Adaptor Protein 3BP2 Is a Potential Ligand of Src Homology 2 and 3 Domains of Lyn-Protein-tyrosine Kinase*

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Koichiro Maeno, Kiyonao Sadaë, Shinkou Kyo, S. M. Shahjahan Miah, Keiko Kawachi-Kamata, Xiujuan Qu, Yuhong Shi, and Hirobei Yamamura

From the Division of Proteomics, Department of Genome Sciences, Kobe University Graduate School of Medicine, 7-5-1 Kusanoki-cho, Chuo-ku, Kobe 650-0017, Japan

Adaptor protein 3BP2, a c-Abl-Src homology 3 (SH3) domain-binding protein, is known to play a regulatory role in T-cell receptor-mediated transcriptional activation of nuclear factor of activated T cell and activator protein 1 by interacting with Syk/ZAP-70 protein-tyrosine kinase. We have previously demonstrated that aggregation of high affinity IgE receptor (FceRI) induces tyrosine phosphorylation of 3BP2, and overexpression of the 3BP2-SH2 domain suppresses antigen-induced degranulation in rat basophilic leukemia RBL-2H3 mast cell line. In this report, we attempt to analyze the biological relevance of 3BP2 tyrosine phosphorylation. By using the transient expression system in COS-7 cells, we have demonstrated that 3BP2 was predominantly phosphorylated on Tyr174, Tyr183, and Tyr446 when it was coexpressed with Syk. An in vitro binding study revealed that phosphorylation of Tyr446 by Syk was likely to create a binding site for the Lyn-SH2 domain in RBL-2H3 cells. In addition, proline-rich region of 3BP2 bound to the Lyn-SH3 domain. Conformational microscopic analysis showed that Lyn and 3BP2 are constitutively colocalized in RBL-2H3 cells. Overexpression of 3BP2 in RBL-2H3 cells resulted in an enhancement of Lyn autophosphorylation. These results suggest that the adaptor protein 3BP2 is a potential regulator of Lyn protein-tyrosine kinase as a ligand of its SH3/SH2 domains in FceRI-mediated signaling in mast cells.

Adaptor protein 3BP2 was originally isolated as a protein-tyrosine kinase c-Abl-Src homology 3 (SH3)1 domain-binding protein of unknown function (1). 3BP2 was also identified as a Syk kinase-interacting protein by the yeast two-hybrid screening (2). Transient overexpression of 3BP2 resulted in a transcriptional activation of nuclear factor of activated T cell and activator protein 1, which is induced by T-cell receptor aggregation. Ser225 and Ser277 of 3BP2 were identified as essential sites for interacting with 14-3-3 to negatively regulate the function of 3BP2 in lymphocytes (3). Moreover, infection of 3BP2 wild type into NK cells by vaccinia virus enhanced cell cytotoxicity. An in vitro binding study suggested that phosphorylation of Tyr183 of 3BP2 could be associated with Vav and phospholipase C-γ (4). In mast cells, overexpression of the 3BP2-SH2 domain suppressed high affinity IgE receptor (FceRI)-mediated tyrosine phosphorylation of phospholipase C-γ, Ca2+ mobilization, and degranulation (5). These findings have demonstrated that 3BP2 plays a critical role in hematopoietic cells.

To propagate the immunoreceptor signal, adaptor proteins contribute to protein-protein and protein-lipid interactions through multiple domains and/or specific phosphotyrosine-containing sequences. Tyrosine phosphorylation of 3BP2 was observed in NK cells and mast cells by cross-linking FcεRI and FceRI, respectively (4, 5). To elucidate the function of 3BP2, it is necessary to determine the protein-tyrosine kinase (PTK) that phosphorylates 3BP2 and its binding partner to assemble a signaling complex through specific phosphotyrosine-containing motifs in 3BP2.

The Src family PTK Lyn is associated with FceRIβ. Upon aggregation of FceRI, Lyn is critical for phosphorylating FceRIβ and β subunits on Tyr residues within the immunoreceptor tyrosine-based activating motif (ITAM) (6–8). By analogy with studies on Hck, Lyn is thought to be activated by the disassembly of the closed intramolecular interaction by (i) CD45-mediated dephosphorylation of C-terminal regulatory Tyr residue, (ii) binding to SH3 and SH2 ligands, and (iii) autophosphorylation of Tyr in the activation loop (9). What is the binding ligand of the SH3 and SH2 domains of Lyn in FceRI signaling pathway? Pull-down experiments using glutathione S-transferase (GST)-Lyn-SH2 fusion protein indicated that there were multiple phosphoproteins interacting with the Lyn-SH2 after the antigen stimulation of RBL-2H3 cells (10). In addition, although the displacement of intramolecular SH3 interaction is not well understood, it seems likely that some aggregation-induced change in an associated molecule provides a higher affinity SH3 ligand that binds to the Lyn-SH3 domain (11). The SH3 domain is directed toward the proline-rich region, but such a ligand has not been identified yet in the FceRI signaling.

