# Tyrosine Phosphorylation of Adaptor Protein 3BP2 Induces T Cell Receptor-Mediated Activation of Transcription Factor†

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ABSTRACT: Molecular adaptors/scaffolds have indispensable roles in the activation of lymphocytes. In this report, we have demonstrated the role of tyrosine phosphorylation of an adaptor protein 3BP2 (c-Abl-SH3 domain binding protein-2, also known as SH3BP2) in T cell receptor (TCR)-mediated activation of transcription factor. Short interfering RNA for 3BP2 suppresses the expression level of endogenous 3BP2 and inhibits TCR-mediated activation of interleukin (IL)-2 promoter and nuclear factor of activated T cells (NFAT) element. Engagement of TCR induces tyrosine phosphorylation and lipid raft translocation of 3BP2. The overexpression studies reveal that substitution of 3BP2-Tyr$^{183}$, Tyr$^{446}$, or Arg$^{486}$ in the SH2 domain suppresses TCR-mediated activation of NFAT. Point mutations of 3BP2 cannot affect the translocation of 3BP2 into the lipid raft. Phosphorylation of Tyr$^{183}$ is required for the interaction with Vav1, the guanine nucleotide exchanging factor of Rac1. In fact, overexpression of dominant-negative form of Rac1 inhibits TCR-mediated activation of NFAT. Phosphorylation of Tyr$^{446}$ recruits the SH2 domain of Lck for the optimal activation of transcription factors. Furthermore, point mutation of Arg$^{486}$ in the 3BP2-SH2 domain that couples ZAP-70 to LAT dramatically reduces NFAT activation. These results suggest that the site-directed functions of 3BP2 induce the activation of transcription factors.

Genetic analysis has revealed that cytoplasmic adaptor proteins, which lack intrinsic enzymatic activities, have indispensable roles in the activation of hematopoietic cells (*1*−*4*). Engagement of T cell receptor (TCR)[1] induces the sequential activation of nonreceptor type protein tyrosine kinases (PTKs) Lck and ZAP-70 that amplify the receptor activating signals in T cells. Binding of Lck to the CD4 and CD8 coreceptors promotes phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) within the ζ subunit of CD3. Dual phosphorylation of ITAM recruits ZAP-70 or related kinase Syk. ZAP-70 is then activated by Lck-mediated tyrosine phosphorylation (*5*). Interaction of T cells with the antigen-presenting cells induces the clustering of TCR and the recruitment of signaling molecules into the lipid raft (*6*, *7*). ZAP-70 phosphorylates the adaptor protein LAT (linker for activation of T cells) to accumulate the signaling molecules such as phospholipase C (PLC)-γ1 in the lipid raft, and to generate $Ca^{2+}$ flux from TCR to the downstream (*8*). Increase in intracellular $Ca^{2+}$ is required to activate the serine/threonine phosphatase calcineurin to dephosphorylate the transcription factor NFAT. Dephosphorylated NFAT translocates to the nucleus, where it participates in interleukin (IL)-2 transcription (*9*). In addition to NFAT, TCR stimulation causes the activation of activator protein 1 (AP-1) and nuclear factor κB (NF-κB), leading to the production of cytokines.

We and others have demonstrated that the adaptor protein c-Abl-Src homology 3 (c-Abl-SH3) domain binding protein-2 (3BP2) is ubiquitously expressed in the human tissues and has a regulatory role in the immune cell responses (*10*−*15*). 3BP2 has the function as a potent inducer of TCR-mediated transcriptional activation of IL-2 promoter and its NFAT and AP-1 elements (*11*). It requires cyclosporin A-sensitive and Ras-dependent signaling pathways. In mast cells, 3BP2 regulates high affinity IgE receptor (FcεRI)-mediated degranulation, suggesting that 3BP2 is a positive regulator of the antigen-induced anaphylaxis (*13*). Furthermore, point mutations of the *3bp2* gene were identified in the human inherited disease cherubism (*16*, *17*). Recently, we have reported that point mutations of 3BP2 in cherubism result in the loss of function (*18*).

3BP2 possesses an N-terminal pleckstrin homology (PH) domain, proline rich regions, and a C-terminal SH2 domain. Engagement of Fc receptors induces tyrosine phosphorylation of 3BP2 (*12*, *13*). A nonreceptor type of PTK Syk could phosphorylate Tyr$^{174}$, Tyr$^{183}$, and Tyr$^{446}$ in 3BP2 (*15*). Phosphorylation of Tyr$^{183}$ in 3BP2 seems to be involved in the cytotoxicity through the interaction with Vav1 and PLC-

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[1] Abbreviations: 3BP2, c-Abl-SH3 domain binding protein-2; TCR, T cell receptor; IL, interleukin; NFAT, nuclear factor of activated T cells; PTKs, protein-tyrosine kinases; LAT, linker for activation of T cells; PLC, phospholipase C; SH, Src homology; FcεRI, high affinity IgE receptor; PH, pleckstrin homology; siRNA, short interfering RNA; HA, hemagglutinin epitope; PMA, phorbol 12-myristate 13-acetate; GST, glutathione S-transferase; WT, wild type.

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$\gamma$1 in natural killer cells (*12*). Proline rich regions and phosphorylated $Tyr^{446}$ in 3BP2 bind to the SH3 and SH2 domains of Lyn to stimulate its kinase activity (*15*). The SH2 domain of 3BP2 binds to LAT, which is the essential molecule to regulate the activation of PLC-$\gamma$ in mast cells (*13*, *19*). Overexpression of the SH2 domain of 3BP2 results in the inhibition of Fc$\epsilon$RI-mediated tyrosine phosphorylation of both PLC-$\gamma$1 and -$\gamma$2, $Ca^{2+}$ mobilization, and degranulation (*13*). These findings suggest that the SH2 domain of 3BP2 regulates $Ca^{2+}$-mediated signals from the immune receptors.

In this report, we have identified an essential role of 3BP2 in TCR-mediated activation of NFAT by utilizing the short interfering RNA (siRNA). Overexpression studies reveal that the specific phosphorylation sites or the SH2 domain of 3BP2 appears to play the critical role in TCR-mediated activation of transcription factor and cytokine gene transcription.

