TOM1L1 Is a Lyn Substrate Involved in FceRI Signaling in Mast Cells*

Juan Zhang¹, Katsuhiko Suzuki¹,², Tomohiro Hitomi³, and Reuben P. Siraganian⁴

From the Receptors and Signal Transduction Section, Oral Infection and Immunity Branch, NIDCR, National Institutes of Health, Bethesda, Maryland 20892

Protein-tyrosine kinase Lyn and Syk are critical for antigen-receptor-induced signal transduction in mast cells. To identify novel Lyn/Syk substrates, we screened an RBL-2H3 bacterial expression library for proteins that were tyrosine phosphorylated with baculoviral expressed Lyn or Syk. Five clones as potential Lyn substrates and eight clones as Syk substrates were identified including known substrates such as SLP-76, LAT, and α-tubulin. A potential substrate of Lyn identified was the molecule Tom1L1, which has several domains thought to be important for membrane trafficking and protein-protein interactions. Because the function of Tom1L1 is unclear, the rat Tom1L1 full-length cDNA was isolated and used to express the protein in COS-1 and RBL-2H3 mast cells. In COS-1 cells, the co-transfection of Tom1L1 and Lyn, but not Syk, resulted in the tyrosine phosphorylation of Tom1L1. In RBL-2H3 mast cells, the overexpressed Tom1L1 was strongly tyrosine phosphorylated in non-stimulated cells, and this phosphorylation was enhanced by FceRI aggregation. By subcellular fractionation, wild-type Tom1L1 was mainly in the cytoplasm with a small fraction constitutively associated with the membrane; this association was markedly reduced in deletion mutants lacking several of the protein interaction domains. The overexpression of Tom1L1 enhanced antigen-induced tumor necrosis factor (TNF) α generation and release. Both protein interaction domains (VHS and the coiled-coil domains) were required for the increased TNFα release, but not the increased TNFα generation. These results suggest that Tom1L1 is a novel protein involved in the FceRI signal transduction for the generation of cytokines.

The aggregation of the high affinity IgE receptor (FceRI) on mast cells and basophils activates multiple signaling pathways that lead to degranulation and the release of mediators of allergic reactions. The earliest biochemical event following FceRI aggregation is the tyrosine phosphorylation of multiple signaling molecules (1, 2). Because FceRI lacks intrinsic enzymatic activity, this phosphorylation is due to the rapid activation of two non-receptor-type protein-tyrosine kinases, Lyn and Syk. In this pathway FceRI aggregation results in Lyn phosphorylating the Tyr residues of the immunoreceptor tyrosine-based activation motifs of the β and γ subunits of FceRI. The tyrosine-phosphorylated immunoreceptor tyrosine-based activation motifs of the β and γ subunits then recruit Lyn and Syk, respectively, through Src homology-2 (SH2) domain-mediated interactions, leading to phosphorylation and activation of Syk (3–5).

Because of their critical role in signal transduction, there is much interest in understanding how Lyn and Syk transfer the signal from FceRI aggregation to downstream events. Several substrates of Lyn and Syk have been identified by different experimental approaches. These include enzymes such as phospholipase Cγ (6), phospholipase D (7), Btk (8–10), Pyk2 (11), Vav (12), phosphatidylinositol 3-kinase (13), and Cbl (14); or adaptor/docking proteins such as SLP-76 (15), LAT (16), HS1 (17), Shc (18), and CLNK/MIST (19). Other substrates of Lyn and Syk include receptor subunits such as FceRIβ and γ subunits, TCRɛ subunit (20), or cytoskeletal components such as α-tubulin (21) and SH3P7 (22).

To identify novel substrates of Lyn and Syk, we used a recently described genetic method for screening a cDNA expression library for proteins that were tyrosine phosphorylated in vitro by these kinases (23). Using an RBL-2H3 expression library, five clones as potential Lyn substrates and eight clones as Syk substrates were isolated including SLP-76, LAT, and α-tubulin. Among these clones, Tom1L1 (target of myb1-like 1), a member of the new TOM protein family of molecules, was identified as a novel Lyn substrate. The TOM family of proteins comprises three members, Tom1, Tom1L1/Srcasm, and Tom1L2 that all contain a VHS (Vps27, Hrs, and STAM domain; GGA, Golgi-localized, and STAM; GAT, GGA and TOM) homology domain that are thought to be involved in vesicular trafficking (24, 25). The function of Tom1L1 in antigen receptor signaling is unclear, although it has been reported that Tom1L1 interacts with Grb2, P85 subunit of phosphatidylinositol 3-kinase, ubiquitin, Tollip, Hrs, and TSG101, and modulates EGF and Src-kinase signaling in keratinocytes (26–31).

---

*This work was supported by the Intramural Research Program of the National Institutes of Health, NIDCR. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Both authors contributed equally to this work.

² Present address: Gunma Institute for Allergy and Asthma, 3233-1 Shinozuka, Ohra-machi, Gunma 370-0615, Japan.

³ Present address: Dept. of Pediatrics, National Hospital Organization-Saga National Hospital, Hinode 1-20-1, Saga, Japan.

⁴ To whom correspondence should be addressed: RAST Section, OIIB, Bldg. 10, Rm. 1N106, NIDCR, National Institutes of Health, Bethesda, MD 20892. Tel.: 301-496-5105; Fax: 301-480-8328; E-mail: rs53x@nih.gov.