We isolated nonreceptor type PTK Syk from porcine spleen (12). Syk is expressed in hematopoietic, epithelial, and endothelial cells (13–16). When the ITAM of FceRIβ subunits is phosphorylated by Lyn, Syk is recruited to the plasma membrane by binding its tandem SH2 domain and is autophosphorylated (17). Syk has multiple autophosphorylation sites. Tyr317, Tyr442, and Tyr546 are located in the linker region of Syk; Tyr519 and Tyr520 are in the activation loop of the kinase.
domain; and Tyr^624 and Tyr^625 are in the C-terminal region (18). Phosphorylation of Tyr^119 and Tyr^120 is critical for the enzymatic activation of Syk (19). Another member of the Syk family PTKs, ZAP-70, has a similar structural feature. However, there are differences in the binding molecules and mechanism of enzymatic activation between Syk and ZAP-70 (13, 14).

In the present study, we provide evidence that Tyr^174, Tyr^183, and Tyr^466 on 3BP2 are phosphorylated by a nonreceptor type PTK, Syk. Phosphorylation of 3BP2 at Tyr^466 creates a binding site for the Lyn-SH2 domain in vitro. Additional interaction between the proline-rich region and Lyn-SH3 may contribute to the constitutive codistribution of both molecules in RBL-2H3 cells. Furthermore, overexpression of 3BP2 resulted in an enhancement of antigen-induced Lyn autophosphorylation in RBL-2H3 cells. On the other hand, overexpression of 3BP2-SH2 resulted in a suppression of Lyn autophosphorylation. Our results suggest that 3BP2 is a potential ligand of Lyn-SH3/SH2 domains that positively regulates the autophosphorylation of Lyn in mast cells. Here, we are proposing the model of a novel Lyn-activating cycle in mast cells.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Protein A-agarose and mouse monoclonal anti-dinitrophenyl IgE (anti-DNP IgE, clone SPE-7) were purchased from Sigma. Anti-phosphotyrosine mAb 4G10 was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-hemagglutinin epitope (HA), anti-3BP2, anti-Syk, and anti-Lyn antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-T7-Tag mAb was from Novagen (Madison, WI). Anti-Pyk2 mAb was from BD Biosciences (San Jose, CA). Anti-porcine Syk antibody was generated as described previously (20). Horseradish peroxidase-conjugated secondary antibodies were from Bio-Rad. Antigen 2, 4-dinitrophenylated bovine serum albumin (BSA) from Sigma. Anti-phosphotyrosine mAb 4G10 was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-dinitrophenyl IgE (anti-DNP IgE, clone SPE-7) were purchased from LSL (Tokyo, Japan) (21).

Construction of cDNA—The HA-tagged expression construct of mouse 3BP2 in pMT3 vector (pMT3-HA-3BP2) was kindly provided by Dr. A. Altman (La Jolla Institute). Point mutations of Tyr174, Tyr183, and Tyr466 of pMT3-HA-3BP2 cDNA to Phe were generated by the site-directed mutagenesis kit (Stratagene, La Jolla, CA). The loss of function mutation of the 3BP2-SH2 domain (Arg^466→Lys) was described previously (5). A loss-of-association mutation (deletion of proline-rich region plus Y446F mutation) was created by PCR. The wild type and mutants of 3BP2 used in this study were shown in Fig. 1, A and B. All mutations were verified by DNA sequencing. Expression constructs of porcine Syk wild-type, kinase-inactive mutant (Lys^396→Arg), and SH2 domain deletion mutant that lacks two SH2 domains and interdomain A (Phe^154→Ala^273) (Syk^ΔSH2) were described previously (20). The cDNAs encoding Lyn, HA-3BP2-SHI3 SH2 domains were maintained as monolayer cultures in DMEM (Sigma) with 10% (v/v) fetal bovine serum. For immunoblotting, samples were separated by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (0.45-μm pore size; Millipore Corp., Bedford, MA). The membranes were incubated with 5% milk in TBST (25 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature. Then membranes were washed once with TBST and incubated with the indicated primary antibodies in TBST for 1 h at 4 °C. After washing four times in TBST, the membranes were incubated with appropriate secondary antibodies (1:5000) for 30 min at room temperature and washed extensively four times in TBST. In all blots, proteins were visualized by the enhanced chemiluminescence (PerkinElmer Life Sciences).

