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Once Phosphorylated, Tyrosines in Carboxyl Terminus of Protein-tyrosine Kinase Syk Interact with Signaling Proteins, Including TULA-2, a Negative Regulator of Mast Cell Degranulation*[S]

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Background: Activation of Syk kinase results in phosphorylation of two conserved tyrosines close to its COOH terminus.
Results: The adaptors Nck, SLP-76, Grb2, and GADS and the negative effector phosphatases SHIP-1 and TULA-2 associate with a phosphorylated peptide based on this sequence.
Conclusion: Phosphorylation of these tyrosines provides a docking site for regulators of FcϵRI signaling.
Significance: TULA-2 negatively regulates FcϵRI signaling.

Activation of the high affinity IgE-binding receptor (FcϵRI) results in the tyrosine phosphorylation of two conserved tyrosines located close to the COOH terminus of the protein-tyrosine kinase Syk. Synthetic peptides representing the last 10 amino acids of the tail of Syk with these two tyrosines either nonphosphorylated or phosphorylated were used to precipitate proteins from mast cell lysates. Proteins specifically precipitated by the phosphorylated peptide were identified by mass spectrometry. These included the adaptor proteins SLP-76, Nck-1, Grb2, and Grb2-related adaptor downstream of Shc (GADS) and the protein phosphatases SHIP-1 and TULA-2 (also known as UBASH3B or STS-1). The presence of these in the precipitates was further confirmed by immunoblotting. Using the peptides as probes in far Western blots showed direct binding of the phosphorylated peptide to Nck-1 and SHIP-1. Immunoprecipitations suggested that there were complexes of these proteins associated with Syk especially after receptor activation; in these complexes are Nck, SHIP-1, SLP-76, Grb2, and TULA-2 (UBASH3B or STS-1). The decreased expression of TULA-2 by treatment of mast cells with siRNA increased the FcϵRI-induced tyrosine phosphorylation of the activation loop tyrosines of Syk and the phosphorylation of phospholipase C-γ2. There was parallel enhancement of the receptor-induced degranulation and activation of nuclear factor for T cells or nuclear factor κB, indicating that TULA-2, like SHIP-1, functions as a negative regulator of FcϵRI signaling in mast cells. Therefore, once phosphorylated, the terminal tyrosines of Syk bind complexes of proteins that are positive and negative regulators of signaling in mast cells.

Mast cell stimulation during allergic inflammation is mediated by the high affinity immunoglobulin E (IgE)-binding receptor (FcϵRI).[2] Cross-linking of these receptors with antigen induces the release of preformed mediators from granules together with the synthesis and release of lipid mediators and cytokines (1–4). FcϵRI, like other members of the multisubunit immune recognition receptors such as those on T and B cells, have a ligand binding domain with no enzymatic activity but are associated with subunits that have immunoreceptor tyrosine-based activation motif (ITAM) sequences within their cytoplasmic domains. Aggregation of FcϵRI results in phosphorylation of the tyrosines of the ITAM in the β and γ subunits of the receptor. These phosphorylated tyrosines then serve as a docking site for the cytoplasmic tyrosine kinase Syk; this binding results in a conformational change and activation of Syk, which is essential for the downstream propagation of signaling in mast cells (4–6). The recruitment to the receptor and the activation of Syk results, directly or indirectly, in the phosphorylation of a number of downstream enzymes and adaptor molecules, including phosphoinositide 3-kinase, phospholipase C-γ (PLC-γ), Vav-1, linker for activation of T cells (LAT), SH2 domain-containing leukocyte protein of 76 kDa (SLP-76), Btk, and Gab2 (2–5, 7). Complexes are formed that are important for signaling; for example, tyrosine-phosphorylated LAT functions as a scaffold providing direct binding sites for growth factor receptor-bound protein 2 (Grb2), Grb2-related adaptor downstream of Shc (GADS), and PLC-γ, and through those proteins, there is association of SLP-76, Nck, and other adaptors. Phospholipase C-γ catalyzes the generation of inositol triphosphate, which

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[2] The abbreviations used are: FcϵRI, high affinity IgE-binding receptor; BMMC, bone marrow-derived mast cells; PLC, phospholipase C; TULA-2, T cell ubiquitin ligand-2; also called STS-1 (suppressor of T cell receptor signaling-1) or UBASH3B (ubiquitin-associated domain (UBA) and SH3 domain-containing protein B); GADS, Grb2-related adaptor downstream of Shc; NFAT, nuclear factor for T cell activation; NFκB, nuclear factor κB; ITAM, immunoreceptor tyrosine-based activation motif; LAT, linker for activation of T cells; SH, Src homology; SLP-76, SH2 domain-containing leukocyte protein of 76 kDa; Grb2, growth factor receptor-bound protein 2; MAP, mitogen-activated protein; p-ITAM, phosphorylated ITAM.

releases Ca^{2+} from intracellular stores and activates store-operated calcium channels for the influx of Ca^{2+} (8).

Syk-mediated tyrosine phosphorylation of Btk, SLP-76, LAT, and PLC-γ is essential to generate signals for sustained Ca^{2+} influx. All these events culminate with mast cell degranulation, phosphorylation of MAP kinases, activation of nuclear factor for T cell activation (NFAT) and nuclear factor κB (NFκB), which turn on cytokine synthesis. The extent of mast cell signaling is also determined by negative regulators; these include the Cbl family of ubiquitin ligases as well as lipid or tyrosine phosphatase SHIP-1/2, PTEN, and SHP1/2 that are recruited or activated by inhibitory receptors (9).

Activation of Syk also leads to phosphorylation of a number of its tyrosines (4, 6). Phosphorylation of these residues regulates the activity of Syk and provides binding sites for other molecules. Among these are Tyr residues in the linker region between the SH2 and kinase domains that when phosphorylated provide a binding site for Cbl, a negative regulator of protein-tyrosine kinases. Similarly, the two adjacent tyrosines in the activation loop of the kinase domain of Syk (Tyr-519/Tyr-520)[3] are critical for downstream propagation of signals after immunoreceptor activation (10). The COOH-terminal region of Syk has three evolutionarily conserved tyrosines (Tyr-623, Tyr-624, and Tyr-625 of the rat sequence), the last two of which are also conserved in human ZAP-70 (11, 12). The last two tyrosines (Tyr-624 and Tyr-625) are phosphorylated in both Syk and ZAP-70 by *in vitro* autophosphorylation or following receptor stimulation (13, 14). Structural and mutational studies suggest that the COOH-terminal tyrosines play a role in regulating Syk function. The crystal structure of ZAP-70 suggests that this family of kinases has an autoinhibitory state where there are interactions of Tyr in the tail with both the kinase and the inter-SH2 region that keeps the kinase in a closed non-active conformation (15). The binding of Syk to the p-ITAM results in a conformational change that exposes the COOH-terminal region (16). This leads to the phosphorylation of the two tyrosines (Tyr-624 and Tyr-625), which then keeps the molecule in an open conformation, allowing for further phosphorylation of other tyrosine residues on Syk both by other tyrosine kinases but mostly by autophosphorylation (17). The mutation of these two tyrosines results in Syk with decreased *in vitro* kinase activity, and when expressed in mast cells, it is less efficient in FcϵRI signaling with reduced mast cell degranulation, decreased phosphorylation of MAP kinases, and lower activation of NFAT and NFκB (12). When B cell signaling is reconstituted in S2 insect cells, Tyr-630 of human Syk (analogous to Tyr-624 of rat Syk) is phosphorylated following B cell receptor activation, and this creates a binding site for SLP-65 (18).