## MATERIALS AND METHODS

*Reagents and Antibodies.* Anti-3BP2, anti-Vav1, and anti-Lck antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CD3 mAb (UCHT1) was obtained from BD Biosciences (San Jose, CA). The horseradish peroxidase-conjugated mouse anti-phosphotyrosine (pTyr) mAb (4G10) and anti-Rac1 mAb were from Upstate Biotechnology (Lake Placid, NY). Sheep polyclonal anti-3BP2 antibody was from Exalpha Biologicals (Watertown, MA) and utilized for the immunoprecipitation of endogenous 3BP2. Anti-hemagglutinin epitope (HA) mAb was from Covance (Princeton, NJ). Phorbol 12-myristate 13-acetate (PMA) was from Sigma (St. Louis, MO). Jurkat T lymphocytes expressing SV40 large T antigen (Jurkat-TAg cells) were maintained in RPMI 1640 medium (Sigma) containing 10% fetal calf serum. Human peripheral blood lymphocytes were isolated by Ficoll−Paque lymphocyte isolation solution (Amersham Biosciences, Piscataway, NJ) from venous blood obtained from healthy volunteers. Primary lymphocytes were washed with PBS twice and suspended in serum-free RPMI 1640 medium prior to cell activation. The expression construct of pMT3-HA-3BP2 was a gift from Dr. Amnon Altman (La Jolla Institute, La Jolla, CA). HA tag was added to the N-terminal of 3BP2 in the pMT3 expression vector. All of the mutations in 3BP2 cDNA were generated by PCR (*15*). Reporter plasmid pIL-2-luc was a gift from Dr. Sho Yamasaki (Chiba University, Japan). Reporter plasmid pNFAT-luc was a gift from Dr. Gerald R. Crabtree (Stanford University, CA). Control plasmid phRL-TK (Renilla-luc) was from Toyobo (Osaka, Japan). The pEFBOS-Flag-Rac1DN and pEFBOS-Flag-Rac1DA, which are the expression plasmids for the dominant-negative (S17N) and dominant-active (G12V) forms of Rac1, were gifts from Dr. Takaya Satoh (Kobe University, Japan).

*Transfection of cDNA and Reporter Gene Assays.* For transient transfection, each cDNA was premixed with FuGENE 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instruction. Twenty-four hours after the transfection, cells were incubated without or with 2 $\mu$g/mL anti-CD3 mAb together with 1 $\mu$g/mL PMA for 5 h. The luciferase activity was determined by the Luciferase Assay System (Promega, Madison, WI). Cells were lysed with the lysis buffer in this system for 15 min at room temperature. Twenty microliters of each cell lysate was reacted with 30 $\mu$L of reaction mixture (LARII) containing the substrate for the measurement of IL-2 or NFAT-luc activity, and then an additional 30 $\mu$L of the reaction mixture (Stop and Glo reagent) containing the substrate for the measurement of Renilla-luc activity, using the luminometer (Lumat LB9501, Berthold, Bad Wildbad, Germany). The activity of each reporter gene was normalized by that of Renilla-luc and expressed as a fold of increase comparing to the activity in nonstimulated cells. Aliquots of cell lysates were utilized for the control immunoblotting.

*RNA Interference.* Two sets of synthetic oligonucleotides involved the sense and antisense target sequences of human 3BP2 with stem-loop sequence; 5′-TCGACCAAACTGTGACAAACTCAAGTCTTCAAGAGAGACTTGAGTTTGTCACAGTTTTTTTTTGGAAG-3′ and 5′-AATTCTTCCAAAAAAAAACTGTGACAAACTCAAGTCTCTCTTGAAGACTTGAGTTTGTCACAGTTTGG-3′ for 3BP2 (1011), and 5′-TCGACCAAACTCAAGTCCTTCCACCTGTTCAAGAGACAGGTGGAAGGACTTGAGTTTTTTTTTGGAAG-3′ and 5′-AATTCTTCCAAAAAAAAACTCAAGTCCTTCCACCTGTCTCTTGAACAGGTGGAAGGACTTGAGTTTGG-3′ for 3BP2 (1021) were phosphorylated by T4 kinase (Takara, Tokyo, Japan), annealed, and ligated into the *Sal*I/*Eco*RI-cleaved backbone of pSilencer 1.0-U6 (Ambion, Austin, TX) (*20*). The resulting siRNA expressing vector targeted the region of 1011−1031 and 1021−1041 nucleotides 3′ to the initiation codon of human 3BP2, which were not homologous to the other known signaling proteins. The control synthetic oligonucleotides were designed by swapping of guanine and cytosine within the target sequence of 3BP2 (1011) and subcloned into the same vector. The control, 3BP2 (1011), or 3BP2 (1021) siRNA expressing vector was transiently transfected into Jurkat-TAg cells using FuGENE 6 reagent.

*Immunoprecipitation and Immunoblotting.* Cells were solubilized in 1% Triton lysis buffer (1% Triton X-100, 50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA, 100 mM NaF, 1 mM $Na_3VO_4$, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2 $\mu$g/mL aprotinin). For immunoprecipitation of 3BP2, cells were solubilized in the denature buffer (1% Triton lysis buffer containing 0.1% SDS and 0.5% deoxycholic acid). After centrifugation, precleared cell lysates were reacted with the indicated antibody prebound to Protein-A-agarose (Sigma) for 1 h at 4 °C. The beads were washed four times with the lysis buffer, and precipitated proteins were eluted by heat treatment at 100 °C for 5 min with the 2×sample buffer. Proteins were separated by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) and transferred onto polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was incubated with 5% milk in TBST (25 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) and then probed with the indicated primary antibodies in TBST. After washing with TBST, the membrane was incubated with the appropriate secondary antibodies and washed extensively in TBST. In all blots, proteins were visualized by the chemiluminescence reagent (Western Lightning, PerkinElmer Life Sciences, Boston, MA) (*13*, *21*). The densitometric analysis was carried out by using the NIH Image software.

*Fractionation of the Lipid Raft.* Cells ($10^7$) were homogenized in 0.025% Triton lysis buffer (0.025% Triton X-100,

150 mM NaCl, 25 mM MES, pH 6.5, 5 mM EDTA, 1 mM $Na_3VO_4$, 1 mM PMSF, and 2 $\mu$g/mL aprotinin), and cell homogenates were fractionated by sucrose density gradient centrifugation (*22*). Nine fractions were collected from the top of the gradient. Fractions 2 and 3 at the 5%/30% sucrose interface contain detergent-insoluble GEMs, and fractions 6 to 9 contain detergent-soluble fractions. In some experiments, proteins in fractions 2 and 3 were collected as lipid raft fractions and concentrated with 0.02% deoxycholic acid and 10% trichloroacetic acid (*23*).