In Vitro Protein Kinase Assay—The washed anti-Lyn immunoprecipitates were incubated in Triton X-100 lysis buffer, and the cell lysates were clarified by the centrifugation. Resulting supernatants were transferred into pSVL expression vector (Amersham Biosciences). pB-tyro-Tagged Btk was gifts from Dr. Kuroski (Kansai Medical University, Japan), and CDNA encoding human Syk was from Dr. B. Mueller-Hilke (Deutsches RheumaForschungs-Zentrum) and subcloned into pSVL expression vector. Cell Culture, Transfection of cDNA, and Cell Activation—Rat basophilic leukemia RBL-2H3 cells and COS-7 cells were maintained as monolayers cultures in DMEM (Sigma) with 10% (v/v) FCS and 10% (v/v) heat-inactivated fetal calf serum. HA-3BP2 wild type (WT) cDNA was stably transfected into RBL-2H3 cells by the LacSwitch II inducible mammalian expression system (Stratagene). Briefly, 40 μg of pMT3-HA-3BP2 cDNA was electroporated into 5 × 10^6 cells by electroporation (950 microfarads, 310 V) using Gene Pulser II (Bio-Rad). Cells resistant to 0.7 mg/ml of hygromycin B and 0.2 mg/ml of active G418 were selected with 0.7 mg/ml hygromycin B and 0.2 mg/ml of active G418. Parental cells by electroporation (950 microfarads, 310 V). Cells were sensitized with anti-DNP IgE (1:5000) and further incubated for 24 h (total of 48 h with IPTG) at 37 °C. Cells were washed twice with ice-cold PBS twice and solubilized in Triton X-100 lysis buffer (1% Triton X-100, 50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA, 100 mM NaF, 1 mM Na_2VO_4, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml aprotinin). Cells were preclayed by the centrifugation, and supernatants were incubated with the indicated antibody prebound to protein A-agarose beads. After rotation for 1 h at 4 °C, the beads were washed four times with the lysis buffer. Immunoprecipitated proteins were eluted by heat treatment at 100 °C for 5 min with 2× sample buffer. Aliquots of the supernatant of first centrifugation were used as detergent-soluble cell lysates.

For immunoblotting, samples were separated by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (0.45-μm pore size; Millipore Corp., Bedford, MA). The membranes were incubated with 5% milk in TBST (25 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature. Then membranes were washed once with TBST and incubated with the indicated primary antibodies in TBST for 1 h at 4 °C. After washing four times in TBST, the membranes were incubated with appropriate secondary antibodies (1:5000) for 30 min at room temperature and washed extensively four times in TBST. In all blots, proteins were visualized by the enhanced chemiluminescence (PerkinElmer Life Sciences).

Pull-down Experiments Using GST Fusion Proteins—The GST-rat Lyn-SH2 domain expression construct was kindly provided by Dr. Heuben P. Siraganian (10). The Lyn-SH3 domain was amplified by PCR, using the primer 5′-GGATCCAGGAGGAAGGACATT-GTG-3′ and 5′-GGCGCGTCGAC/GCTTCGTTCTAAGGTGTT-3′ (the restriction enzyme digestion sites are underlined). The pGEX PCR product was subcloned into the pGEX-4T-3 (Amersham Biosciences) to make a domain in-frame with upstream GST. The GST-fusion protein-expressing constructs were solubilized in Triton X-100 lysis buffer, and the cell lysates were cleared by the centrifugation. Resulting supernatants were incubated with glutathione-Sepharose 4B beads (Amersham Biosciences) for 1 h at 4 °C. The beads were washed four times with prechilled PBS buffer, and precipitated proteins were eluted by heat treatment at 100 °C for 5 min with 2× sample buffer.
with PBS, fixed with 4% paraformaldehyde in PBS for 10 min, and permeabilized with 0.2% Triton X-100 in PBS for 10 min. Then cells were washed four times for 2 min in PBS and blocked with 3% BSA in PBS. For double staining, cells were reacted with rabbit anti-Lyn antibody and anti-HA mAb (Covance, Princeton, NJ) for 1 h, washed three times with 0.5% Triton X-100 in PBS, and then incubated with the secondary antibody (Alexa Fluor 488 goat anti-mouse IgG (green) and Alexa Fluor 594 goat anti-rabbit IgG (red) (Molecular Probes, Inc., Eugene, OR) for 30 min. After the extensive washing, cells were mounted using SlowFade-Light (Molecular Probes) and analyzed by using confocal imaging system LSM5 Pascal (Carl Zeiss, Jena, Germany) (22).