⁵ The abbreviations used are: SH2, Src homology domain 2; FceRI, the high affinity receptor for IgE; RACE, rapid amplification of cDNA ends; Tom1L1, target of myb1-like 1; HA, hemagglutinin; VHS, domain found in Vps27, Hrs, and STAM; GAT, GGA and TOM domain; GGA, Golgi-localized, γ-ear containing, ARF-binding protein; EGF, epidermal growth factor; TNFα, tumor necrosis factor α; MAP, mitogen-activated protein; siRNA, small interfering RNA.
TOM1L1 a Lyn Substrate in FcεRI Signaling

Because TOM1L1 was isolated as a potential substrate of Lyn, it was important to investigate whether this molecule is involved in antigen receptor signaling. To study the function of TOM1L1 in FcεRI signal transduction, the full-length cDNA of rat TOM1L1 was isolated from rat basophilic leukemia RBL-2H3 cells, a model system for basophils and mast cells. TOM1L1 protein was overexpressed in COS-1 and RBL-2H3 cells. Our experiments suggested that FcεRI stimulation increased the tyrosine phosphorylation of TOM1L1, probably through Lyn; and overexpression of TOM1L1 enhanced antigen-stimulated TNFα synthesis and release. Furthermore, the VHS domain of TOM1L1 was required for the enhanced TNFα release. These results suggested that TOM1L1 is a novel Lyn substrate, and is involved in the FcεRI signaling in mast cells.

EXPERIMENTAL PROCEDURES

Materials and Antibodies—The RBL-2H3 cDNA of rat TOM1L1 library has been described previously (32). The pBacPAK vector was from Clontech (Mountain View, CA), and pSVL vector was from Amersham Biosciences. A pBluescript vector containing the hemagglutinin (HA) sequence was kindly provided by Dr. Nicholas Ryba (NIDCR, National Institutes of Health). The pSV2-Neo vector expressing the neomycin resistance gene was from ATCC (Rockville, MD). pEAK 12 vector was from Edge-Biosystems (Gaithersburg, MD). The polyclonal anti-HA tag (HA probe, Y-11) antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), and the horseradish peroxidase-conjugated anti-phosphotyrosine monoclonal antibody (4G10) was from Upstate Biotechnology, Inc. (Lake Placid, NY). The rabbit anti-mouse TOM1L1 antibody was kindly provided by Dr. Seykora, University of Pennsylvania, Philadelphia, PA (29). All other antibodies used were previously described (33, 34).

Construction of Recombinant Baculoviral Syk and Lyn—The cDNAs containing the open reading frame of rat Syk and Lyn were ligated into pBacPAK vector for transfer to baculovirus (Syk was ligated into BamHI and EcoRI sites; whereas Lyn was ligated into BamHI and XbaI sites). For expression of baculoviral proteins, suspension cultures of Sf9 insect cells were infected at a multiplicity of infection of 1 to 10 with recombinant baculovirus at a cell density of 1 × 10⁶ cells/ml using serum-free medium Sf9-II (Invitrogen).

Phosphorylation Screening—The technique used for phosphorylation screening was a modification of the method described by Lock et al. (23). Briefly, the Escherichia coli plates of a RBL-2H3 cDNA Agt11 expression library were overlaid for 12–14 h at 37 °C with nitrocellulose filter soaked in 10 mM isopropl 1-thio-β-d-galactopyranoside. The filters were then blocked with blocking solution (20 mM Tris, pH 7.4, 150 mM NaCl, and 3% bovine serum albumin), washed in Triton wash buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, 1 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride), rinsed in kinase reaction buffer (20 mM HEPES pH 7.4, 10 mM MgCl₂, 2 mM MnCl₂, 1 mM Na₃VO₄, 5 mM NaF, 2 mM dithiothreitol, and 0.1% Triton X-100), and incubated for 60 min at 30 °C in kinase reaction buffer containing baculovirus-derived Lyn or Syk, respectively, and 250 μM ATP. After incubation, the filters were washed with kinase wash buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA, 20 mM NaF, and 1 mM Na₃VO₄), and incubated in stripping buffer (62.5 mM Tris, pH 7.4, 2% SDS, 100 mM 2-mercaptoethanol) at 50 °C for 30 min to remove possible associated phosphoproteins, including Lyn or Syk derived from Sf9 cells. The filters were then washed with TBST (20 mM Tris, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) and tyrosine-phosphorylated proteins were detected by immunoblotting with anti-phosphotyrosine monoclonal antibody, 4G10. The positive clones were plaque-purified by successive rounds of phosphorylation screening. The cDNA inserts were amplified by polymerase chain reaction using Tag DNA polymerase HF (Invitrogen) and Agt11 forward and reverse primers, and then subcloned into pCRII TOPO TA cloning vector (Invitrogen) and sequenced.

Phosphorylation Assay of the Fusion Proteins Produced in Agt11 Recombinants—Crude lysates were prepared from Agt11 recombinant lysogens, separated by SDS-PAGE, and electropher-transferred to nitrocellulose membrane filters. After blocking and washing, the membranes were incubated for 60 min at 30 °C in kinase reaction buffer containing 25 μM unlabeled ATP and 1/10 volume of a cell lysate from uninfected Sf9 cells to mask phosphorylation by Sf9 cell-derived kinases. Following washing in kinase reaction buffer without ATP, membranes were incubated for 60 min at 30 °C with gentle shaking in kinase reaction buffer containing 25 μM unlabeled ATP, 5 μCi/ml [γ-32P]ATP, and 1/10 to 1/50 volume of Sf9 cell lysates containing baculovirus-derived Lyn or Syk, respectively. After washing, the signals were visualized by autoradiography.

5′-Rapid Amplification of cDNA Ends (RACE)—The unknown sequence at the 5′-end of rat TOM1L1 was isolated by 5′-RACE (Invitrogen) using the sequence information from the Agt11 clone we identified (clone L6). RNA was isolated from RBL-2H3 cells using an RNeasy Mini Kit (Qiagen, Valencia, CA). First strand cDNA was synthesized using a gene-specific antisense primer, 5′-TGTTGCCCCTAAGAAGTG-3′. Following the addition of homopolymeric tail to the 3′-end of the CDNA, two rounds of PCR were performed. The gene-specific antisense primer, 5′-CCACGGAGAAGATGATCCAC-3′, and AAP primer (Invitrogen) were used for the primary PCR, and the gene-specific antisense primer, 5′-GTTCATTGTCTCAGCATTCAC-3′, and UAP primer (Invitrogen) were used for the secondary (nested) PCR. The 5′-RACE product was subcloned into pCR2.1 (Invitrogen) and sequenced.