*Generation of GST-Fusion Proteins and Pull-Down Experiments.* The cDNA for human Lck-SH2 domain ($Ala^{119}$-$Cys^{224}$) was isolated from Jurkat-TAg cells by RT-PCR. The resulting PCR product was subcloned into pGEX-4T.3 (Amersham) to make the domain in-frame with upstream glutathione S-transferase (GST) and verified by DNA sequencing. Jurkat-TAg cells transfected with the different kinds of cDNA were solubilized in the binding buffer (1% Nonidet P-40, 50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA, 100 mM NaF, 1 mM $Na_3VO_4$, 1 mM PMSF, and 2 $\mu$g/mL aprotinin) (*15*). The postnuclear cell lysates were precleared by 20 $\mu$g of GST prebound to glutathione-Sepharose 4B beads (Amersham). After the centrifugation, the resulting supernatants were incubated with 20 $\mu$g of GST-Lck-SH2 domain prebound to glutathione-Sepharose 4B beads for 1 h at 4 °C. The beads were washed four times with the binding buffer, and precipitated proteins were eluted by heat treatment at 100 °C for 5 min with the 2× sample buffer. Binding proteins were separated by SDS−PAGE and analyzed by immunoblotting.

## RESULTS

*The Short Interfering RNA (siRNA) for 3BP2 Suppresses TCR-Mediated Activation of Cytokine Promoter.* Since the phenotype of 3BP2 knock out mice has not been reported yet, we attempted to analyze the essential function of 3BP2 in T lymphocytes by using the siRNA for 3BP2. We designed short hairpin RNAs to silence the endogenous human 3BP2 mRNA under U6 RNA promoter. When Jurkat-TAg cells were transfected with 3BP2 siRNA expression vectors, the amount of 3BP2 protein was significantly suppressed compared to the control siRNA transfected cells (Figure 1A). Densitometric analysis of this data proved that the suppressions of the endogenous 3BP2 by 3BP2 siRNA were 69% (3BP2 siRNA 1011) and 53% (1021), respectively. On the basis of this result, we analyzed the role of 3BP2 on TCR-mediated transcriptional activation of cytokine promoters by using two kinds of 3BP2 siRNA compared to the control siRNA. Transient transfection of 3BP2 siRNA into Jurkat-TAg cells resulted in the suppression of TCR-mediated activation of IL-2 promoter, paralleled to the knock down level of endogenous 3BP2 (Figure 1B, upper panel). Next we tested the effect of these 3BP2 siRNA on TCR-mediated activation of transcription factor. TCR-mediated activation of NFAT was suppressed by 3BP2 siRNA (Figure 1B, lower panel). Thus, 3BP2 siRNA inhibits TCR-mediated transcriptional activation of NFAT, and therefore IL-2 promoter. These results demonstrated that the adaptor protein 3BP2 participates in the TCR-mediated cytokine synthesis.

*Tyrosine Phosphorylation of 3BP2 Is Required for the Activation of NFAT.* Adaptor protein 3BP2 is a substrate of

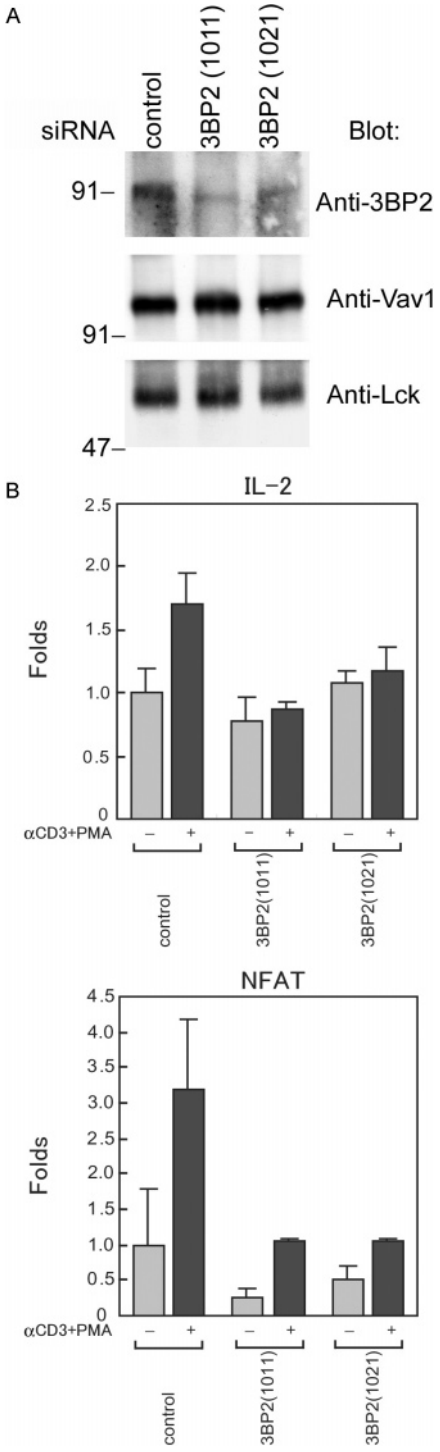


FIGURE 1: 3BP2 siRNA suppresses TCR-mediated transcriptional activation of IL-2 and NFAT. (A) Compared to the control siRNA, two kinds of 3BP2 siRNA (1011 and 1021) suppressed the expression of endogenous 3BP2 at 69% (3BP2 siRNA 1011) and 53% (1021), respectively. The densitometric analysis was carried out by using the NIH Image software, using anti-Vav1 and anti-Lck blots as an internal control. (B) 3BP2 siRNA (1011 and 1021) suppressed transcriptional activation of IL-2 and NFAT. Jurkat-TAg cells ($5 \times 10^5$ cells) were transfected with 0.2 $\mu$g of either the control siRNA or 3BP2 siRNA (1011), together with 0.15 $\mu$g of the indicated reporter construct and 0.03 $\mu$g of phRL-TK. Twenty-four hours after transfection, cells were stimulated without (−) or with anti-CD3 mAb (αCD3) together with PMA for 5 h. The cell lysates were analyzed by immunoblotting and the dual-luciferase assay. The relative activities of each reporter gene were normalized by that of phRL-TK. The results are the mean values ± SD from 3 independent experiments.