RESULTS

Tyrosine Kinase Syk Phosphorylated Tyr174, Tyr183, and Tyr446 in 3BP2.—We previously reported the FcεRI-mediated tyrosine phosphorylation of the adaptor protein 3BP2 in RBL-2H3 mast cells (5). 3BP2 is rapidly tyrosine-phosphorylated and reached a peak 1 min after stimulation, suggesting that proximal nonreceptor type PTKs may contribute to tyrosine phosphorylation of 3BP2. In COS-7 cells, 3BP2 was tyrosine-phosphorylated by Syk, Lyn, or Btk among PTKs known to be activated after FcεRI aggregation in mast cells (Fig. 1C). In particular, 3BP2 was predominantly phosphorylated by Syk, which is known to play an essential role in B-cell development and Fc receptor-mediated activation of hematopoietic cells (13, 14). Based on this finding, we attempted to identify the tyrosine residues in 3BP2 phosphorylated by Syk. Previously, the phage display study showed that Syk prefers the Asp-Tyr-Glu (DYE) sequence in its substrate (23). 3BP2 has two tyrosines, Tyr174 and Tyr446, located in the DYE sequence (Fig. 1). In addition, an in vitro binding study suggested that phosphorylation of Tyr183 located in Tyr-X-X-Pro motif creates a binding site for the Vav-SH2 domain (Fig. 1) (4). Therefore, we generated a series of 3BP2 mutations in which each Tyr was substituted to Phe (Fig. 1B). Substitution of all three Tyr residues to Phe (3BP2–3F) abrogated Syk-mediated phosphorylation in COS-7 cells, suggesting that some of those residues are the phosphorylation sites by Syk (Fig. 1D, lanes 3 and 4). Compared with 3BP2–3F, the 3BP2 mutants in which two Tyr residues were substituted for Phe (174Y, 183Y, and 446Y) were almost tyrosine-phosphorylated by Syk in COS-7 cells (Fig. 1D, lanes 5–7). This indicates that Syk phosphorylates all three Tyr residues in 3BP2. The coexpression of 3BP2 with Lyn or Btk resulted in a weak tyrosine phosphorylation of 3BP2 (Fig. 1C). Similar to Syk, Lyn or Btk could phosphorylate Tyr174, Tyr183, and Tyr446 in COS-7 cells (data not shown). Expressions of the individual PTKs and 3BP2 mutants were confirmed by the immunoblotting (Fig. 1, C and D). These results demonstrate that Tyr174, Tyr183, and Tyr446 in 3BP2 are the putative tyrosine phosphorylation sites by nonreceptor type PTKs, in particular Syk.

3BP2–Tyr446 Was a Major Binding Site to the Lyn-SH2 Domain.—To examine the role of 3BP2 tyrosine phosphorylation, we utilized the RBL-2H3 cells in which the expression of HA-3BP2 is induced by the addition of IPTG. As we reported previously, aggregation of FcεRI induced a rapid tyrosine phosphorylation of 3BP2 in RBL-2H3 cells (Fig. 2A) (5). Phosphorylation of 3BP2 was visible at 30 s after antigen stimulation and sustained to 10 min. To identify the binding molecule(s) of 3BP2, pull-down experiments were carried out by using various GST-SH2 fusion proteins against mast cells expressing HA-3BP2 (data not shown). Among them, GST-Lyn-SH2 reacted with HA-3BP2 from cell lysates from antigen-stimulated cells, suggesting that antigen-induced tyrosine phosphorylation of 3BP2 creates the binding site for Lyn-SH2 in mast cells (Fig. 2B).

To examine the mechanism of this interaction, a reconstitution system using COS-7 cells was utilized by transient transfection of cDNA. 3BP2-WT, but not 3BP2–3F, was bound to Lyn-SH2 when it was coexpressed with Syk (Fig. 2C, lane 2 versus lane 4). Phosphorylation of 3BP2 by Lyn could not induce enough interaction with Lyn-SH2 in COS-7 cells, due to the low magnitude of 3BP2 tyrosine phosphorylation as shown in Fig. 1C (Fig. 2C, lane 6). Based on these observations, we then attempted to identify the corresponding Tyr residue(s) that associated with Lyn-SH2 by using this system. The 3BP2–WT, 3BP2–3F, and 3BP2 mutants in which two Tyr residues were substituted for Phe (174Y, 183Y, and 446Y) (Fig. 1B) was cotransfected with Syk, and each cell lysate was incubated with GST-Lyn-SH2 (Fig. 2D). Among three Tyr residues, the binding of 3BP2–446Y for Lyn-SH2 was similar to that of wild type (Fig. 2D, lane 2 versus lane 10). 3BP2–183Y caused a weak binding with Lyn-SH2, and 3BP2–174Y did not associate with Lyn-SH2 (Fig. 2D, lanes 8 and 6, respectively). This result indicates that phosphorylation of Tyr446 by Syk creates binding site for Lyn-SH2. For further confirmation, another pull-down experiment was performed. 3BP2–WT, 3BP2–3F, or 3BP2 mutants in which one Tyr residue was substituted for Phe (Y174F, Y183F, Y446F) (Fig. 1B) was cotransfected with Syk, and each cell lysate was incubated with GST-Lyn-SH2 (Fig. 2E). Compared with the 3BP2–WT, expression of 3BP2 Y446F with Syk resulted in a decreased interaction with Lyn-SH2 (Fig. 2E, lane 2 versus lane 10). A point mutation of Tyr174 or Tyr183 into Phe did not decrease binding with Lyn-SH2 (Fig. 2E, lanes 6 and 8). In the experiments using COS-7 cells, the expression amounts of PTK and the different kinds of 3BP2 were confirmed by the immunoblotting of cell lysates (Fig. 2, C–E, right panel). Therefore, these results suggest that antigen stimulation induces tyrosine phosphorylation of 3BP2, causing the interaction with Lyn. Moreover, among three putative tyrosine phosphorylation sites in 3BP2, phosphorylation of Tyr446 seemed to be the major site contributing to the interaction with Lyn-SH2 upon FcεRI aggregation in mast cells.