In the present study, we investigated whether the two tyrosines in the tail region of Syk that are phosphorylated after receptor activation can be docking sites for other proteins. A synthetic peptide representing the last 10 amino acids of the tail of Syk with these two tyrosines phosphorylated precipitated signaling proteins from mast cell lysates. These included the adaptors known to be important for signaling, including SLP-76, Nck, Grb2, GADS, and the phosphatases SHIP-1 and TULA-2 (also called UBASH3B or STS-1). Part of this interaction was a result of complexes of these proteins forming after receptor activation. Among these proteins, SLP-76, Grb2, and GADS are required for signaling as mast cells deficient in these molecules display severely diminished degranulation and cytokine production following FcϵRI aggregation (19, 20). TULA-1 (also called UBASH3A or STS-2) and TULA-2 are protein-tyrosine phosphatases that play a role in regulating T cell receptor-mediated signaling (21–23) as well as other signaling pathways (24–28). TULA-2 dephosphorylates various tyrosine-phosphorylated proteins, including Src and Syk family tyrosine kinases (22, 29, 30). By using siRNA to decrease its expression, TULA-2 was shown in the present experiments to be a negative regulator of signaling in mast cells. The results indicate that tyrosine residues in the tail of Syk not only directly regulate Syk enzymatic activity as shown previously, but also once phosphorylated, these tyrosines function as docking sites for positive and negative regulators of signal transduction in mast cells.

EXPERIMENTAL PROCEDURES

Materials and Antibodies—Mouse IL-3 and stem cell factor were purchased from Invitrogen. UltraLink Resin neutravidin-coated beads were from Pierce. Anti-SHIP-1 (P1C1) was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-SLP-76 (LCP2), anti-Nck (Nck-1), and anti-Grb2 were from Epitomics (Burlingame, CA). Anti-Grb2 was from United States Biological (Swampscott, MA). Anti-TULA-1 (UBASH3A or STS-2) and anti-Nck (1A1) were from Novus Biologicals (Littleton, CO). Anti-TULA-2 (UBASH3B or STS-1) was from Rockland Immunochemicals (Gilbertsville, PA). All other materials were as described previously (12).

Cell Culture, cDNA Transfection, and Cell Activation—The RBL-2H3 mast cell line was cultured as monolayers as described previously (12, 31). Bone marrow-derived mast cells (BMMC) were prepared from C57Bl/6 mice and used for experiments starting with the 4th week of culture (32). The growth factor-dependent MC9 NFAT-GFP and MC9 NFκB-GFP mouse reporter mast cell lines were cultured as described previously (33).

Transient Transfection with siRNA—siGENOME ON-TARGETplus SMARTpool targeting Syk or TULA-2, each containing four siRNA duplexes per gene, were purchased from Dharmacon (Lafayette, CO). For control, a pool containing four siRNAs with minimal effect on known mouse genes (ON-TARGETplus siCONTROL non-targeting pool, catalog number D-001810-10-20) was also from Dharmacon. Transfection was carried out using the Lonza Nucleofector 96-well Shuttle System as described previously (33). In all experiments, efficiency of protein knockdown was determined by immunoblotting total cell lysates at 24, 48, and 72 h after transfection.

Immunoprecipitation and Immunoblotting—RBL-2H3 cells were sensitized with antigen-specific IgE during overnight culture and then stimulated with antigen as described previously (12). At the indicated times, the monolayers were rinsed with ice-cold PBS containing 2 mM Na_3VO_4 and protease inhibitors (2 mM PMSF, 200 μM 4(2aminoethyl)benzenesulfonyl fluoride,

[3] The amino acid numbering system is for the rat Syk sequence, NCBI accession number NP_036890.

76 milliunits/ml aprotinin, 50 μg/ml leupeptin, and 5 μM pepstatin A) and solubilized in Triton lysis buffer (1% Triton, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, protease inhibitors, and Na_3VO_4). Immunoprecipitations and immunoblotting were as described previously (12).

Pulldown with Synthetic Peptides—Two biotinylated synthetic peptides containing the last 10 COOH-terminal amino acids of rat Syk were from Bionexus (Oakland, CA); one of these peptides was non-phosphorylated (YYY), whereas the other was phosphorylated (YPP) at tyrosines representing Tyr-624 and Tyr-625 (Fig. 1*A*). Lysates from 2×10^7 RBL-2H3 cells were prepared as described above in Triton lysis buffer, and the post-nuclear supernatants were precleared with 50 μl of neutravidin-coated beads. After gentle rotation at 4 °C for 60 min, the beads were removed by centrifugation (200 × *g* at 4 °C), and the supernatant was incubated with 50 μl of neutravidin beads coupled with 1 μM biotinylated YYY peptide. After a 60-min incubation as described above, the supernatant was collected and then incubated for 60 min with 50 μl of neutravidin beads coupled with 1 μM biotinylated YPP peptide. The beads containing the precipitated proteins were washed four times with ice-cold lysis buffer. For Western blotting experiments, the bound proteins were eluted by boiling for 5 min with SDS-PAGE sample buffer and analyzed as described above. In some experiments, these beads were used for two-dimensional differential in-gel electrophoresis as describe below.

Two-dimensional Differential In-gel Electrophoresis and Protein Identification—The two-dimensional differential in-gel electrophoresis was performed by Applied Biomics (Hayward, CA). Briefly, proteins precipitated by the YYY peptide were labeled with Cy3 (green), and proteins precipitated by the YPP peptide were labeled with Cy5 (red). After isoelectric focusing in the first dimension and SDS-PAGE in the second dimension, protein spots were selected that showed preferential binding to the phosphorylated peptide. The spots were picked up and analyzed by mass spectrometry (MALDI-TOF/TOF).

Far Western Blotting—Proteins precipitated using the synthetic peptides (YYY and YPP) were eluted from the beads and separated by 10% SDS-PAGE. After transfer, the membranes containing the proteins were incubated for 1 h with blocking buffer (10 mM Tris-HCl, pH 7.4, 155 mM NaCl, 4% BSA, 0.6 μg/ml biotin, and 0.015% Tween 20). Separately, 0.2 μg of each biotinylated peptide was incubated with 1.5 μg of HRP-conjugated streptavidin with gentle rotation for 1 h, and then 0.6 μg of biotin (200-fold molar excess) was added to block the HRP-conjugated streptavidin not associated with the biotinylated peptide. The blots were probed with 0.01 μg/ml YYY or YPP biotinylated peptides prebound to HRP-conjugated streptavidin. After that, the membranes were washed several times with Tris buffer (10 mM Tris-HCl, pH 7.4, 155 mM NaCl, and 0.015% Tween 20) and visualized by enhanced chemiluminescence.

Measurement of β-Hexosaminidase Release—The β-hexosaminidase release was determined in transfected BMMC that were sensitized for 24 h with 0.3 μg/ml antigen-specific IgE (anti-trinitrophenyl mAb TIB-142) as described previously (32). The percentage of β-hexosaminidase release in control cells as a percentage of the total content was 24.0 ± 2.4% at 24 h, 28.8 ± 1.8% at 48 h, and 20.9 ± 2.3% at 72 h. (mean ± S.D.).

Flow Cytometric Measurements of NFAT and NFκB Activation—Fluorescence of the GFP reporter was used as a marker of FcεRI-induced NFAT or NFκB activation in the MC9 NFAT or MC9 NFκB reporter cell lines as described previously (33).

Data Analysis—Statistical analysis was by non-parametric *t* test using GraphPad Prism 5 software, and the *p* value is represented as * ($p < 0.05$), ** ($p < 0.005$), and *** ($p < 0.0005$).