Construction of Expression Vectors—The coding region of rat TOM1L1 cDNA was amplified by reverse transcriptase-PCR using RBL-2H3 mRNA. The PCR primers were 5′-TGACCTCGAGCTCTGAGCTACCATGGCGTTG-3′ for the 5′-end, and 5′-TACGATATCCGGATCCATGCCATTGCTTTGAC-3′ for the 3′-end. To add the HA tag sequence to TOM1L1, the PCR product was digested with Xhol and EcoRV and ligated into the pBluescript-HA plasmid. The sequence of the VHS domain (amino acid positions 12–154) or the coiled-coil motif (amino acid positions 260–298) was deleted using a QuickChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). The primers used for deletion were 5′-CCGCGATCTGCTACCCCCCTGAGATGAGG-3′/5′-CTCCATCAAAGGGCTAGGGATGCCGC-3′ for the VHS domain deletion (ΔVHS); and for the coiled-coil motif deletion (ΔCC), the primers were
TABLE 1
cDNA clones identified by expression screening for Lyn substrates

| Clone | ORF (amino acid) | Identity/homology | GenBank accession No. | ORF (amino acid) |
|-------|------------------|-------------------|----------------------|------------------|
| L1<sup>a</sup> | 90 | Hypothetical protein (DKFZp444K1421) | NM_032141 | 558 |
| L2<sup>b</sup> | 444 | SLP-76 SH2 domain-containing leukocyte protein of 76 kDa | U20159 | 533 |
| L6 | 73 | TOM1L1 (target of Myb1-like 1) | AJ100701 | 476 |
| L7<sup>c</sup> (3) | 169 | Sup35p (chromatin structural protein homolog) | U88539 | 1,082 |
| L14 | 25 | MCA-32 (mast cell antigen-32) | U39546 | 268 |

<sup>a</sup> Refers to the open reading frame (ORF) of the cDNA that is expressed in the fusion molecule in Agt11.

<sup>b</sup> L1, -7, or -2 are the same clone as Lyn substrates S6, -9, and -10, respectively.

<sup>c</sup> The number of times each clone was identified by independent screenings is shown in parentheses.

5’-GCTGGTGTTAGGCCGACAGCC-3’/5’-GGCTTCCGCGAACTCCACACGGC-3’. After verification of the constructs by DNA sequencing, the pBluescript plasmids containing the wild-type TOM1L1-HA cDNA or its mutants were digested with XhoI and BamHI, and subcloned into the pSVL expression vector. To construct pEAK expression plasmid, the wild-type TOM1L1-HA and its mutants were amplified by PCR, digested with XhoI and BamHI, and subcloned into the pSVL plasmid constructs by DNA sequencing, the pBluescript plasmids containing the wild-type TOM1L1-HA cDNA or its mutants were amplified by PCR, and inserted into pEAK 12 vector.

Cell Culture, cDNA Transfection, and Cell Activation—COS-1 and RBL-2H3 cells and their transfected cultures were as described previously (34). COS-1 cells were co-transfected with 1.5 μg of pSVL-TOM1L1-HA with the indicated combination of 1.5 μg of pSVL-Lyn or pSVL-Syk plasmids using FuGENE 6 Transfection Reagent (Roche Diagnostics Co.) and analyzed ~48 h post-transfection. The C4A2 Syk-negative variant of the RBL-2H3 cells were stably transfected with pSVL wild-type Syk plus pSV2-neo (Syk) or pSV2-neo only (vector), and stable clones were isolated. For transient transfection, RBL-2H3 cells were transfected with different forms of pEAK-TOM1L1-HA using the Amaxa system. Mast cell activation was induced by incubating cells with IgE and then stimulating with antigen.

Immunoprecipitation and Immunoblotting—For preparing cell lysates, RBL-2H3 cells were first washed with ice-cold phosphate-buffered saline containing 1 mM Na<sub>2</sub>VO<sub>4</sub>, 90 milli-units/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride and then lysed at 4 °C in Triton lysis buffer (1% Triton X-100, 50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA, 100 mM NaF, 10 mM phenylmethylsulfonyl fluoride, 90 milli-units/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A). In COS-1 cells experiments, cells were solubilized with RIPA buffer (Triton lysis buffer containing 1% deoxycholate and 0.1% SDS). Lysates were clarified by centrifugation at 15,000 × g for 15 min. Lysates of S9 cells expressing Lyn or Syk were prepared essentially as described above except that the cells were harvested by centrifugation prior to lysis. For immunoprecipitation, postnuclear supernatants were first precleared by mixing with protein A-agarose beads (Sigma) and then immunoprecipitated with antibodies prebound to protein A-agarose beads. After gentle rotation at 4 °C for 2 h, the beads were washed four times, and then precipitated proteins were eluted by boiling for 5 min with 2× SDS-PAGE sample buffer. In some experiments, total cell lysates were immunoprecipitated with anti-phosphotyrosine antibody 4G10 coupled to agarose beads, and after washing the bound proteins were eluted with 100 mM phenyl phosphate. Total cell lysates and immunoprecipitated proteins were separated by SDS-PAGE, then transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA). The blots were probed with individual primary antibodies, and then incubated with horseradish peroxidase-conjugated donkey anti-mouse or rabbit antibodies. Proteins were visualized by the enhanced chemiluminescence reagent (Renown, PerkinElmer Life Sciences).

