, SLAP-130–SKAP55-mediated regulation of MIST targeting to FcεRI-associated Src-PTKs demonstrated in the present study would provide a basis to understand the independent and interdependent roles of these PTKs in FcεRI-mediated mast cell activation.

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