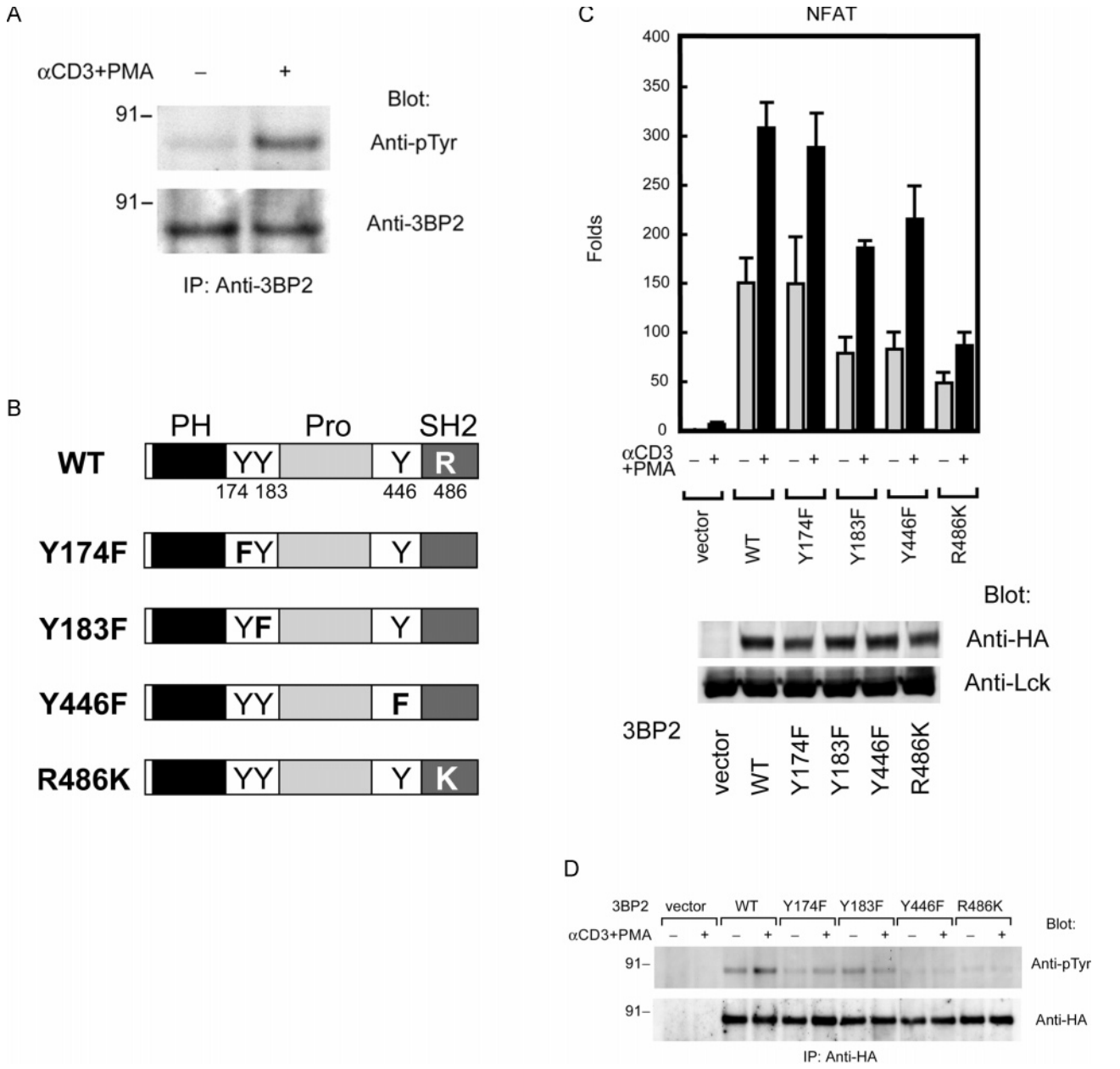


FIGURE 2: 3BP2 promotes the transcriptional activation of NFAT through $Tyr^{183}$, $Tyr^{446}$, and the SH2 domain. (A) Tyrosine phosphorylation of endogenous 3BP2. Jurkat-TAg cells ($6 \times 10^6$) were stimulated without (−) or with anti-CD3 mAb (αCD3) together with PMA for 1 min (+). Cells were solubilized in the denature buffer, and cell lysates were immunoprecipitated with anti-3BP2 antibody. The immunoprecipitates were analyzed by immunoblotting with anti-pTyr and anti-3BP2 antibodies. (B) Schematic diagram of 3BP2 constructs used in this experiment. $Tyr^{174}$ was substituted for phenylalanine (Y174F), $Tyr^{183}$ for phenylalanine (Y183F), and $Tyr^{446}$ for phenylalanine (Y446F), respectively. $Arg^{486}$ in the SH2 domain was substituted for lysine (R486K). (C) 3BP2 promotes the transcriptional activation of NFAT through $Tyr^{183}$, $Tyr^{446}$, and the SH2 domain. Jurkat-TAg cells ($5 \times 10^5$) were cotransfected with 0.2 $\mu$g of 3BP2 cDNA, 0.15 $\mu$g of NFAT reporter construct, and 0.03 $\mu$g of phRL-TK. Twenty-four hours after transfection, cells were stimulated without (−) or with anti-CD3 mAb (αCD3) together with PMA (+) for 5 h. The cell lysates were analyzed by the dual-luciferase assay. The relative activities of each reporter gene were normalized by that of phRL-TK. (C, lower panel) Nonstimulated cell lysates for the reporter gene assay were analyzed by immunoblotting with anti-HA and anti-Lck mAbs. The results are the mean values ± SD from 3 independent experiments. (D) Tyrosine phosphorylation of 3BP2. Jurkat-TAg cells were transfected with 1 $\mu$g of various 3BP2 cDNA. Forty-eight hours after transfection, cells were stimulated without (−) or with anti-CD3 mAb (αCD3) together with PMA for 1 min (+), and then solubilized in the denature buffer. Cell lysates were immunoprecipitated with anti-HA mAb. The immunoprecipitates were analyzed by immunoblotting with anti-phosphotyrosine (pTyr) and anti-HA mAbs.