Subcellular Fractionation—For the preparation of cytosolic and membrane fractions, cells were washed with ice-cold phosphate-buffered saline and re-suspended in hypotonic buffer (42 mM KCl, 10 mM HEPES, pH 7.4, 5 mM MgCl<sub>2</sub>, 1 mM Na<sub>2</sub>VO<sub>4</sub> and protease inhibitors). Cell homogenates were again centrifuged (10 min at 200 × g), and the supernatants were again centrifuged for 30 min at 100,000 × g (35). Supernatants of the second centrifugation were collected as the cytosolic fraction. The pellet was washed once with hypotonic buffer, then re-suspended in Laemmli sample buffer and collected as the membrane fraction. Equal volumes of the different fractions were subjected to SDS-PAGE and immunoblotted with different antibodies.

Cytokine Measurements—Cells sensitized with IgE were stimulated with or without antigen for 2 h at 37 °C. After stimulation, the supernatants and cells were collected separately, and analyzed for TNFα and MCP1 by specific enzyme-linked immunosorbent assay kits according to the manufacturer’s instruction (BIOSOURCE International, Camarillo, CA).

RESULTS
Identification of Lyn/Syk Substrates—To identify novel substrates of Lyn and Syk, we screened a cDNA expression library for proteins that were tyrosine-phosphorylated in vitro by these kinases. Recombinant proteins expressed in phage plaques of an RBL-2H3 Agt11 expression library were immobilized on nitrocellulose filters, incubated with baculovirally expressed Lyn or Syk, and the tyrosine-phosphorylated proteins were detected with an anti-phosphotyrosine antibody. By this screening, eight clones as potential Lyn substrates (S clones), and five clones as potential Lyn substrates (L clones) were identified (Tables 1 and 2). Conceptual translation showed that each clone, except S15, contained an insert that corresponded to a partial cDNA with an open reading frame. S7 was a part of α-tubulin, which has already been shown to be an in vitro substrate of Syk (21). Several clones were identified as substrates of both Lyn and Syk. For example, the Lyn substrates L1, L7, and L2 were the same as the Syk substrate clones S6, S9, and S10, respectively. S10 (same clone as L2) and S14 were the rat homologues of the adaptor proteins SLP-76 and LAT, respectively, which are known as molecules phosphorylated downstream of Syk (17, 18). S9 (same clone as L7) matches a portion of the chromatin structural protein homologue (Supt5hp). S22 corresponds to the 5′ part of Mlx, a Max-like bHLHZip family tran-
TOM1L1 a Lyn Substrate in FcεRI Signaling

TABLE 2
cDNA clones identified by expression screening for Syk substrates

| Clone | ORF* (amino acid) | Identity/homology | GenBank accession No. | ORF (amino acid) |
|-------|-------------------|-------------------|----------------------|------------------|
| S6    | 90                | Hypothetical protein (DKFZp434K1421) | NM_032141 | 558 |
| S7    | 37                | S-α-Tubulin       | VN1226 | 451 |
| S8    | 136               | Hypothetical protein (FLJ10199) | AK001061 | 253 |
| S9    | (2) 169           | Sup35hp (chromatin structural protein homolog) | U88539 | 1,082 |
| S10   | (6) 444           | SLP-76 SH2 domain-containing leukocyte protein of 76 kDa | U20159 | 533 |
| S14   | (2) 18            | LAT (linker for activation of T cells) | AJ01184 | 242 |
| S15   | 15                | LAP2 (lamina-associated polypeptide 2) 3’ non-coding region | U18314 | 452 |
| S22   | (2) 69            | Mix (max-like bHLHZip protein) | NM_013383 | 244 |

*Refers to the open reading frame (ORF) of the cDNA that is expressed in the fusion molecule in Agt11.
* S6, S9, -9, -10 are the same clone as Lyn substrates L1, L7, and -2, respectively.
* The number of times each clone was identified by independent screenings is shown in parentheses.

Confirmation of Lyn/Syk Substrates by Phosphorylation Assay—Because the baculovirus-infected S9 cell lysates were used as a source of Lyn or Syk in our screening, it was possible that S9-derived kinase, but not Lyn or Syk, might have caused some of these phosphorylations. To identify the protein-tyrosine kinases that induced the phosphorylation of these clones, the phosphorylation assay was performed using the fusion proteins produced from Agt11 recombinants. Crude lysates prepared from the different potential clones were separated by SDS-PAGE, and proteins were transferred to nitrocellulose filters. The membranes were incubated with unlabeled ATP to allow for autophosphorylation, and with lysates of uninfected S9 cells to permit phosphorylation by S9 cell-derived kinases. The filters were then incubated with lysates containing baculoviral expressed Syk or Lyn in the presence of [γ-32P]ATP (Fig. 1). Cdb3, a known substrate of Syk, subcloned into Agt11 vector was included as a control. As shown in the first lane of Fig. 1, Cdb3 was phosphorylated by Lyn, and only slightly by Syk. So the specificity of Syk and Lyn was detected by this assay. Among the three clones identified by both Syk and Lyn in the library screening (Tables 1 and 2), clone S6 (same as L1; a hypothetical protein) was phosphorylated more by Lyn (fifth lane); whereas clones S10 (SLP-76, same as L2) and S9 (Sup35hp, same as L7) were phosphorylated more by Syk (fourth and sixth lanes). Furthermore, clones S7 (α-tubulin) and S15 (LAT), which have been reported to be substrates of Syk, were phosphorylated by both Syk and Lyn (seventh and ninth lanes). On the other hand, the phosphorylation of clones L6 (TOM1L1) and L14 (MCA-32) were mainly by Lyn (second and third lanes). However, clone S8 (a hypothetical protein, eighth lane), was not phosphorylated in this assay with Syk although it had been detected in library screening with Syk. This clone might have been phosphorylated by S9-derived kinase in the library screening.