Phosphorylation of Tyr446 but Not the SH2 Domain of 3BP2 Contributed to the Interaction of Lyn with Syk.—GST-Lyn-SH2 binds to Syk in antigen-stimulated RBL-2H3 mast cells (10). A pull-down experiment demonstrated that Syk was precipitated with GST-Lyn-SH2 when it was coexpressed with 3BP2–WT in COS-7 cells (Fig. 3A, lane 2). Kinase-inactive form of Syk was poorly precipitated with Lyn-SH2 (Fig. 3A, lane 3). Also, a point mutation of Tyr446 of 3BP2 caused the same result, suggesting that phosphorylation of Tyr446 of 3BP2 by Syk is required for the full-scale binding of Syk with Lyn-SH2 (Fig. 3A, lane 4). Thus, 3BP2 is necessary for Syk to associate with Lyn-SH2 in COS-7 cells, because Syk alone could not phosphorylate the corresponding Tyr residue to interact with Lyn-SH2, unlike in antigen-stimulated mast cells (Fig. 3A, lane 1) (10). Interestingly, a point mutation of Arg486 into Lys in 3BP2–SH2 had no effect on the association with Syk, although 3BP2–SH2 was shown to directly associate with Syk in yeast (Fig. 3A, lane 5) (2).

Truncation of the SH2 domain of Syk resulted in an increase in the kinase activity and tyrosine phosphorylation of Syk (24). Unlike the wild type, the SH2 domain-truncated form of Syk (SykΔSH2) was capable of associating with Lyn-SH2, independent of 3BP2 tyrosine phosphorylation (Fig. 3B, lanes 1 and 2). The multiple bands of SykΔSH2 seem to be due to its autophasphorylation, since SykΔSH2 possesses seven putative autophasphorylation sites (Tyr317, Tyr342, Tyr346, Tyr319, Tyr520, Tyr525, and Tyr529) (3F). 3BP2, lanes 1 and 2, lower panel) (18, 25). This suggests that Lyn-SH2 could bind to the autophasphorylation site of SykΔSH2 in COS-7 cells. To interact with Lyn-SH2, wild type Syk requires 3BP2; however, Syk with multiple phosphorylation does not require 3BP2. The expres-
sion amounts of Syk, 3BP2, and their mutants were confirmed by the immunoblotting of cell lysates (Fig. 3. A and B, right panel). Therefore, these results suggest that 3BP2 stimulates tyrosine phosphorylation of Syk, indirectly or directly. Phosphorylation of Tyr446 is critical for this hypothetical function of 3BP2.

**Proline-rich Region of 3BP2 Associated with Lyn-SH3 Domain**—3BP2 was first isolated as a c-Abl-SH3 domain-binding protein via its proline-rich region (1). Also, 3BP2 was capable of binding to SH3 domain of Grb2, Nck, Src, or Fyn (1, 2). Since we have demonstrated that 3BP2 binds to the Lyn-SH2 domain, we then tested whether the SH3 domain of Lyn could interact with 3BP2. The binding of 3BP2 with the Lyn-SH3 domain was observed when GST-Lyn-SH3 domain reacted with cell lysates from both unstimulated and antigen-stimulated mast cells expressing HA-3BP2 (Fig. 4A). The expression of HA-3BP2 was confirmed by the immunoblotting of cell lysates (Fig. 4A, bottom panel). To examine the mechanism of this interaction, a reconstitution system using COS-7 cells was utilized. Whereas the Lyn-SH2 bound to 3BP2 when it was
coexpressed with Syk, Lyn-SH3 could bind to 3BP2 without phosphorylation by Syk (Fig. 4B). Substitution of Tyr446 did not affect the interaction of both molecules. The interaction between 3BP2 and Lyn-SH3 was dramatically decreased by the deletion of the proline-rich region in 3BP2 (Fig. 4C). 3BP2 with the deletion of the proline-rich region and a Y446F mutation (H9004 Pro Y446F) was poorly bound to Lyn-SH3. Therefore, this result indicated that proline-rich region could associate with Lyn-SH3 domain. The expression amounts of Syk and the different kinds of 3BP2 were confirmed by the immunoblotting of cell lysates (Fig. 4, B and C, right panel). Similarly, both Lyn-SH2 and SH3 domains bound to the endogenous 3BP2 IPTG for 48 h to induce the expression of HA-3BP2 were sensitized with anti-DNP IgE and then stimulated by 30 ng/ml antigen (Ag) DNP-BSA for the indicated times. A, cells were solubilized in 1% Triton X-100 lysis buffer, and cell lysates were immunoprecipitated (IP) with anti-HA antibody. Precipitated proteins were analyzed by immunoblotting (IB) with anti-Tyr(P) (pTyr) mAb and anti-HA antibody. Similar results were obtained when the other clones were examined. B, IPTG-treated (+) or untreated (−) cells were sensitized with anti-DNP IgE. Either unstimulated (−) or antigen-stimulated cells (30 ng/ml DNP-BSA for 1 min) (+) were solubilized in the binding buffer. Cell lysates were re-steamed with either GST or GST-Lyn-SH2 prebound to glutathione-Sepharose 4B beads, and binding proteins were analyzed by immunoblotting with anti-HA antibody. Similar results were obtained when the other clones were examined. C–E, each 1 μg of PTK and various 3BP2 cDNA were cotransfected into COS-7 cells, as indicated. 48 h after transfection, cells were solubilized in the binding buffer. Cell lysates were incubated with either GST or GST-Lyn-SH2 prebound to the beads. Binding proteins and cell lysates were separated by 8% SDS-PAGE and analyzed by immunoblotting with anti-HA antibody.