PTKs in Fc receptor signaling (*12*, *13*). We have demonstrated that the antigen stimulation induces FcεRI-mediated tyrosine phosphorylation of 3BP2 in the rat basophilic leukemia RBL-2H3 cells (*13*). Syk could phosphorylate in $Tyr^{174}$, $Tyr^{183}$, and $Tyr^{446}$ in 3BP2 (*15*). Therefore, we tested whether the endogenous 3BP2 is tyrosine phosphorylated by TCR stimulation (Figure 2A). Engagement of TCR caused tyrosine phosphorylation of endogenous 3BP2 in Jurkat-TAg cells. To analyze the function of tyrosine phosphorylation of 3BP2 in T cells, various mutant forms of 3BP2 cDNA were generated and cotransfected with reporter gene constructs into Jurkat-TAg cells (Figure 2B). Overexpression of 3BP2 wild-type stimulated transcriptional activation of NFAT (Figure 2C). Among three putative phosphorylation

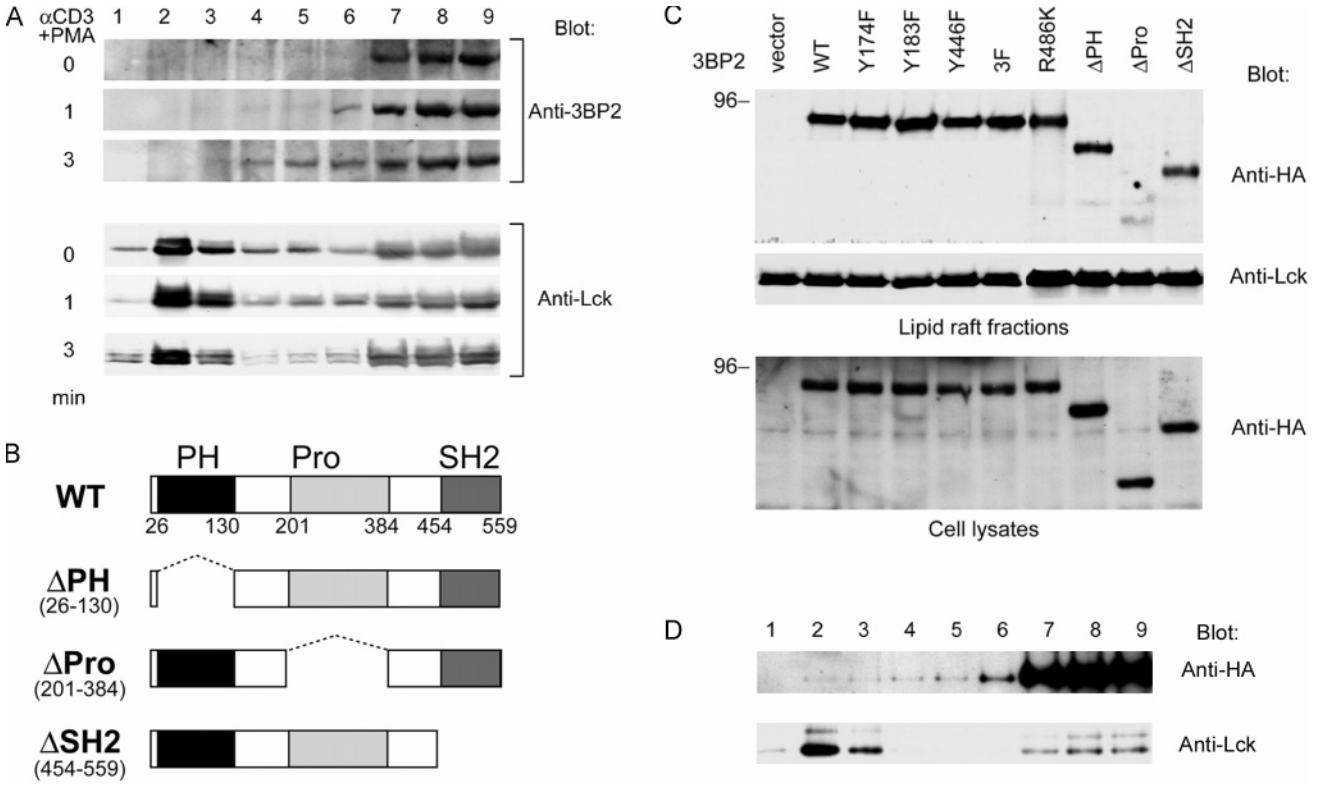


FIGURE 3: 3BP2 associates with the lipid raft. (A) TCR-stimulation induces the translocation of 3BP2 into the lipid raft. Jurkat-TAg cells ($10^7$) were stimulated without or with anti-CD3 mAb (αCD3) together with PMA for 1 and 3 min. Sucrose density gradient fractions of Jurkat-TAg cells were analyzed by immunoblotting with anti-3BP2 and anti-Lck antibodies. (B) Schematic diagram of 3BP2 constructs with domain-deletion mutation used in this experiment. (C) Overexpression of 3BP2 induces its association with the lipid raft. Jurkat-TAg cells ($2.5 \times 10^6$) were transfected with 1 $\mu$g of either vector or various 3BP2 constructs as indicated. Forty-eight hours after transfection, cells lysates were separated by the sucrose density gradient centrifugation. Fractions 2 and 3 were collected and analyzed as the lipid raft fraction. The lipid raft fractions and whole cell lysates were analyzed by immunoblotting with anti-HA and anti-Lck mAbs. Anti-Lck blots were utilized as a control of the lipid raft fractionation in this experiment. (D) Subcellular localization of overexpressed 3BP2. Jurkat-TAg cells overexpressing HA-3BP2 ($10^7$) were fractionated by the sucrose density gradient centrifugation and analyzed by immunoblotting with anti-HA and anti-Lck antibodies.

sites in 3BP2, substitution of $Tyr^{183}$ or $Tyr^{446}$ dramatically suppressed the activation of NFAT whereas mutation of $Tyr^{174}$ had no effect. Point mutation in the SH2 domain ($Arg^{486}$ to Lys) also decreased TCR-mediated activation of NFAT. This supports the evidence that 3BP2-mediated activation of NFAT is sensitive to cyclosporin A (*11*). Collectively, substitution of $Tyr^{183}$, $Tyr^{446}$, or $Arg^{486}$ of 3BP2 suppresses the activation of NFAT (Figure 2C). Substitution of each $Tyr^{174}$, $Tyr^{183}$, $Tyr^{446}$, or $Arg^{486}$ decreased tyrosine phosphorylation of 3BP2 (Figure 2D). The SH2 domain of 3BP2 associates with ZAP-70 in T cells (*11*). Presumably, 3BP2 may be phosphorylated by ZAP-70, a member of Syk-family PTK, in T cells.