Isolation of Rat TOM1L1 cDNA—As mentioned above, TOM1L1 was identified as a novel Lyn substrate by the in vitro phosphorylation screening. Based on the sequence information of clone L6, we used the 5’−RACE system to amplify the 5’−end fragment of rat-TOM1L1 from RBL-2H3 mRNA. The full-length cDNA encodes a protein with 474 amino acids, which contains a VHS domain thought to be important for membrane trafficking and a GAT region that is present in the protein family of GGAs (Golgi associated, γ-adaptin ear domain homology, ARF-interacting proteins) (Fig. 2A). The nucleotide sequence of the coding region of rat-TOM1L1 had 83.4% homology with human TOM1L1 cDNA (39). At the protein level, there was 84.1% homology between rat and human TOM1L1; whereas the VHS domain (93.0%) and GAT region (97.4%) have particularly higher homology. Rat TOM1L1 protein contains 11 tyrosine residues, similar to the human and mouse homolog, three of these tyrosines have SH2 domain-recognition motifs, 

Y134L/DFLD for the SH2 (N) of phospholipase C-γ, Y441EVM for the SH2 (N) of p85 subunit of phosphatidylinositol 3-kinase, and Y457EEI for the SH2 of Src family kinases (37).

The endogenous expression of TOM1L1 in RBL-2H3 mast cells was tested by Northern and Western blot analysis. Fig. 2B
shows that using a cDNA probe corresponding to the coding region of rat TOM1L1, the messenger RNA of TOM1L1 was easily detected in RBL-2H3 cells. By using a specific anti-mouse TOM1L1 antibody, TOM1L1 protein (molecular mass at 53 kDa) was detected in both MC9 mouse and the RBL-2H3 rat mast cells (Fig. 2C). This result further confirmed the expression of TOM1L1 in mast cells.

Syk Is Not Required for TOM1L1 Tyrosine Phosphorylation—To examine whether TOM1L1 was phosphorylated by Lyn in vivo, COS-1 cells were co-transfected with HA-tagged TOM1L1 and Lyn and/or Syk (Fig. 3A). The co-expression of TOM1L1 with Lyn, but not Syk, induced significant tyrosine phosphorylation of TOM1L1 (third and fourth lanes). However, the tyrosine phosphorylation of TOM1L1 was enhanced when the molecule was co-transfected with both Lyn and Syk (fifth lane). This result suggested that Lyn is primarily responsible for the tyrosine phosphorylation of TOM1L1, whereas Syk can enhance the Lyn-initiated TOM1L1 phosphorylation.

To examine the function of TOM1L1 in mast cell responses, we investigated whether IgE-receptor stimulation could regulate the tyrosine phosphorylation of TOM1L1. RBL-2H3 cells were activated with IgE plus antigen, and then the tyrosine-phosphorylated proteins were immunoprecipitated by anti-phosphotyrosine antibody 4G10, and analyzed by immunoblotting with anti-TOM1L1 antibody. The results shown in Fig. 3B indicated that TOM1L1 was already tyrosine phosphorylated before receptor stimulation; whereas FcεRI aggregation further enhanced the tyrosine phosphorylation of TOM1L1.

As mentioned before, TOM1L1 was isolated as a substrate of Lyn, but not Syk, in our in vitro screening. However, the results
obtained from COS-1 cell co-expression suggested that Syk could enhance TOM1L1 phosphorylation initiated by Lyn. To further study the role of Syk in TOM1L1 regulation, a Syk-negative variant of the RBL-2H3 cells was stably transfected with wild-type Syk or vector only, and stable cloned lines were isolated (data not shown). Immunoblot analysis of the Syk-reconstituted or vector control lines showed similar expression of TOM1L1 (data not shown). These stably transfected cells were stimulated by IgE plus antigen, and their tyrosine-phosphorylated proteins were immunoprecipitated by 4G10 and blotted by anti-TOM1L1. The results (Fig. 3C) indicated that tyrosine phosphorylation of TOM1L1 was similar among the different Syk reconstituted or vector control lines, which suggested that Syk did not play a role in TOM1L1 tyrosine phosphorylation. Therefore, in the physiologically relevant cells, the tyrosine phosphorylation of TOM1L1 does not require Syk.

TOM1L1 Overexpression Enhanced FcεRI-induced TNFα Release and Synthesis—To study the function of TOM1L1 in FcεRI signaling, RBL-2H3 cells were transiently transfected with HA-tagged wild-type TOM1L1, or its deletion mutants TOM1L1-ΔVHS and TOM1L1-ΔCC (Fig. 4A). Western blotting confirmed that there was similar protein expression after transfection of the different forms of TOM1L1, with ~20-fold overexpression compared with the endogenous level (Fig. 4B and data not shown). These transiently transfected cells were used for the following functional studies.

Degranulation is one of the major functional responses of mast cells to antigen stimulation. Therefore, we compared the FcεRI-induced degranulation in cells transfected by vector only, or by the different forms of TOM1L1. After IgE sensitization the cells were stimulated with concentrations of antigen from 1 to 100 ng/ml. The assays of both histamine and β-hexosaminidase release indicated that overexpression of wild-type TOM1L1 or its deletion mutants TOM1L1-ΔVHS and TOM1L1-ΔCC had no discernible effect on antigen-induced mast cell degranulation (data not shown).

In human primary keratinocytes, TOM1L1 is capable of promoting transcriptional events downstream of the EGF-RAS-MAP kinase pathway (29). Therefore, it is possible that TOM1L1 may play a role in FcεRI-induced cytokine synthesis and release. TNFα and MCP1 assays were performed to test this hypothesis. As showed in Fig. 4C, the overexpression of wild-type TOM1L1 had no effect on the basal release of TNFα, but it did enhance receptor-induced TNFα release when cells were