**Fig. 2.** Phosphorylation of Tyr446 in 3BP2 by Syk created a major binding site to the Lyn-SH2 domain. A, the stably transfected RBL-2H3 cells (5 × 10⁶) pretreated with (+) or without (−) 1 mM IPTG for 48 h to induce the expression of HA-3BP2 were sensitized with anti-DNP IgE and then stimulated by 30 ng/ml antigen (Ag) DNP-BSA for the indicated times. A, cells were solubilized in 1% Triton X-100 lysis buffer, and cell lysates were immunoprecipitated (IP) with anti-HA antibody. Precipitated proteins were analyzed by immunoblotting (IB) with anti-Tyr(P) (pTyr) mAb and anti-HA antibody. Similar results were obtained when the other clones were examined. B, IPTG-treated (+) or untreated (−) cells were sensitized with anti-DNP IgE. Either unstimulated (−) or antigen-stimulated cells (30 ng/ml DNP-BSA for 1 min) (+) were solubilized in the binding buffer. Cell lysates were re-steamed with either GST or GST-Lyn-SH2 prebound to glutathione-Sepharose 4B beads, and binding proteins were analyzed by immunoblotting with anti-HA antibody. Similar results were obtained when the other clones were examined. C–E, each 1 μg of PTK and various 3BP2 cDNA were cotransfected into COS-7 cells, as indicated. 48 h after transfection, cells were solubilized in the binding buffer. Cell lysates were incubated with either GST or GST-Lyn-SH2 prebound to the beads. Binding proteins and cell lysates were separated by 8% SDS-PAGE and analyzed by immunoblotting with anti-HA antibody.

**Fig. 3.** Phosphorylation of 3BP2 stimulated the association of Lyn-SH2 with Syk. A and B, HA-tagged wild-type or various mutant forms of 3BP2 cDNA was cotransfected with porcine Syk wild-type (WT), kinase-inactive form (KI), or SH2 domain-truncated form (SH2) cDNA into COS-7 cells. After 48 h, cells were solubilized in the binding buffer and reacted with GST-Lyn-SH2 prebound to the beads. Binding proteins and cell lysates were separated by 8% SDS-PAGE and analyzed by immunoblotting (IB) with anti-HA and anti-porcine Syk antibodies.
FIG. 4. Interaction of proline-rich region with Lyn-SH3 might contribute their codistribution in RBL-2H3 cells. A, stably transfected RBL-2H3 cells (5 × 10⁶) pretreated with (+) or without IPTG (−) were sensitized with anti-DNP IgE. Unstimulated (−) or antigen-stimulated cells (30 ng/ml DNP-BSA for 1 min) (+) were solubilized in the binding buffer. B and C, COS-7 cells were transfected with Syk and various 3BP2 cDNA, as indicated. 48 h after transfection, cells were solubilized in the binding buffer. A–C, cell lysates were reacted with GST, GST-Lyn-SH2, or GST-Lyn-SH3 prebound to the beads. Binding proteins and cell lysates were analyzed by immunoblotting (IB) with anti-HA and anti-Syk antibodies. D, parental RBL-2H3 cells (2 × 10⁶) were sensitized with anti-DNP IgE. Unstimulated (−) or antigen-stimulated cells (+) were solubilized in the binding buffer, and cell lysates were reacted with GST, GST-Lyn-SH2, or GST-Lyn-SH3 prebound to the beads. Binding proteins and cell lysates were analyzed by immunoblotting with anti-3BP2 antibody. E, immunohistochemical analysis. RBL-2H3 cells expressing HA-3BP2 cDNA were analyzed with anti-Lyn and anti-HA antibodies. Left, localization of 3BP2 (anti-HA, green); middle, Lyn (anti-Lyn, red); right, merged image in unstimulated (top) and antigen-stimulated cells (bottom).
Association of Lyn with 3BP2

(Fig. 4D). The binding of the endogenous 3BP2 with Lyn-SH2 was observed when the cells were stimulated with the antigen, whereas the interaction with Lyn-SH3 domain was also detected both in nonstimulated and stimulated cells. Immunohistochemical analysis by laser confocal microscopy revealed that 3BP2 and Lyn were colocalized in the plasma membrane in unstimulated and antigen-stimulated RBL-2H3 cells (Fig. 4E).