RESULTS

Proteins Are Specifically Precipitated by Tyrosine-phosphorylated Synthetic Peptide Corresponding to Syk COOH-terminal Region—There are three adjacent tyrosines (623–625 in rat Syk) in the COOH-terminal region of Syk that are highly conserved with the exception of Tyr-623, which is absent in human ZAP-70. The two terminal residues, Tyr-624 and Tyr-625, are autophosphorylated *in vitro*, and they are phosphorylated in mast cells after FcεRI activation (13, 14). Previously, we observed that these residues are important for regulating the enzymatic activity of Syk and that their mutation results in decreased kinase activity and less efficient FcεRI signaling (12). Once phosphorylated, these tyrosines in Syk could also provide docking sites for proteins with SH2 domains (15, 18). To investigate such interactions, we used two synthetic peptides that encompass the Syk COOH-terminal region; in one of the peptides, the two tyrosines representing Tyr-624 and Tyr-625 were phosphorylated (Fig. 1*A*). These peptides were used to precipitate proteins from lysates of non-stimulated RBL-2H3 mast cells. Total cell lysates were precleared with nude neutravidin beads and with the non-phosphorylated YYY peptide prebound to beads and then incubated with the YPP phosphorylated peptide. Proteins that bound to the beads were eluted and separated by SDS-PAGE; the phosphorylated YPP peptide specifically precipitated protein bands of ~145, 76, 49, and 38 kDa that were not precipitated with YYY peptide (Fig. 1*B*). This result suggested that the phosphorylation of Tyr-624 and Tyr-625 of Syk creates a potential docking site for proteins that could contribute to Syk-mediated signaling.

Identification of Proteins That Bind to Phosphorylated Tyr-624 and Tyr-625 of Syk COOH-terminal Region—A mass spectrometry approach was used to identify the proteins specifically precipitated by the phosphorylated Syk COOH-terminal peptide. Proteins were precipitated as in Fig. 1; those bound to the YYY peptide were labeled with Cy3, and those bound to the YPP peptide were labeled with Cy5. After isoelectric focusing in the first dimension and SDS-PAGE in the second dimension, protein spots were selected that showed preferential binding to the phosphorylated peptide and analyzed by mass spectrometry (Table 1). A number of signaling molecules were identified that bound to the phosphorylated peptide; these included the adaptor proteins SLP-76, Nck-1, Grb2, and GADS and the protein phosphatases SHIP-1 and TULA-2 (also known as UBASH3B or STS-1). There was also association of the transport protein Sec23B, which is involved in vesicular traffic (34). The SLP-76, Nck-1, Grb2, and GADS adaptors are known to contribute to Syk-mediated signaling after immunoreceptor activation by participation in signaling complexes (3). SHIP-1 has been identified as a negative regulator of the FcεRI response (35), and

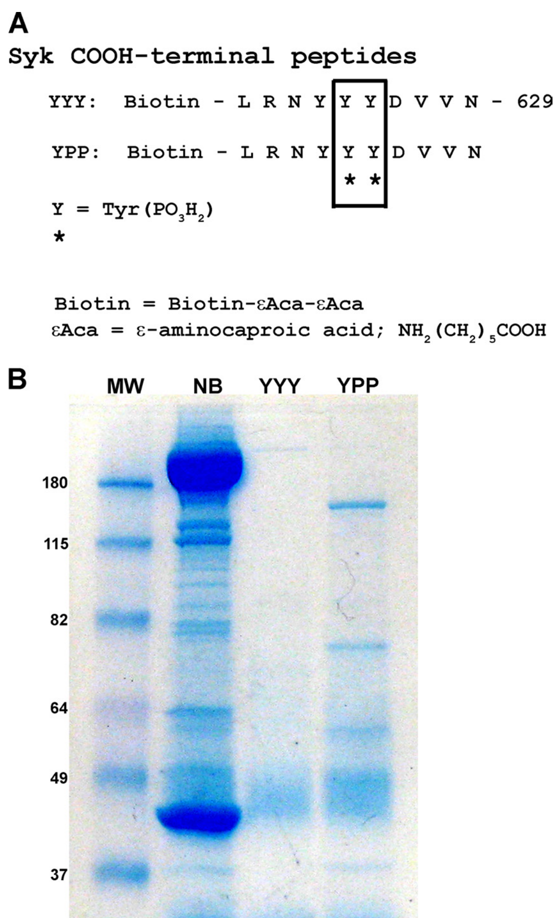

FIGURE 1. **Proteins are specifically precipitated by tyrosine-phosphorylated synthetic peptide corresponding to Syk COOH-terminal region.** *A*, the peptides encompassed the last 10 amino acids of the COOH terminus of Syk; the two terminal tyrosines (Tyr-624 and Tyr-625 of the rat sequence) that are phosphorylated *in vitro* or after receptor activation are *boxed*. The YYY is the non-phosphorylated peptide, whereas YPP is the diphosphorylated peptide. *B*, total lysates from non-stimulated RBL-2H3 mast cells were first precleared with neutravidin-coated beads (*NB*) and then incubated with beads prebound with the non-phosphorylated peptide (YYY). After that, the supernatants were incubated with beads containing the diphosphorylated peptide (YPP). The precipitated proteins were eluted by boiling and separated by SDS-PAGE, and the gels were stained with Coomassie blue. *MW*, molecular weight. The broad 40–49-kDa band in the eluates is neutravidin from the beads. Similar results were observed in five independent experiments.

UBASH3A (TULA-2) has been shown to negatively regulate T cell signaling and to associate with and dephosphorylate Syk when both are co-expressed in HEK293 cells (29). These results indicate that phosphorylated Tyr-624 and Tyr-625 of the Syk COOH-terminal region are a potential docking site for adaptor proteins and for protein-tyrosine phosphatases.

Tyrosine-phosphorylated Syk COOH-terminal Peptide Precipitates SHIP-1, SLP-76, TULA-2, Nck, and Grb2—Most of the proteins identified by mass spectrometry as binding specifically to the phosphorylated Syk COOH-terminal peptide had molecular weights similar to those of the bands visualized by protein staining (Fig. 1*B*). To validate the results obtained by mass spectrometry, proteins precipitated from RBL-2H3 cell lysates with the naked beads or with YYY or YPP peptide were analyzed by immunoblotting with specific antibodies (Fig. 2). This confirmed that the phosphatases SHIP-1 and TULA-2 and the adaptor proteins SLP-76, Nck-1, and Grb2 were specifically precipitated with the phosphorylated peptide representing Tyr-624 and Tyr-625 of Syk (YPP) but not with non-phosphorylated YYY peptide. However, because of the lack of antibodies, we could not confirm the presence in the precipitates of the other proteins that were identified by mass spectrometry. Nck-1 and Nck-2 are homologous proteins suggesting that Nck-2 could also bind to the YPP peptide; this was confirmed by immunoblotting (data not shown). Altogether, these immunoblotting results indicated that the adaptor proteins SLP-76, Nck-1, Nck-2, and Grb2 and the phosphatases SHIP-1 and TULA-2 bind to the phosphorylated Syk COOH-terminal peptide.