overexpression of TOM1L1 in RBL-2H3 cells. Total cell lysates (10⁶ cell equivalents/lane) prepared from cells transfected with different forms of TOM1L1, empty vector, or a control vector that expressed an HA-tagged non-related protein were immunoblotted with anti-HA antibody. C, FcεRI-stimulated TNFα release and synthesis. The cells transfected with the indicated constructs were incubated with IgE and stimulated by the different concentrations of antigen for 2 h. The synthesized and released TNFα were assayed by enzyme-linked immunosorbent assay. Data represent the mean ± S.D. from three independent experiments. The paired t test was used for statistical analysis. *, p < 0.05. The averaged values for vector control were as follows: for release, values were undetectable for non-stimulated cells; with 10 ng/ml antigen, values were 146 pg/1 × 10⁶ cells; with 100 ng/ml antigen, values were 80 pg/1 × 10⁶ cells. For the intracellular content, values were 54 pg/1 × 10⁶ cells; with 10 ng/ml antigen, values were 168 pg/1 × 10⁶ cells; and with 100 ng/ml antigen, values were 118 pg/1 × 10⁶ cells.
stimulated with two different concentrations of antigen. In contrast, the mutant TOM1L1, which lacks the VHS domain, had no effect on both basal and antigen-stimulated release of TNFα. In contrast, the overexpression of another deletion mutant, TOM1L1-ΔCC, still enhanced FcεRI-initiated TNFα release; even though this mutant of TOM1L1 was less potent than the native form. The results were different when the intracellular content of TNFα was assayed. In un-stimulated cells, none of the different forms of TOM1L1 had any effect on TNFα generation. However, following antigen stimulation, the cells transfected by wild-type or different mutant TOM1L1 synthesized more TNFα than that of control cells (transfected by vector only). These results indicate that TOM1L1 plays a positive regulatory role on antigen-induced TNFα generation and release, and that the VHS domain is required for TNFα release, but not for synthesis.

The effect of TOM1L1 overexpression on MCP1 was not as dramatic. The overexpression of both wild-type TOM1L1 and the coiled-coil deletion form, but not VHS deletion mutant, increased FcεRI-induced MCP1 generation and release; however, these increases were not statistically significant (data not shown).

**VHS Domain Is Essential for Tyrosine Phosphorylation and Membrane Localization of TOM1L1**—One of the earliest events following FcεRI stimulation is the tyrosine phosphorylation of cellular proteins. Therefore, the effect of different TOM1L1 on receptor-induced protein tyrosine phosphorylation was examined using the anti-phosphotyrosine antibody 4G10. As shown in Fig. 5, overexpressed TOM1L1 was constitutively tyrosine phosphorylated in non-stimulated cells. This strong tyrosine phosphorylation was specific for TOM1L1, because no phosphorylation was detected for an overexpressed non-relevant control protein that contains 7 tyrosine residues (third and fourth lanes, control cells). Compared with the cells transfected by vector only, except for the strong phosphorylation band of overexpressed TOM1L1, the transfection of different TOM1L1 had no noticeable effects on the tyrosine phosphorylation of other cellular proteins before or after FcεRI stimulation. The result shown in Fig. 5 indicated that wild-type TOM1L1 had strong basal phosphorylation, and this was enhanced by receptor stimulation. Further experiments suggested that FcεRI-enhanced TOM1L1 phosphorylation lasted for more than 80 min (data not shown). In contrast, deletion of the VHS domain abolished the basal tyrosine phosphorylation of TOM1L1, and dramatically delayed and reduced the receptor-initiated TOM1L1 phosphorylation. In comparison to the wild-type TOM1L1, the mutant that lacks the coiled-coil region still had basal phosphorylation, but its signal strength was clearly reduced.

VHS domain-containing proteins are often localized to membranes and this domain is thought to play a role in membrane binding. We tested the intracellular localization of TOM1L1 by subcellular fractionation (Fig. 6). In the transfected RBL-2H3 cells, HA-tagged wild-type TOM1L1 was mainly localized in the cytoplasm, with a small portion constitutively associated with the membrane fraction. Antigen stimulation had minimal effects on the intracellular localization of TOM1L1. The ΔCC mutation somewhat reduced the membrane localization of TOM1L1, whereas deletion of the VHS domain totally abolished the ability of TOM1L1 to associate with cell membranes.

**DISCUSSION**

Protein tyrosine phosphorylation is one of the earliest biochemical reactions following FcεRI stimulation. Among the important tyrosine kinases for these phosphorylations are Lyn
TOM1L1 a Lyn Substrate in FceRI Signaling

and Syk. To search for novel substrates of these kinases, a mast cell cDNA expression library was screened by a phosphorylation assay using recombinant baculoviral Syk and Lyn. Five clones as potential Lyn substrates and eight clones as Syk substrates were identified including TOM1L1, a member of a new family of molecules. Full-length cDNA of rat TOM1L1 was isolated and used to express the protein in COS cells and RBL-2H3 mast cells. TOM1L1 was tyrosine phosphorylated by Lyn in COS cells, and was already phosphorylated in non-stimulated RBL-2H3 cells. FceRI aggregation further enhanced tyrosine phosphorylation of TOM1L1. Furthermore, in mast cells, Syk was not required for the tyrosine phosphorylation of TOM1L1 before or after receptor aggregation. The overexpression of wild-type TOM1L1 in RBL-2H3 cells enhanced FceRI-induced TNFα generation and release. The VHS domain, but not the coiled-coil region of TOM1L1 was required for the function of the protein and its membrane localization.

Because protein tyrosine phosphorylation plays a critical role in signal transduction from many receptors, there has been much effort in identifying phosphorylated proteins. Lock et al. (23) screened a 3T3 fibroblast cDNA expression library using a phosphorylation assay to isolate substrates of Src. In the present study, we used a similar method to isolate potential substrates of Syk and Lyn. To increase the possibility of isolating signaling molecules involved in the antigen receptor pathways, we used a mast cell cDNA expression library. There were several proteins identified by screening with Syk/Lyn, such as SLP-76, LAT, and α-tubulin, which had previously been shown to be substrates of these kinases. These results suggest that our screening method is effective for isolating potential substrates of Syk and Lyn.