Since 3BP2 was not tyrosine-phosphorylated in unstimulated cells, the interaction of the 3BP2 proline-rich region with Lyn-SH3 may contribute to the colocalization of both molecules in mast cells.

Overexpression of 3BP2-stimulated Autophosphorylation of Lyn—Finally, we attempted to examine the functional role of the interaction of 3BP2 with Lyn. Cells with or without an overexpression of 3BP2 wild type were stimulated with an antigen, and cell lysates were immunoprecipitated with anti-Lyn antibody. Anti-Lyn immunoprecipitates were subjected to an in vitro protein kinase assay. Overexpression of 3BP2 resulted in an increase in the autophosphorylation of Lyn upon antigen stimulation in RBL-2H3 cells (Fig. 5A). The densitometric analysis revealed that IPTG-induced expression of 3BP2 resulted in a 1.7-fold increase in the kinase activity of Lyn after antigen stimulation compared with that of unstimulated cells, whereas the value was 1.2-fold in control cells (Fig. 5A). Furthermore, overexpression of 3BP2-SH2, the dominant-negative form of 3BP2, resulted in the decrease (0.3–0.7-fold) in the autophosphorylation of Lyn (Fig. 5B). Expression of HA-3BP2-WT and HA-3BP2-SH2 was confirmed by the immunoblotting of cell lysates (Fig. 5, A and B, lower panels). Therefore, these results indicated that overexpression of 3BP2-WT enhances the autophosphorylation of Lyn to increase in its kinase activity. Interaction of Lyn with the endogenous 3BP2 positively regulates the kinase activity of Lyn.

DISCUSSION

We previously demonstrated that the SH2 domain of 3BP2 positively regulates FcεRI-mediated tyrosine phosphorylation of phospholipase C-γ, calcium mobilization, and subsequent degranulation in RBL-2H3 cells (5). In this report, we have shown that the proline-rich region and phosphorylated Tyr446 of 3BP2 bound to Lyn-SH3 and -SH2 domains, respectively (Figs. 2 and 4). This suggests that 3BP2 is a ligand of Lyn in FcεRI signaling (Fig. 6A). In unstimulated cells, a proline-rich region may guide 3BP2 close to the target molecule, and then the receptor aggregation creates the additional high affinity docking site by phosphorylating Tyr446. Perhaps a pleckstrin homology domain associates with phosphatidylinositol 3,4,5-trisphosphate in glycolipid-enriched microdomains contributing to the localization of 3BP2 in unstimulated cells. In COS-7 cells, the expression of 3BP2 enhanced Syk tyrosine phosphorylation (Fig. 3A, lanes 1 and 2). One possible explanation is that 3BP2 activates the endogenous Lyn to phosphorylate Tyr446 in the linker region of Syk, which could bind to the Lyn-SH2 in COS-7 cells because orthologous phosphorylation site in ZAP-70 Tyr219 is a binding site for the SH2 domain of Lck, a member of Src family PTKs (26, 27). However, recent findings revealed that phosphorylation of Tyr446 is indispensable for FcεRI-mediated mast cell activation (28). Another possible explanation is that 3BP2 activates Lyn to phosphorylate the endogenous ITAM-bearing protein, which then recruits and activates Syk. Phosphorylation of Tyr219 and Tyr220 in its activation loop of Syk creates the binding sites to the SH2 domain of Lck (29). All together, these observations suggest that 3BP2 is a putative ligand of Lyn-SH3/SH2 domains inducing enzymatic activation of Lyn in RBL-2H3 cells (Fig. 6A). An adaptor protein is thought to function to accumulate signaling molecules by interacting with their signaling domains and specific motifs containing phosphotyrosyl residues. Our present study reveals that an adaptor molecule has a role to induce enzymatic activation of PTKs, presumably by inducing conformational alterations (Fig. 6A).