Nck and SHIP-1 Bind Directly to Tyrosine-phosphorylated Syk COOH-terminal Peptide—The precipitation of these proteins with the synthetic peptide could be the result of either direct interaction with phosphorylated Tyr-624/625 of Syk or due to complex formation where one of the proteins is binding and then interacting with other proteins. Far Western blotting was therefore used to determine whether the interactions were due to direct or indirect binding (Fig. 3). The phosphorylated YPP peptide bound to two bands, a prominent signal at 49 kDa and a weaker signal at 145 kDa, whereas the non-phosphorylated YYY peptide did not bind. The membranes were also blotted with specific antibodies that identified that the strongly reacting 49-kDa band was Nck, whereas the 145-kDa band was SHIP-1. Although TULA-2 and the other proteins were on the membrane, they did not directly interact with the YPP peptide. Therefore, these results indicate that the adaptor Nck-1 and the phosphatase SHIP-1 directly bind to phosphorylated Tyr-624/625 of Syk, whereas the other proteins are precipitated due to complex formation with these two molecules.

Analyses of Phosphorylated Syk-interacting Protein in Cells—The *in vitro* experiments with synthetic peptides and cell lysates strongly suggested that phosphorylation of Syk Tyr-624 and Tyr-625 creates a docking site that recruits SLP-76, Nck-1, Grb2, SHIP-1, and TULA-2. Immunoprecipitation from antigen-activated or non-activated RBL-2H3 cells followed by immunoblotting was used to determine whether in cells there was interaction of these proteins both before and after receptor stimulation (Fig. 4). As would be expected if the interactions depend on the phosphorylation of these tyrosines in Syk, most co-immunoprecipitation occurred in the lysates from activated cells. When Syk was immunoprecipitated, there was co-precipitation of SHIP-1 and SLP-76 only from stimulated cells, whereas Nck and Grb2 were co-precipitated with Syk from non-stimulated cells with increased association after cell stimulation. Immunoprecipitation of Nck-1 showed association with SHIP-1, SLP-76, TULA-2, and Grb2 after receptor stimulation with much less association with TULA-2 and Grb2 in non-activated cells. There was also co-precipitation of SHIP-1 and Nck with Grb2. In other immunoprecipitations, anti-SHIP-1 showed that SHIP-1 associated with Syk, Nck-1, and TULA-2 most prominently in stimulated cells, whereas anti-TULA-2 co-precipitated SHIP-1, Nck-1, and Grb2 (Fig. 4*B*). In all these experiments, the precipitates were also blotted with anti-Syk, and when not shown in the figure, Syk was not detected. Although we did not detect the co-precipitation of TULA-2 with Syk under these conditions, there was still

TABLE 1

Identification of proteins that bound to phosphorylated Tyr-624 and Tyr-625 of tail region of Syk

Proteins were precipitated as in Fig. 1 with a synthetic peptide based on the last 10 amino acids of Syk in which the two tyrosines were either non-phosphorylated (YYY) or phosphorylated (YPP). The proteins precipitated by the YYY peptide were labeled with Cy3 (green), and proteins from the YPP peptide were labeled with Cy5 (red). After isoelectric focusing in the first dimension and SDS-PAGE in the second dimension, protein spots were selected that showed preferential binding to the phosphorylated peptide and analyzed by mass spectrometry (MALDI-TOF/TOF). The table is the summary of data where more than four peptides were identified.

Protein name	Symbol	Accession no. (GI:)	Molecular mass	pI	Peptide count	C.I.[a]
			kDa			*%*
Inositol-polyphosphate 5-phosphatase D	SHIP-1	9506813	133,509	7.04	40	100
Predicted: RIKEN cDNA 2810457I06	UBASH3B	109483356	71,467	6.40	15	100
Lymphocyte cytosolic protein 2	SLP-76	18426850	59,485	5.90	10	100
Sec23 homolog B	Sec23B	157817612	50,348	7.50	11	99.9
Nck adaptor protein 1	Nck	157820985	42,849	6.06	14	100
Grb2-related adaptor protein 2	GADS	77917586	37,313	6.20	23	100
Growth factor receptor-bound protein 2 isoform 1	Grb2	914957	23,541	6.30	15	100

[a] C.I. indicates the confidence of protein identification where scores above 95% are significant.

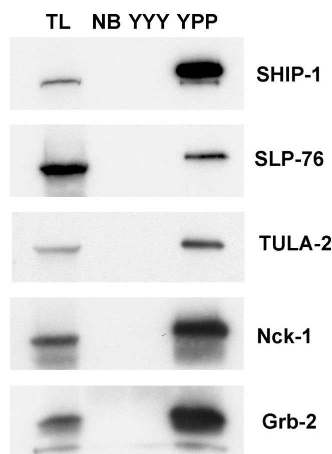

FIGURE 2. **Tyrosine-phosphorylated Syk COOH-terminal peptide precipitates SHIP-1, SLP-76, TULA-2, Nck, and Grb2.** Proteins were precipitated with naked beads (*NB*) or with peptide YYY or YPP prebound to beads from lysates of 10^8 non-stimulated RBL-2H3 cells as described in Fig. 1. The eluted proteins were separated by 10% SDS-PAGE gels, and then after transfer, the membranes containing the total lysates and precipitated proteins were blotted with the indicated antibodies. *TL*, total cell lysate (5×10^5 cells/lane) collected before the preclearing step. Similar results were observed in five independent experiments.

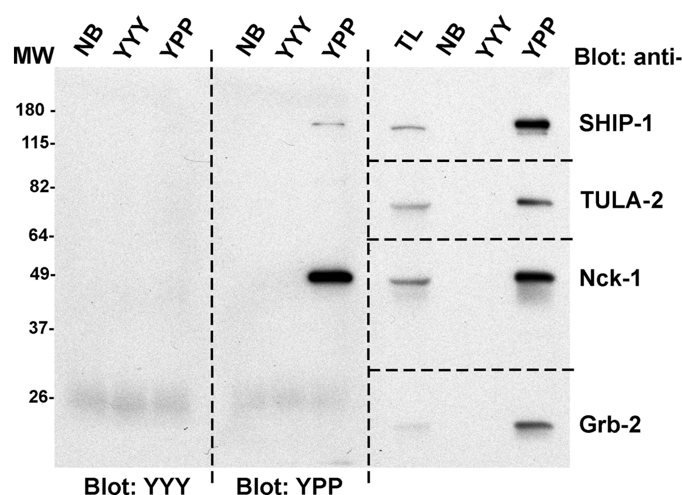

FIGURE 3. **Tyrosine-phosphorylated Syk COOH-terminal peptide directly binds Nck and SHIP-1.** Proteins were precipitated with YYY, YPP, or naked beads (*NB*) from lysates of non-stimulated RBL-2H3 cells as described in Fig. 1. Proteins were then separated by SDS-PAGE on 10% gels, and after transfer, the membrane was cut for blotting as indicated by the *dashed lines*. The membranes used for far Western blotting were probed using YYY or YPP peptides prebound to streptavidin-HPR. Total cell lysate (*TL*) and precipitated proteins were also immunoblotted with anti-SHIP-1, anti-TULA-2, anti-Nck, and anti-Gbr-2. Then the membrane was put back together for protein visualization by enhanced chemiluminescence. Similar results were observed in three independent experiments.

TULA-2 association with SHIP-1, Nck-1, and Grb2 especially in the precipitates from activated cells. This suggests that the binding of TULA-2 to phosphorylated Syk could be indirectly mediated by SHIP-1 or Nck. Together with the peptide studies, these results suggest that after receptor activation, which results in phosphorylation of these COOH-terminal tyrosines of Syk, there is binding of complexes of proteins; Nck, SHIP-1, SLP-76, Grb2, and TULA-2 is one complex that associates through the Syk-Nck interaction, whereas another complex of SHIP-1, Nck, Grb2, and TULA-2 binds Syk through either Nck or SHIP-1.