TOM1L1 is one of the clones that we identified in the phosphorylation screen with Lyn. The gene of TOM1L1 was identified as a human paralog of the avian TOM1 (39). However, the function of this protein is still not very clear. By the yeast two-hybrid interaction screen of a murine keratinocyte library, Seykora et al. (26) found that TOM1L1 interacts with Fyn. In the murine brain, the distribution of TOM1L1 mRNA is correlated with that of Fyn, and the result of co-transfecting Fyn and TOM1L1 suggests that Fyn phosphorylates TOM1L1 (26). Recently, it was observed that EGF receptor stimulation induces the tyrosine phosphorylation of TOM1L1 in keratinocytes. In these cells, overexpression of TOM1L1 activates endogenous Fyn and Src, modulates the activity of p44/42 MAP kinases, and promotes the transcriptional events downstream of the EGF-RAS-MAP kinase pathway involving Elk-1 (29). In the current study, we observed that Lyn, but not Syk, was responsible for phosphorylating TOM1L1. Furthermore, our experiments with RBL-2H3 cells suggests that like the EGF receptor, FceRI stimulation also enhanced the tyrosine phosphorylation of TOM1L1, even though the latter does not possess intrinsic kinase activity.

TOM1L1 contains a VHS domain thought to be important for membrane trafficking and a GAT (GGA and TOM) region, which is also found in the GGA (Golgi-localizing, γ-adaptin ear domain homology, ARF-binding protein) family of proteins. In contrast to GGA proteins, the VHS domain of TOM1L1 does not bind to the ACP (acidic amino acid cluster-LI, (dileucine)) motif found in cargo receptors that cycle between the trans-Golgi network and endosomes, and the GAT domain of TOM1L1 does not bind to ARF. The GAT region of GGA is sufficient to target the reporter green fluorescent protein to the Golgi complex, and to cause dissociation of AP-1 from Golgi complex at high expression levels. However, it has been reported that the GAT domain of TOM1L1 interacts with Tollip (Toll-interacting protein), and also weakly associates with ubiquitin (27). In the current experiments, we observed that in RBL-2H3 mast cells, the deletion of the VHS domain abolished membrane association and the basal tyrosine phosphorylation of this protein. In antigen-stimulated cells, time course experiments showed that the VHS-deleted TOM1L1 was tyrosine phosphorylated at a slower rate and to a lesser extent compared with that of the wild-type molecule. Furthermore, when overexpressed in RBL cells, wild-type, but not the VHS domain mutant TOM1L1, enhanced the antigen-induced TNFα release. In contrast, the deletion of the coiled-coil region that is located in the GAT domain had less effect on the function of TOM1L1. These results suggest that the protein-protein interactions of TOM1L1 play a role in TNFα release from mast cells.

Although the overexpression of wild-type TOM1L1 in RBL-2H3 cells enhanced FceRI-induced TNFα generation and release, there did not appear to be effects when its expression was decreased. Four different TOM1L1 siRNAs were tested in mast cell lines that have an NFAT or NfκB reporter systems. By real time PCR, two of these siRNAs efficiently reduced the TOM1L1 mRNA level to 30–40% of the controls (60–70% knockdown). However, there was no significant change in antigen-induced induction of NFAT or NfκB activation in these cells with decreased TOM1L1 expression. Similarly in other experiments there was no phenotypic changes after siRNA-induced efficient protein knockdown (>60% decrease by immunoblotting) of more than half of the molecules known to be involved in FceRI-signal transduction. However, the lack of a change in phenotype in the siRNA TOM1L1-transfected cells does not necessarily mean that this molecule is not involved in these signaling pathways. It is possible that there is an excess of this targeted protein so that even a decrease to this extent leaves enough to function in these pathways. Alternatively, there could be redundancy of signaling molecules, the function of TOM1L1 being replaced by other proteins with similar function. Therefore, even though the siRNA of TOM1L1 failed to result in a change in phenotype, the strong antigen-induced tyrosine phosphorylation of this molecule, its phosphorylation by Lyn, and the phenotype changes induced by overexpression all suggest a role of TOM1L1 in FceRI signaling.

In conclusion, by phosphorylation screening of a RBL-2H3 mast cell expression library, several potential substrates of Lyn/Syk were identified including TOM1L1, which was phosphorylated primarily by Lyn. In RBL-2H3 cells, TOM1L1 showed strong basal tyrosine phosphorylation, which was further enhanced by FceRI aggregation. The overexpression of wild-type TOM1L1 increased antigen-induced TNFα release and

---

6 J. Zhang, M. Mendoza, and R. P. Siraganian, unpublished observations.
TOM1L1 a Lyn Substrate in FcεRI Signaling

Acknowledgments—We thank Drs. Anna Cristina Grodzki and Kawa Amin for reviewing the manuscript and helpful suggestions. We also thank Greta Bader for histamine analysis and Mary Mendoza for technical support.