*Localization of 3BP2 in the Lipid Rafts Itself Is Not Enough To Induce the Activation of Transcription Factor.* Engagement of TCR induces the accumulations of activated signaling molecules in the lipid raft. Therefore, next we examined the intracellular localization of 3BP2 in T cells. Jurkat-TAg cells were stimulated with anti-CD3 mAb together with PMA, and cell lysates were fractionated by sucrose density gradient centrifugation (Figure 3A). In nonstimulated cells, the endogenous 3BP2 was localized in the detergent-soluble fractions. After the engagement of TCR, some of 3BP2 was translocated into the low-density detergent-insoluble fractions (lipid raft).

To analyze the mechanism of 3BP2 translocation into the lipid raft, a series of deletion mutants of 3BP2 cDNA were constructed and transfected into Jurkat-TAg cells (Figure 3B). Overexpression of 3BP2 wild-type resulted in its localization into the lipid raft (Figure 3C). Immunohistochemical analysis also provided the evidence that 3BP2 wild type was colocalized with cholera toxin B, the marker of the lipid raft in Jurkat-TAg cells (data not shown). Substitution of each tyrosine residue or point mutation in the SH2 domain could not affect the localization of 3BP2 in the lipid raft (Figure 3C). The gradient fractionation of the overexpressed 3BP2 shows the similar pattern of endogenous 3BP2 in the TCR-stimulated cells (Figure 3D). However, deletion of proline rich regions (ΔPro), but neither N-terminal PH (ΔPH) nor C-terminal SH2 domain (ΔSH2), abrogated the localization of 3BP2 in the lipid raft (Figure 3C). This result demonstrates that the localization of some 3BP2 in the lipid raft itself is not enough to induce the activation of transcription factors. 3BP2-mediated activation of transcription factors still requires tyrosine phosphorylation and SH2 domain of 3BP2.

*Phosphorylation of $Tyr^{183}$ in 3BP2 Recruits Vav1.* TCR stimulation induces tyrosine phosphorylation of 3BP2 on $Tyr^{174}$, $Tyr^{183}$, and $Tyr^{446}$ (Figure 2D). While substitution of $Tyr^{174}$ could not affect TCR-mediated activation of NFAT, $Tyr^{183}$ appears to be important for the transcriptional activation of NFAT (Figure 2C). Therefore, we tested the function of $Tyr^{183}$ in TCR signaling. The in vitro binding study using the phosphorylated synthetic polypeptide suggests that phosphorylated $Tyr^{183}$ is a binding site to Vav1 in NK cells (*12*).