Decreased Expression of TULA-2 Enhances FcεRI-induced Degranulation and NFAT or NFκB Activation—The TULA family of proteins, which has two members, TULA-1 and TULA-2, has recently emerged as negative regulators of receptor signaling in T cells and platelets (21, 22, 36). The present results strongly suggested that TULA-2 is in complexes that associate with phosphorylated Syk, but its function in mast cells has not been described; therefore, an siRNA approach was used to investigate the role of TULA-2 in FcεRI signaling. Bone marrow-derived mouse mast cells were treated with a pool of four siRNAs targeting TULA-2, and IgE-FcεRI-mediated degranulation was determined for 3 days post-transfection. As Syk is essential for FcεRI-induced degranulation, siRNA targeting Syk was used as a control. Knockdown of TULA-2 significantly enhanced the β-hexosaminidase release at all 3 days post-transfection with a maximum increase of 36 ± 17.6% ($n = 5$) on day 3 (Fig. 5*A*). On all 3 days after transfection, there was decreased TULA-2 protein expression (Fig. 5*B*). As previously reported, treatment with siRNA for Syk efficiently reduced protein expression and FcεRI-mediated degranulation (Fig. 5, *A* and *B*). These results indicate that TULA-2 is a negative regulator of FcεRI-mediated degranulation.

Aggregation of FcεRI also leads to Syk-dependent activation of the transcription factors NFAT and NFκB. To investigate the role of TULA-2 in these pathways, reporter mast cell lines were used in which NFAT or NFκB control the expression of GFP. The cells were treated with a pool of four siRNAs for TULA-2, and FcεRI-induced GFP expression was determined by FACS analyses for 3 days post-transfection. There was enhanced receptor-induced activation of both NFAT and NFκB at all 3 days of testing when cells were treated with siRNA for TULA-2

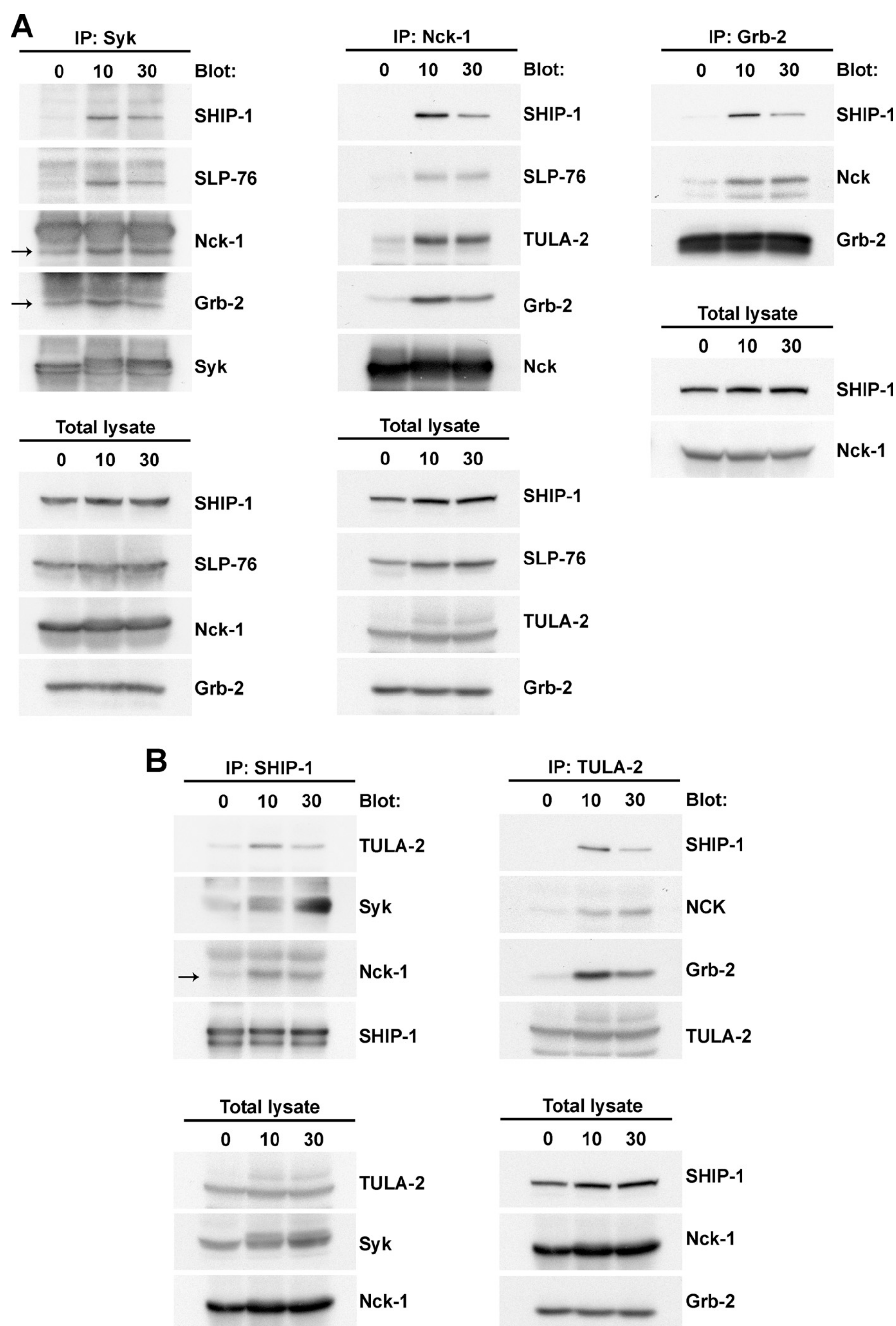

FIGURE 4. **Analysis of phosphorylated Syk-interacting proteins in cells.** RBL-2H3 cells were sensitized with IgE and then stimulated for the indicated times (in minutes) with antigen. *A*, Syk, Nck, and Grb2 were immunoprecipitated (*IP*) with the respective antibodies, and the proteins were eluted and separated by SDS-PAGE on 10% gels. Total cell lysates and precipitates were analyzed by immunoblotting with the indicated antibodies. *B*, the phosphatases SHIP-1 and TULA-2 were immunoprecipitated with the indicated antibodies. The immunoprecipitates and total cell lysates were analyzed by immunoblotting as described above. Similar results were observed in three experiments.

(Fig. 5, *C* and *E*) that paralleled decreased expression of TULA-2 during all 3 days of testing (Fig. 5, *D* and *F*). As expected, the knockdown of Syk efficiently reduced FcεRI-induced NFAT and NFκB activation (Fig. 5, *C–E*). To further validate these results, we tested the capacity of each of the single siRNAs from the original pool that targets TULA-2 (supplemental Figs. 1 and 2). Three of the four siRNAs efficiently decreased the expression of TULA-2, and only these three increased NFAT or NFκB activation after receptor stimulation. The knockdown of TULA-2 did not show any compensatory changes in the level of TULA-1, the other member of this family (supplemental Figs. 1 and 2). Thus, TULA-2 negatively regulates FcεRI-induced NFAT and NFκB activation.