REFERENCES

1. Benhamou, M., Gutkind, J. S., Robbins, K. C., and Siraganian, R. P. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 5327–5330
2. Benhamou, M., Ryba, N. J., Kihara, H., Nishikata, H., and Siraganian, R. P. (1993) J. Biol. Chem. 268, 23318–23324
3. Kihara, H., and Siraganian, R. P. (1994) J. Biol. Chem. 269, 22427–22432
4. Minoguchi, K., Benhamou, M., Swaim, W. D., Kawakami, Y., and Siraganian, R. P. (1992) J. Biol. Chem. 267, 16902–16908
5. Joubin, M. H., Adamczewski, M., Numerof, R., Letourneau, O., Vallee, A., and Kinet, J. P. (1994) J. Biol. Chem. 269, 5918–5925
6. Takata, M., Sabe, H., Hata, A., Inazu, T., Homma, Y., Nukada, T., Yamamura, H., and Kurotsuchi, T. (1994) EMBO J. 13, 1341–1349
7. Hitomi, T., Yanagi, S., Inatome, R., and Yamamura, H. (1999) FEBS Lett. 445, 371–374
8. Rawlings, D. J., Scharenberg, A. M., Park, H., Wahl, M. I., Lin, S. Q., Kato, R. M., Fluckiger, A. C., Witte, O. N., and Kinet, J. P. (1996) Science 271, 822–825
9. Kawakami, Y., Kitaura, J., Hartman, S. E., Maeda-Yamamoto, M., Kawakami, T., and Siraganian, R. P. (1999) J. Biol. Chem. 274, 32573–32581
10. Okazaki, H., Zhang, J., Hamawy, M. M., and Siraganian, R. P. (1997) J. Biol. Chem. 272, 32443–32447
11. Teramoto, H., Salem, P., Robbins, K. C., Bustelo, X. R., and Gutkind, J. S. (1997) J. Biol. Chem. 272, 10751–10755
12. Beitz, L. O., Fruman, D. A., Igarashi, T., Cantley, L. C., and Scharenberg, A. M. (1999) J. Biol. Chem. 274, 32662–32666
13. Lubner, M. L., Rao, N., Lill, N. L., Andoniou, C. E., Miyake, S., Clark, E. A., Drucker, R., and Band, H. (1998) J. Biol. Chem. 273, 35273–35281
14. Hendricks-Taylor, L. R., Motto, D. G., Zhang, J., Siraganian, R. P., and Koretzky, G. A. (1997) J. Biol. Chem. 272, 1363–1367
15. Saitoh, S., Arudchandran, R., Manetz, T. S., Zhang, W. G., Sommers, C. L., Love, P. E., Rivera, J., and Samelson, L. E. (2000) Immunity 12, 525–535
16. Yamashita, Y., Fukuda, T., Nishizumi, H., Inazu, T., Higashi, K., Kitamura, D., Ishida, Y., Yamamura, H., Watanabe, T., and Yamamoto, T. (1997) J. Exp. Med. 185, 1387–1392
17. Jabril-Cuenod, B., Zhang, C., Scharenberg, A. M., Paolini, R., Numerof, R., Beaven, M. A., and Kinet, J. P. (1996) J. Biol. Chem. 271, 16268–16272
18. Goitsuka, R., Kanazashi, H., Sasunuma, H., Fujimura, Y., Hidaka, Y., Tatsu, A., Ra, C., Hayashi, K., and Kitamura, D. (2000) Int. Immunol. 12, 573–580
19. Latour, S., Fourmell, M., and Veillette, A. (1997) Mol. Cell. Biol. 17, 4434–4441
20. Peters, J. D., Furlong, M. T., Asai, D. J., Harrison, M. L., and Geahlen, R. L. (1996) J. Biol. Chem. 271, 4755–4762
21. Larbolette, O., Wollscheid, B., Schweikert, L., Nielsen, P. J., and Wienands, J. (1999) Mol. Cell. Biol. 19, 1539–1546
22. Lock, P., Abram, C. L., Gibson, T., and Courtenide, S. A. (1998) EMBO J. 17, 4346–4357
23. Ohk, O., and Lehto, V. P. (1998) FEBS Lett. 440, 255–257
24. Ohk, O., Poussu, A., Mao, Y., Quioco, F., and Lehto, V. P. (2002) FEBS Lett. 513, 19–23
25. Seykora, J. T., Mei, L., Dotto, G. P., and Stein, P. L. (2002) J. Biol. Chem. 277, 2812–2822
26. Katoh, Y., Shiba, Y., Mitsuhashi, H., Yanagida, Y., Takatsu, H., and Nakayama, K. (2004) J. Biol. Chem. 279, 24435–24443
27. Puettitano, R. (2005) J. Biol. Chem. 280, 9258–9264
28. Li, W., Marshall, C., Mei, L., Dzubow, L., Schmuits, C., Dans, M., and Seykora, J. (2005) J. Biol. Chem. 280, 6036–6046
29. Katoh, Y., Imakagura, H., Futatsumori, M., and Nakayama, K. (2006) Biochem. Biophys. Res. Commun. 341, 143–149
30. Franco, M., Furstost, O., Simon, V., Benistant, C., Hong, W. J., and Roche, S. (2006) Mol. Cell. Biol. 26, 1932–1947
31. Nishikata, H., Oliver, C., Mergenhagen, S. E., and Siraganian, R. P. (1992) J. Immunol. 149, 862–870
32. Basciano, L. K., Berenstein, E. H., Mkak, L., and Siraganian, R. P. (1986) J. Biol. Chem. 261, 11823–11831
33. Zhang, J., Berenstein, E. H., Evans, R. L., and Siraganian, R. P. (1996) J. Exp. Med. 184, 71–79
34. Deckert, M., Tarte-Deckert, S., Hernandez, J., Rottapel, R., and Haltman, A. (1998) Immunity 9, 595–605
35. Billin, A. N., Eilers, A. L., Queva, C., and Ayer, D. E. (1999) J. Biol. Chem. 274, 36344–36350
36. Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., Neel, B. G., Birge, R. B., Fajardo, J. E., Chou, M. M., Hanafusa, H., Schaffhausen, B., and Cantley, L. C. (1993) Cell 72, 767–778
37. Songyang, Z., Shoelson, S. E., McClade, J., Oliver, P., Pawson, T., Bustelo, X. R., Barbacid, M., Sabe, H., Hanafusa, H., Yi, T., Ren, R., Baltimore, D., Ratnofsky, S., Feldman, R. A., and Cantley, L. C. (1994) Mol. Cell. Biol. 14, 2777–2785
38. Seroussi, E., Kedra, D., Cost-Alimova, M., Sandberg-Nordqvist, A. C., Fransson, I., Jacobs, J. F., Fu, Y., Pan, H., Qi, B., and Cantley, L. C. (2006) J. Biol. Chem. 281, 28125–28132
39. Pirozzi, G., Terry, R. W., Epstein, D., and Labow, M. A. (1995) J. Immunol. 155, 5811–5818