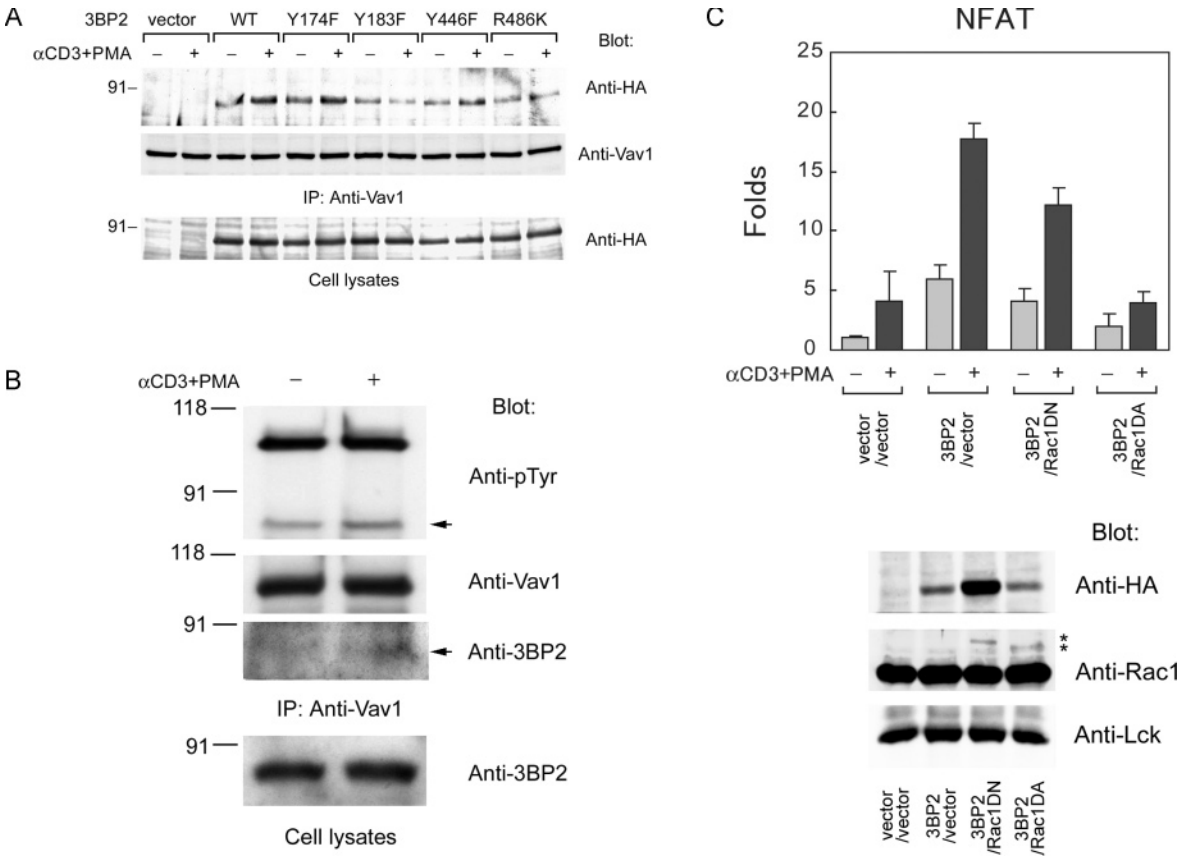


FIGURE 4: Tyr[183] in 3BP2 associates with Vav1. (A) Tyr[183] in 3BP2 is required for the interaction with Vav1. Jurkat-TAg cells ($2.5 \times 10^6$) were transfected with 1 $\mu$g of various 3BP2 cDNA. Forty-eight hours after transfection, cells were stimulated without (−) or with anti-CD3 mAb (αCD3) together with PMA for 3 min (+), and cell lysates were immunoprecipitated with anti-Vav1 antibody. The immunoprecipitates and cell lysates were analyzed by immunoblotting with anti-HA and anti-Vav1 antibodies. (B) Peripheral blood lymphocytes ($2 \times 10^7$) were stimulated without (−) or with anti-CD3 mAb (αCD3) together with PMA for 3 min (+), and cell lysates were immunoprecipitated with anti-Vav1 antibody. The immunoprecipitates and cell lysates were analyzed by immunoblotting with anti-pTyr, anti-3BP2, and anti-Vav1 antibodies. Arrows indicate the position of endogenous 3BP2. (C) 3BP2 requires Rac1 for the transcriptional activation of NFAT. Jurkat-TAg cells ($5 \times 10^5$) were cotransfected with 0.2 $\mu$g of 3BP2 cDNA (pMT3-HA-3BP2 cDNA) or pMT3 vector and 0.2 $\mu$g of either the dominant-negative form of Rac1 (pEFBOS-Flag-Rac1DN), the dominant-active form of Rac1 (pEFBOS-Flag-Rac1DA), or pEFBOS vector, together with 0.15 $\mu$g of NFAT reporter construct and 0.03 $\mu$g of phRL-TK. Twenty-four hours after transfection, cells were stimulated without (−) or with anti-CD3 mAb (αCD3) together with PMA (+) for 5 h. The cell lysates were analyzed by the dual-luciferase assay. The relative activities of each reporter gene were normalized by that of phRL-TK. The results are the mean values ± SD from 3 independent experiments. (C, lower panel) Nonstimulated cell lysates for the reporter gene assay were analyzed by immunoblotting with anti-HA, anti-Rac1, and anti-Lck antibodies. Asterisks indicate the expression of Flag-tagged Rac1DN and Flag-tagged Rac1DA.

Infection of 3BP2 into natural killer cells by vaccinia virus enhances the cell cytotoxicity through Tyr[183] (*12*). In addition, we have demonstrated that 3BP2-Tyr[183] binds to the SH2 domain of Vav1 in vitro (*18*). Point mutation of Tyr[183] abrogated the interaction of 3BP2 with Vav1-SH2 domain. Thus, we attempted to analyze whether this interaction is observed in intact T cells (Figure 4A). Immunoprecipitation experiments revealed that overexpression of 3BP2 induces the association with Vav1, and this association was enhanced by the TCR stimulation. TCR-induced association of 3BP2 with Vav1 was also observed in human peripheral lymphocytes (Figure 4B). This induced association was abrogated by the substitution of Tyr[183] or Arg[486] of 3BP2. Substitution of Tyr[446] reduced the coprecipitation, suggesting that Tyr[446] is involved in the optimum interaction of 3BP2-Vav1 in T cells. The SH2 domain of 3BP2 was shown to associate with ZAP-70 (*11*). Therefore, this result also suggests that ZAP-70 participates in phosphorylating Tyr[183] of 3BP2 in T cells.

Moreover, overexpression of the dominant-negative form of Rac1 (Rac1DN) suppressed TCR-mediated activation of NFAT (Figure 4C). Although cotransfection with Rac1DN resulted in the higher expression of 3BP2, the transcriptional activity of NFAT was significantly suppressed in the cells expressing Rac1DN (Figure 4C, lower panel). Therefore, TCR-mediated activation of NFAT requires the interaction of Tyr[183] in 3BP2 with the Vav1-Rac1 pathway in Jurkat-TAg cells. We could not obtain the additional effect on NFAT activation by the cotransfection of 3BP2 with Rac1DA (Figure 4C, lower panel). It could be due to the effect of cytoskeletal reorganization or exhaust of the downstream signaling molecules by the expression of Rac1DA.

*Phosphorylation of Tyr[446] in 3BP2 Recruits Lck.* We have demonstrated that phosphorylation of Tyr[446] in 3BP2 creates the binding site to the SH2 domain of Lyn to induce its enzymatic activation after the engagement of FcεRI (*15*). Thus, we tested whether Tyr[446] in 3BP2 is capable of binding to the SH2 domain of Lck in T cells (Figure 5). Unlike wild type, substitution of Tyr[446] or Arg[486] abrogated the interaction of 3BP2 with the SH2 domain of Lck (Figure 5). In addition to the Src family PTK, yeast and mammalian two-hybrid experiments demonstrated that 3BP2 directly binds to Syk family PTK, and this interaction requires its kinase activity.

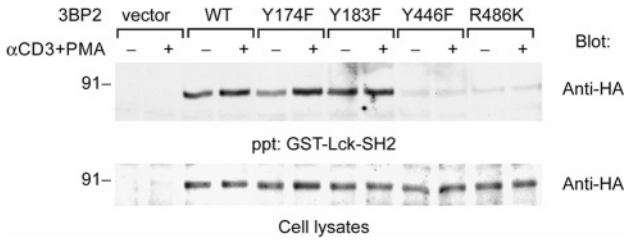


FIGURE 5: Tyr[446] in 3BP2 associates with Lck. Jurkat-TAg cells ($2.5 \times 10^6$) were transfected with 1 $\mu$g of various 3BP2 cDNA. Forty-eight hours after transfection, cells were stimulated without (−) or with anti-CD3 mAb (αCD3) together with PMA for 3 min (+) and solubilized in the binding buffer. Nonstimulated or stimulated cell lysates were precleared by GST prebound to glutathione-Sepharose 4B beads and then incubated with GST-Lck-SH2 domain prebound to glutathione-Sepharose 4B beads. Binding proteins and cell lysates were analyzed by immunoblotting with anti-HA mAb.