Enhanced Syk Activation Loop and Phospholipase C-γ2 Tyrosine Phosphorylation by TULA-2 Knockdown—Phosphorylation of the activation loop tyrosines (Tyr-519/Tyr-520) is essential for Syk-mediated FcεRI responses in mast cells (10), and screening using a combinatorial phosphotyrosine peptide library suggests that TULA-2 can dephosphorylate these residues (37). To investigate whether this occurs in FcεRI-medi-

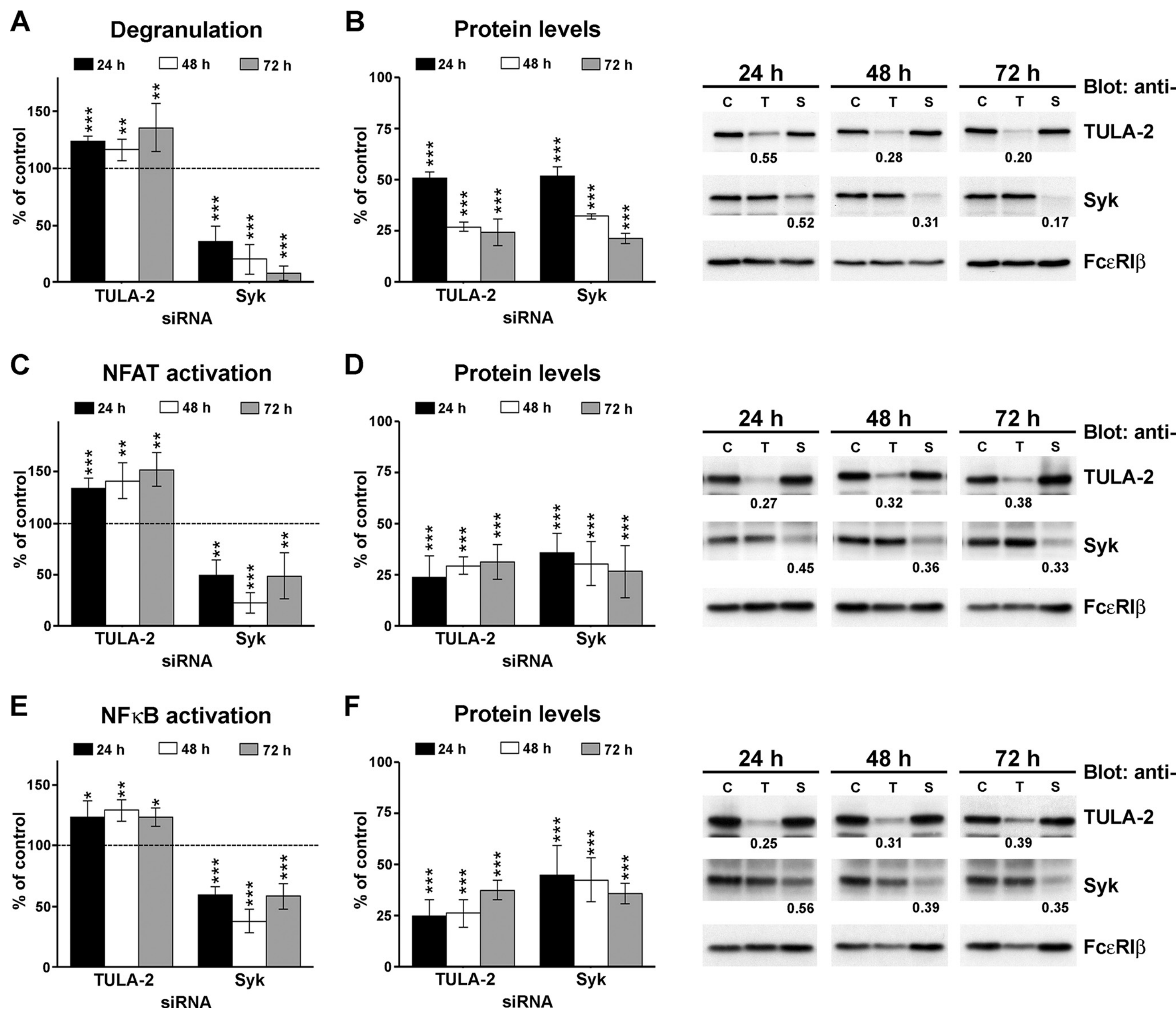

FIGURE 5. **Decreased expression of TULA-2 enhances FcϵRI-induced degranulation and NFAT and NFκB activation.** *A*, BMMC were treated with siRNA specific for TULA-2 or Syk or with siCONTROL, and 24, 48, and 72 h later, the IgE antigen-induced β-hexosaminidase release was determined. Degranulation of cells treated with siRNA for Syk or TULA-2 is presented as the percentage of the release determined for cells transfected with control siRNA (*dashed line*). *B*, the efficiency of protein knockdown in cells treated with control, TULA-2, or Syk siRNA (indicated as *C*, *T*, or *S*, respectively) was determined by immunoblotting as shown (*right*). Quantification of protein levels was determined by densitometry after normalization for gel loading from blots probed with anti-FcϵRIβ and expressed as the percentage of controls (*left*). Results are mean ± S.D. from four independent experiments. *C* and *E*, MC9 NFAT-GFP cells or MC9 NFκB-GFP cells were treated with siRNA specific for TULA-2 or Syk or with siCONTROL and stimulated with antigen at the indicated times, and NFAT or NFκB activation was determined by FACS analysis. The results are mean ± S.D. of three independent experiments expressed as the percentage of cells transfected with control siRNA (*dashed line*). Expression of the target proteins in the cells was determined by immunoblotting (*D* and *F*) similar to that in BMMC. Results are mean ± S.D. from three independent experiments. *, $p < 0.05$; **, $p < 0.005$; and ***, $p < 0.0005$.

ated reactions, we treated BMMC with siRNA targeting TULA-2 or siRNA control for 72 h and then analyzed the antigen-induced phosphorylation of the activation loop tyrosines of Syk (Fig. 6*A*). There was significantly increased phosphorylation of the activation loop tyrosines of Syk in cells treated with siRNA for TULA-2 compared with cells transfected with siRNA control. These results suggest that TULA-2, by controlling phosphorylation of these tyrosines, plays a role in regulating the activity of Syk.

Activation of Syk propagates downstream signals, one of which is the tyrosine phosphorylation and activation of phospholipase C-γ2. There was significantly increased phosphorylation of PLC-γ2 after receptor activation in BMMC treated with siRNA of TULA-2 compared with cells transfected with siRNA controls (Fig. 6*B*). However, there were only minor increases in total cellular protein tyrosine phosphorylations by antigen activation in TULA-2 siRNA-treated cells (supplemental Fig. 3A) but without detectable changes in the phosphorylation of ERK1/2 or p38 MAP kinases (supplemental Fig. 3B). In contrast, Syk siRNA-treated cells showed a reduction in phosphorylation of PLC-γ2, total cellular proteins, and the MAP kinases. These results indicate that TULA-2 is a phosphatase that, by regulating the phosphorylation of the activation loop tyrosines of Syk, controls its enzymatic activity, thereby modifying the downstream signals that result in degranulation and the activation of NFAT/NFκB transcription factors. These data