Aggregation of FcεRI induces activation of Lyn, which then phosphorylates tyrosine residues in the ITAM of FcεRIβ and -γ subunits to recruit and activate downstream Syk. Similarly, genetic studies have demonstrated that an expression of Lyn is necessary to activate Syk to trigger calcium mobilization in avian B-cells (30). These observations indicate that Lyn is upstream of Syk (Fig. 6B, left) (31). It is expected that 3BP2 is the downstream substrate of Syk. When 3BP2 is tyrosine-phosphorylated by Syk, it mediates feedback regulation of the FcεRI-mediated signaling pathway by stimulating or sustaining the kinase activity of Lyn (Fig. 6B, left). Therefore, 3BP2 may function to multiply the antigen-induced mast cell-activating signal. Interestingly, there is an evidence of the alternative activation mechanism of Syk (Fig. 6B, right). Syk is associated with the T-cell receptor prior to the stimulation, and CD3 engagement induces activation of Syk, independent of Lck or CD45 (32, 33). In addition, Syk augments tyrosine phosphorylation of CD3ζ to stimulate the kinase activity of Syk by itself (34). We have demonstrated that 3BP2 is preferentially tyrosine-phosphorylated by Syk in COS-7 cells. Phosphorylation of Tyr446 of 3BP2 by Syk probably functions as a molecular switch to activate Lyn (Fig. 6A). Some of Syk in the different compartmentalized fractions could have a regulatory role for
hematopoietic cells. The present study suggests the existence of the four-step model of Lyn activation cycle shown in Fig. 6C. First, genetic study by Yanagi et al. (36) demonstrated that disruption of the CD45 gene in avian B cells resulted in an increase in phosphorylation of both tyrosine residues in the C-terminal regulatory site and in the activation loop. Kinase activity of Lyn was suppressed in the CD45-negative cells, although both of the tyrosine residues were hyperphosphorylated (36). In mast cells, genetic studies demonstrated that CD45 is not required for FceRI-mediated degranulation (37). These observations indicate that CD45 is a prerequisite for Lyn prior to activation, presumably by dephosphorylation of both autophosphorylation and C-terminal negative regulatory tyrosines to displace an intramolecular interaction (Fig. 6C) (11, 36). Second, we have demonstrated that 3BP2 is a ligand of SH3/SH2 domains of Lyn. Inhibition of the function of Lyn by its SH3 domain binding peptide demonstrates the importance of the Lyn-SH3 domain in mast cell activation (38). Immunohistochemical analysis by laser confocal microscopy reveals that 3BP2 is distributed in the plasma membrane together with Lyn prior to stimulation (Fig. 4E). In the resting state, the Lyn-SH3 domain can be associated with the proline-rich region of 3BP2. It is expected that 3BP2 interacts with the target molecule via its proline-rich region, which allows for the efficient and rapid activation of Lyn upon FceRI aggregation. Third, phosphorylation of Tyr446 might create a high affinity binding site to the Lyn-SH2 domain, and presumably this binding causes the conformational change of Lyn, leading to the autophosphorylation. Syk, Lyn itself, or Btk is a candidate PTK to phosphorylate Tyr446 of 3BP2. Our results suggest the possibility that Lyn could be downstream of some other PTKs at the initial state of mast cell activation. Autophosphorylated Lyn elevates its kinase activity for propagating the downstream signals. Fourth, the activation of Lyn was terminated by the phosphorylation of C-terminal tyrosine by Csk. Lyn with a double tyrosine phosphorylation is inactive (36). Alternatively, some of the activated Lyn was modified by mult ubiquitination by c-Cbl after FceRI stimulation. Taken together, we concluded that 3BP2 might be one of the regulatory factors of the Lyn activation cycle in FceRI signaling.

Although the phenotype of 3BP2 knockout mice has not been reported yet, there is evidence that mutation of the 3BP2 gene cause the human disease cherubism. Cherubism is an autosomal dominant inherited disease characterized by multiple symmetrical cysts in the mandible and the maxilla, excessive bone degradation, and typical facial swelling. The cysts are filled with a fibrous tissue mass containing multinucleated, osteoclastic giant cells. Studies of 12 families of this disease detected a single amino acid mutation in exon nine of the 3BP2 gene (39). 3BP2 could be implicated in the balance of osteoclast and osteoblast activities that are essential for normal tooth eruption. Although the abnormal signals in cherubism have not yet been identified, it is expected that Tyr446-mediated function of 3BP2 could be affected by these mutations, since all mutations identified are located near Tyr446. Although 3BP2 was preferentially detected in spleen, peripheral blood leukocyte, and thymus, the exact role of 3BP2 in hematopoietic/lymphoid cell signaling remains unclear (2, 3). So far, immunological defects due to the mutations of 3BP2 observed in cherubism have not yet been reported.

The present study has demonstrated that 3BP2 interacts with Lyn and may possibly promote the catalytic activity of Lyn. In addition to integrating signaling molecule at the ap-

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*a* S. Kyo, K. Sada, and H. Yamamura, unpublished observation.
propriate location, an “adaptor” protein 3BP2 has a regulatory role in PTK as a ligand binding to the SH3/SH2 domains of Lyn, similar to the function of FceRIα as a ligand of Syk PTK in mast cells.

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Adaptor Protein 3BP2 Is a Potential Ligand of Src Homology 2 and 3 Domains of Lyn Protein-tyrosine Kinase
Koichiro Maeno, Kiyonao Sada, Shinkou Kyo, S. M. Shahjahan Miah, Keiko Kawauchi-Kamata, Xiujuan Qu, Yuhong Shi and Hirohei Yamamura

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