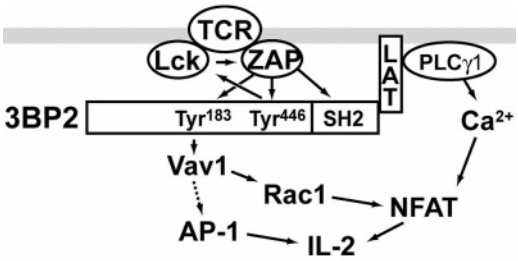


FIGURE 6: Role of 3BP2 in TCR signaling. TCR-mediated sequential activation of Lck and ZAP-70 contributes tyrosine phosphorylation of Tyr[183] and Tyr[446] of 3BP2. Phosphorylated Tyr[183] binds to Vav1-SH2. Phosphorylated Tyr[446] binds to Lck-SH2 for the optimum tyrosine phosphorylation of 3BP2. 3BP2-SH2 domain couples activated ZAP-70 to LAT and PLC-$\gamma$. 3BP2 mediates both $Ca^{2+}$- and Vav1-Rac1-dependent pathways for activation of NFAT. Expression of 3BP2 is required for the transcriptional activation of NFAT and IL-2 promoter.

As described above, the SH2 domain of 3BP2 was shown to associate with ZAP-70 in T cells (*11*). Thus, this result clearly demonstrates that Tyr[446] is phosporylated by ZAP-70. Also, substitution of Tyr[446] resulted in the partial suppression of 3BP2-Vav1 association, suggesting that Lck is involved in phosphorylating Tyr[183] in 3BP2 (Figure 4A). Phosphorylated Tyr[446] may recruit Lck to contribute to the optimum response of 3BP2-mediated activation of transcription factors. Furthermore, it is possible that 3BP2 connects Lck to Vav in the lipid raft and induces tyrosine phosphorylation of Vav by Lck. Therefore, 3BP2 is a potential adaptor to connect proximal PTKs to Vav1 in immune receptor signals.

## DISCUSSION

3BP2 functions to connect TCR signals to NFAT through Tyr[183], Tyr[446], and the SH2 domain (Figure 6). The SH2 domain of 3BP2 plays an important role in TCR-mediated activation of NFAT (Figure 2C). The SH2 domain of 3BP2 couples ZAP-70 to LAT signaling complexes in T cells (*11*). Also, we have demonstrated that the SH2 domain of 3BP2 binds to LAT in mast cells and overexpression of the SH2 domain of 3BP2 suppresses Fc$\epsilon$RI-mediated tyrosine phosphorylation of both PLC-$\gamma$1 and -$\gamma$2, $Ca^{2+}$ mobilization, and degranulation (*13*). Thus, the SH2 domain of 3BP2 seems to regulate $Ca^{2+}$-mediated signaling. The present study has demonstrated that phosphorylation of Tyr[183] and Tyr[446] is necessary for the potential activation of NFAT (Figure 2C).

Inhibition of the site-directed functions of 3BP2 may have therapeutic potential by regulating the activity of the transcription factors and subsequent cytokine production. For activation of NFAT, 3BP2 mediates both calcineurin- and Vav1-Rac1-dependent pathways (Figure 6). The SH2 domain of 3BP2 may regulate $Ca^{2+}$ mobilization, calcium-sensitive phosphatase calcineurin, and NFAT activation (*11*). More recently, 3BP2 was shown to associate with Vav to regulate NFAT in B cell antigen receptor signaling (*24*). Therefore, TCR signals through the SH2 domain of 3BP2 might be sensitive to FK506. This raises the idea to develop the immunosuppressive compound that regulates TCR-mediated activation of transcription factors through phosphorylation of Tyr[183] and/or Tyr[446] of 3BP2, besides FK506.

The molecules that connect the proximal PTKs and Vav1 have not been determined yet in TCR signaling. Engagement of TCR induces tyrosine phosphorylation of Vav1 by Lck, paralleled to its guanine nucleotide exchanging activity (*25*). Genetic analysis by using the knock-in mice revealed that the mutation of Tyr[315] of ZAP-70, which was shown to associate with Vav1, does not attenuate TCR-mediated tyrosine phosphorylation of Vav1 in T lymphocyte (*26*). Similarly, the mutation of the corresponding Tyr[342] in Syk does not affect Fc$\epsilon$RI-stimulated tyrosine phosphorylation of Vav1 in mast cells (*27*). These findings suggest that Vav1 is not a direct substrate of Syk/ZAP-70. Rather, ZAP-70 is essential for the recruitment of Vav1 into the lipid raft (*28*). We have shown that 3BP2-Tyr[183] associates with Vav1 in Jurkat-TAg cells (Figure 4A). ZAP-70 may phosphorylate 3BP2-Tyr[183] located within the Tyr-X-X-Pro motif to recruit the SH2 domain of Vav1 (*29*). Since the engagement of TCR induces the translocation of 3BP2 into the lipid raft (Figure 3), it is likely that 3BP2 recruits Vav1 into the lipid raft to be tyrosine phosphorylated by Lck. Therefore, it is conclusive that 3BP2 acts as one of the adaptors to connect proximal PTKs to Vav1 in the lipid raft for functional TCR signaling. 3BP2 may translocate into the immunological synapse for the downstream signal (*7*).

It is expected that serine/threonine kinases are involved in controlling TCR-mediated transcriptional activation of NFAT (*14*). Phosphorylation of 3BP2-Ser[277] negatively regulates the activation of NFAT by interacting with 14-3-3 (*14*). In mast cells, overexpression of 3BP2 with a point mutation of Ser[277] resulted in an increase in Fc$\epsilon$RI-mediated degranulation. However, overexpression of cherubism mutant forms of 3BP2 suppressed mast cell degranulation, although they lost the ability to interact with 14-3-3 (*18*). These results suggest the possibility that negative reulation by serine/threonine kinase could be a secondary event through the process of TCR-mediated activation of NFAT.

In summary, we have demonstrated that an adaptor protein 3BP2 regulates transcription factors through its tyrosine phosphorylation and SH2 domain (Figure 6). 3BP2 is the essential proximal inducer of the TCR-mediated activation of NFAT. Controlling the expression and site-directed functions of 3BP2 may be useful for regulation of cytokine production in the pathological reactions. Here, we propose that 3BP2 has therapeutic potential for transplantation immunity and acute inflammations.

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