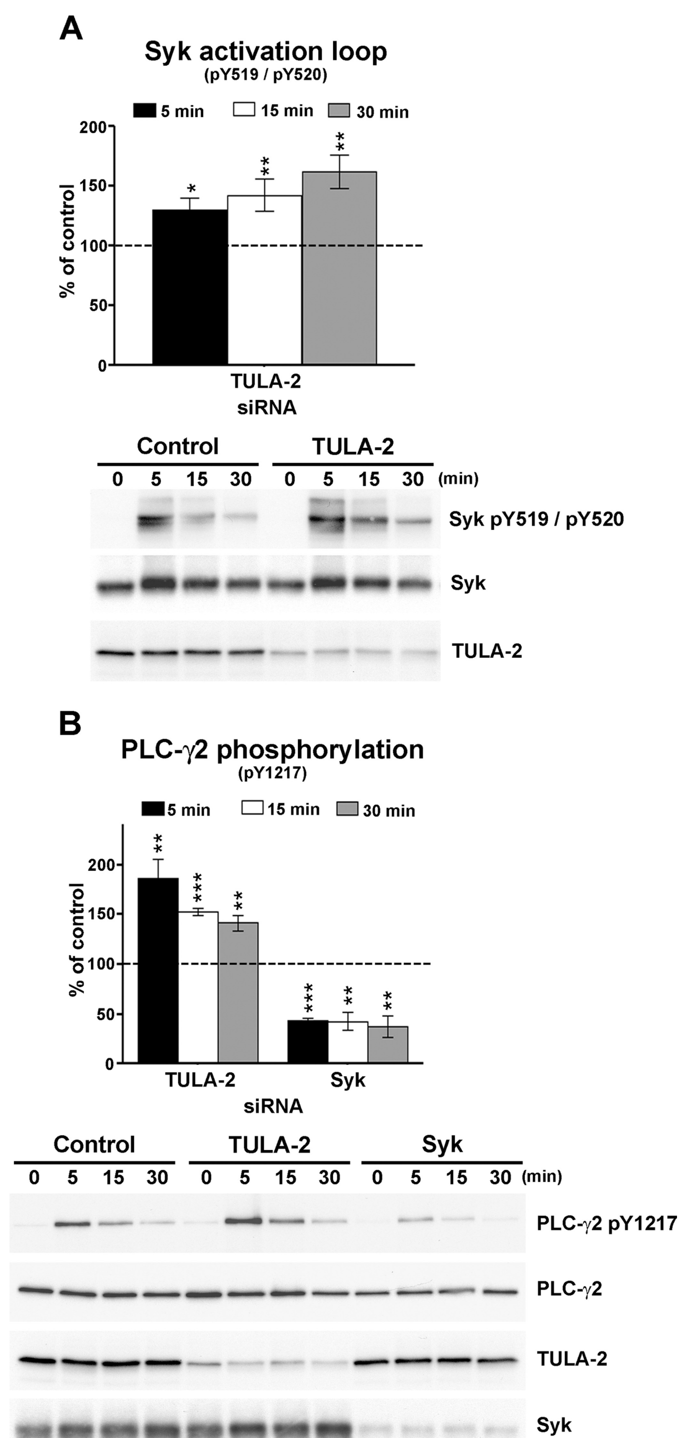

FIGURE 6. **Syk activation loop and PLC-γ2 phosphorylations are enhanced by reduced TULA-2 expression.** *A*, BMMC were treated with siRNA specific for TULA-2 or Syk or with siCONTROL and 72 h later were stimulated with IgE antigen for the indicated times. Proteins were analyzed by immunoblotting with antibodies to Syk activation loop (Tyr(P)-519/Tyr(P)-520) or TULA-2. After stripping, the membranes were reblotted with anti-Syk and anti-TULA-2. *B*, BMMC treated with siRNA specific for TULA-2 or Syk or with siCONTROL was analyzed by immunoblotting with specific anti-PLC-γ2 Tyr(P)-1217 or TULA-2. After stripping, the membranes were blotted with antibodies to PLC-γ2, TULA-2, or Syk. Quantification of protein phosphorylation was determined by densitometry after normalization for gel loading and expressed as percentage of controls (*dashed line*). Results are mean ± S.D. from three independent experiments. *, $p < 0.05$; **, $p < 0.005$; and ***, $p < 0.0005$.

indicate that TULA-2 functions as a negative regulator of FcεRI signaling in mast cells.

DISCUSSION

We demonstrate herein that once phosphorylated tyrosines in the COOH-terminal region of Syk bind complexes of adaptor proteins and phosphatases that are involved in FcεRI signaling (Fig. 7). Aggregation of FcεRI results in the phosphorylation by Lyn of the tyrosines in the ITAM (p-ITAM) of subunits of the receptor. The p-ITAM recruits Syk from the cytoplasm to the membrane, and their interaction results in conformational change and activation of Syk. Structural and mutational studies suggest that the COOH-terminal tyrosines of Syk maintain the kinase in an inactive autoinhibitory state due to interactions of Tyr in the tail with both the kinase and the inter-SH2 region (15, 38). The conformational change of Syk exposes its terminal region, resulting in the phosphorylation of Tyr-624 and Tyr-625, which then keeps the molecule in an open conformation, allowing for further phosphorylation of other tyrosine residues on Syk both by other tyrosine kinases but mostly by autophosphorylation. Previously, we showed that mutation of these tyrosine residues in the tail of Syk decreased the capacity of Syk to signal by regulating its enzymatic activity. Here we demonstrate a further function namely as a docking site for adaptor proteins and phosphatases. The interaction of proteins such as Nck and SHIP-1 with these phosphorylated tyrosine residues would further stabilize the active state but also regulate the extent of the signal. Collectively, these data establish the important role of phosphorylation of tyrosines in the tail region in regulating the signal transduction function of Syk.

The adaptor protein Nck and the lipid phosphatase SHIP-1 directly bind to the phosphorylated tyrosines of the tail region of Syk with further interaction through complex formation of other proteins, including SLP-76, TULA-2, Grb2, and GADS. These adaptors and effectors probably also associate with other phosphorylated tyrosine residues on Syk such as those in the linker region. These proteins control signaling in mast cells; SLP-76, Grb2, and GADS are required for optimal response, whereas SHIP-1 and TULA-2 are negative regulators. The SH2 domain of Nck and SHIP-1 bind to sequences that are similar to what is found in the terminal region of Syk. Thus, Nck-1/Nck-2 preferentially bind to pYD(E/D)V, whereas SHIP preferentially binds to pY(Y/S/T)L(L/N) where pY is phosphotyrosine (39–42). The further association of proteins with the phosphorylated Syk terminal region probably depends on complex formation due to SH2 and SH3 interactions such as the SH3 domains in Nck, GADS, Grb2, and TULA-2 interacting with a proline-rich motif of SLP-76 (43) or Grb2 binding of SHIP-1 (44, 45). There was minimal if any co-immunoprecipitation of these proteins from non-stimulated cells where the terminal tyrosines of Syk would not be phosphorylated (Fig. 4). After FcεRI stimulation, there was co-immunoprecipitation of proteins, suggesting that these were related to the tyrosine phosphorylation occurring in Syk and in other proteins. Immunoprecipitation with different antibodies showed co-precipitation of all the proteins that were detected with the synthetic peptide. However, it is impossible to determine from the immunoprecipitation results whether all of these proteins were in one large

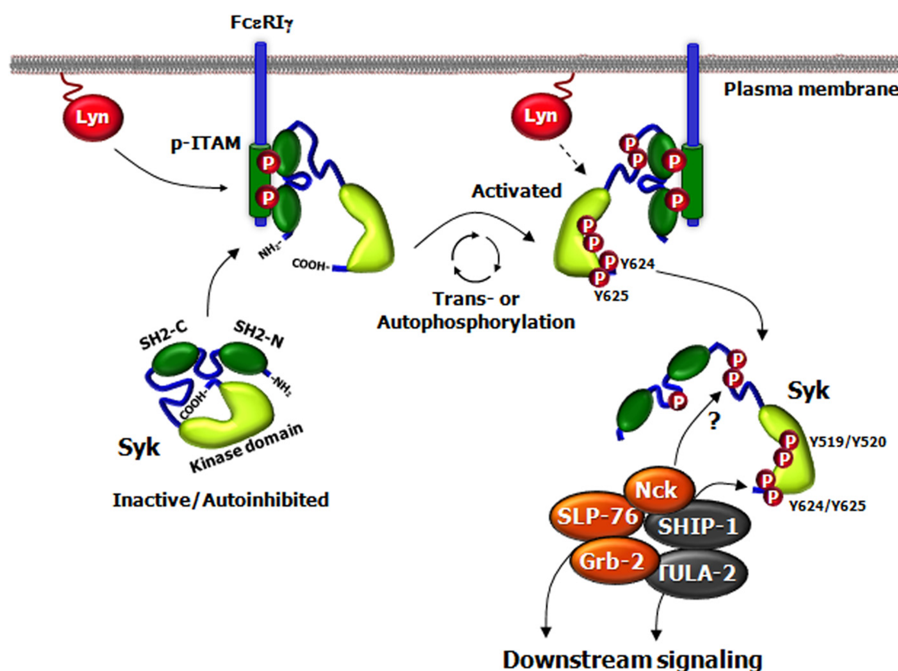

FIGURE 7. **Schematic model of function of phosphorylated Tyr-624 and Tyr-625 as docking sites for proteins in Syk-mediated signaling.** Aggregation of FcεRI results in the phosphorylation by Lyn of the tyrosines in the ITAM (p-ITAM) of the β and γ subunits of the receptor (only the γ subunit is shown). This recruits Syk from the cytoplasm; the binding of Syk to the p-ITAM results in its conformational change and activation. Syk becomes tyrosine-phosphorylated by either auto- or trans-phosphorylation with a small contribution by other kinases. Among these phosphorylated sites are Tyr-624 and Tyr-625 in the Syk COOH-terminal region. The phosphorylation of these tyrosines increases Syk kinase activity and creates a docking site for the direct binding of the adaptor protein Nck and the phosphatase SHIP-1. This then allows for further association of proteins that include the adaptors SLP-76, GADS, and Grb2 and tyrosine phosphatase TULA-2. These proteins could also bind to other phosphorylated tyrosine residues such as those in the linker region of Syk.

complex or were in several smaller complexes with distinctive compositions.

The adaptor SLP-76 is important in mast cell signaling; cells deficient in SLP-76 develop normally but exhibit defective responses to FcεRI activation (19). SLP-76 associates with Nck (46) and in the present experiments was co-immunoprecipitated with Nck and Syk. SLP-76 recruited to Syk is then tyrosine-phosphorylated (47, 48). The SLP-76 homologue SLP-65, which is expressed in B cells, binds by its SH2 domain to the COOH-terminal phosphorylated tyrosines of Syk (18, 38). Both Nck and SLP-76 associate with Grb2 and other signaling molecules; SLP-76 has a central proline region that constitutively binds GADS (43) and an SH2 domain in the COOH-terminal region that bind phosphorylated proteins (3). These interactions are important for signaling in mast cells as the expression of SLP-76 with a mutation in the binding sites for Nck or GADS results in defective degranulation and cytokine responses (49, 50). The proline-rich region in SLP-76 could also provide a binding site for the SH3 domain of TULA-2. Interaction of SLP-76 and Nck together with other molecules such as Vav, Cdc42, and Wiskott-Aldrich syndrome protein contributes to actin polymerization and cytoskeleton rearrangements, which are important for mast cell granule release (51–53).

The inositol polyphosphatase SHIP-1 negatively regulates mast cell signaling by opposing the activity of phosphoinositide 3-kinase (54). There are increased FcεRI-induced degranulation, NFκB activation, and IL-6 production in BMMC from SHIP-1-deficient mice (35, 55). The recruitment of SHIP-1 to the membrane by binding to Syk or the subunit β of FcεRI would allow SHIP-1 to act on its substrate generated by phosphoinositide 3-kinase activation (56).

The TULA family of protein-tyrosine phosphatases has been identified as negative regulators of signaling pathways (21, 22, 25–28, 30, 36). The two members of this family, TULA-1 and TULA-2, have an NH_2-terminal ubiquitin association domain that can bind mono- and polyubiquitin, a central SH3 domain, and a COOH-terminal phosphoglycerate mutase domain with tyrosine phosphatase activity (21, 25, 26, 57). Despite their high degree of homology, there are differences between the two members of this family: TULA-1 has lower phosphatase activity than TULA-2 (22, 29, 37, 58, 59), and TULA-2 decreases the kinase activity of Syk, whereas TULA-1 increases it (29). TULA-2 dephosphorylates various tyrosine-phosphorylated proteins, including Src and Syk family tyrosine kinases (22, 29, 30). *In vitro*, TULA-2 but not TULA-1 is highly active toward peptides corresponding to the phosphorylated tyrosines in the linker region of Syk, and in cells, overexpression of TULA-2 results in a decrease in tyrosine phosphorylation of Syk on the same residues (37). Both TULA-1 and TULA-2 have to be absent for the hyper-responsive response to T cell receptor stimulation and increased cytokine secretion together with the increased tyrosine phosphorylation of the Syk family kinase ZAP-70 (21–23). However, re-expression of TULA-2 in the double deficient T cells leads to a substantial reversal of the phenotype, indicating that TULA-2 is responsible for the major part for the phenotype and that this requires its phosphatase activity (22). TULA-2 associates with Syk in HEK293T cells overexpressing Syk and TULA-2 (29) and in platelets (36).

There is an increase in collagen receptor Glycoprotein VI-induced Syk phosphorylation in cells from TULA-double deficient mice (36). In the present experiments, we found that although both TULA family members were expressed in mast cells the decreased expression of TULA-2 alone was enough to enhance FcεRI-induced activation. Therefore, unlike in T cells, in mast cells, the knockdown of TULA-2 alone of this family of phosphatases has an effect on signal transduction. Although the decreased expression of TULA-2 enhanced FcεRI-induced degranulation and signaling to NFAT/NFκB, it did not increase MAP kinase phosphorylations. This suggests that TULA-2 does not regulate pathways to MAP kinases.

PLC-γ2 is a direct target for Syk phosphorylation during receptor activation in immune cells and therefore Syk regulates the products of PLC-γ activation, inositol trisphosphate and diacylglycerol, which trigger calcium influx and protein kinase C activation that are critical for downstream events. The decrease in TULA-2 expression by siRNA transfection in BMMC resulted in increased tyrosine phosphorylation of PLC-γ2 with increased degranulation and NFAT/NFκB activation. The increased tyrosine phosphorylation of PLC-γ2 after FcεRI stimulation in TULA-2 siRNA-treated cells was most probably a result of the increased activity of Syk.

Syk mutated at these COOH-terminal Tyr residues is less efficient in degranulation and NFAT and NFκB activation (12). This mutated Syk has decreased enzymatic activity and reduced binding to phosphorylated ITAM. Mutation of these tyrosines also results as shown here in the loss of the binding sites for adaptor molecules that are positive or negative regulators of immunoreceptor signaling. Mutated Syk was less efficient in signaling, indicating that, on balance, changes in enzymatic activity and the loss of binding of adaptors that are positive enhancers such as SLP-76 are more important than the loss of recruitment of the negative regulators SHIP-1 and TULA-2.

In conclusion, the results further our understanding of the role of Syk in immunoreceptor signaling. Binding of Syk to phosphorylated ITAM induces conformational changes that decrease its autoinhibitory form, exposing the COOH-terminal region of Syk and switching the protein from an inactive to active form. This allows phosphorylation of the two COOH-terminal tyrosines, which maintains the active state; these residues also create docking sites that directly bind adaptors and regulators of signaling.

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