The Chaperone Protein 14-3-3 Interacts with 3BP2/SH3BP2 and Regulates Its Adapter Function*  

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Isabelle Foucault†‡, Yun-Cai Liu†, Alain Bernard‡, and Marcel Deckert§∥  

From INSERM U343 and Laboratoire d’Immunologie, Hôpital de l’Archet I, Nice 06202, France and the Division of Cell Biology, La Jolla Institute for Allergy and Immunology, San Diego, California 92121  

Lymphocyte stimulation by immunoreceptors is achieved through the activation of multiple signaling pathways leading to cytokine gene transcription. Adapter proteins are critical signaling components that can integrate multiple pathways by allowing the assembly of multimolecular signaling complexes. We previously showed that the cytoplasmic adapter 3BP2 (also known as SH3BP2) promotes NFAT/AP-1 transcriptional activities in T cells through the activation of Ras- and calcineurin-dependent pathways. However, the molecular mechanisms by which 3BP2/SH3BP2 regulates cell signaling and activation remain poorly documented. In this study, using a combination of yeast two-hybrid analysis and biochemical approaches, we present evidence for a physical interaction between 3BP2 and the cytoplasmic protein 14-3-3. This interaction was direct and constitutively detected in yeast and in mammalian cells. Phorbol ester, pervanadate, and forskolin/isobutylmethylxanthine stimulations enhanced this interaction, as well as co-expression of constitutive active mutants of serine/threonine kinases, including protein kinase C. We found that dephosphorylation of 3BP2 by alkaline phosphatase disrupted its interaction with 14-3-3 and that 3BP2 was a substrate of purified protein kinase C in vitro, suggesting that the phosphorylation of 3BP2 by upstream kinases was required for 14-3-3 binding. Using deletion mutants of 3BP2, two 14-3-3 binding domains were mapped to two proline-rich (residues 201–240 and 270–310) domains of 3BP2. These domains were shown to contain two 14-3-3 consensus binding motifs. We identified residues Ser225 and Ser277 of 3BP2 as being essential for interaction with 14-3-3 family proteins, optimal 3BP2 serine phosphorylation, and then for 3BP2-dependent function. Indeed, a 3BP2 mutant protein incapable of binding 14-3-3 showed increased capacity to stimulate NFAT transcriptional activities, suggesting that 14-3-3 binding to 3BP2 negatively regulates 3BP2 adapter function in lymphocytes.

Antigen receptor engagement on lymphocytes induces the activation of the protein-tyrosine kinases (PTKs) of the Src family of tyrosine kinases (PTKs), c-Src, and Syk families. In turn, the activated PTKs promote the assembly of intracellular protein complexes that transduce signals to the cytoplasm and nucleus (1). In recent years, it has become apparent that adapter molecules play critical roles in the coupling of receptor-proximal events, such as protein-tyrosine kinases activation, to specific signaling processes linked to cell growth and differentiation (2, 3). Adapter proteins lack intrinsic catalytic activity, but they possess motifs and domains capable of mediating protein-protein and protein-lipid interactions (4). The assembly of large signaling complexes, through the multiple interactions stabilized by adapters, allows the activation of downstream effectors, including phospholipase Cγ (PLCγ), guanine nucleotide exchange factors of the Vav family, and phosphatidylinositol 3-kinase, which couple extracellular signals to small GTPases signaling, cytoskeletal reorganization, gene expression, and, ultimately, to lymphocyte activation and functions (1, 5). For example, by SH2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) and by linker for activation of T cells (LAT), two adapter molecules expressed in T cells, are required for proper activation of PLCγ, intracellular calcium fluxes, and Ras stimulation during T cell activation (6, 7). In an analogous manner, the B cell linker protein (BLNK) is required for tyrosine phosphorylation of PLCγ and activation of Jun N-terminal kinase in activated B lymphocytes (8).

We previously showed that the cytoplasmic adapter protein 3BP2 (also known as SH3-binding protein 2 or SH3BP2) positively regulates T cell signaling (9). 3BP2 has been originally described as an in vitro binding partner of the SH3 domain of the PTK c-Abl (10). 3BP2 is composed of a N-terminal pleckstrin homology domain, a central proline-rich (PR) region that interacts with c-Abl, and a C-terminal SH2 domain. Human 3BP2 transcripts are ubiquitously expressed but can be preferentially found in spleen, in peripheral blood leukocytes, and, to a lesser extent, in thymus (9, 11). During a screen for Syk kinase-interacting proteins in lymphocytes, we found a direct, activation-induced interaction of 3BP2 with the SH2 domain of Syk and Zap-70. In addition, the SH2 domain of 3BP2 was also found to associate in vitro several tyrosine-phosphorylated proteins including PLCγ1, c-Cbl, and LAT, proteins that have all been implicated in T cell signaling pathways (9). We also showed that 3BP2 was a potent inducer of both basal and TCR-mediated activities of NFAT and AP-1, two transcription factors involved in interleukin 2 gene transcription. Furthermore, 3BP2-induced gene activation in T cell required the activation of a cyclosporin A-sensitive signaling pathway and of a Ras-dependent signaling pathway (9). More recently, 3BP2 was shown to also have a positive regulatory
role during the development of NK cell-mediated cytotoxicity through the binding of several signaling molecules like PLCγ1 and Vav1 (12) and to regulate FceRI-mediated degranulation in basophilic cells (13). Finally, mutations in the $3p2i/sh3bp2$ gene have been linked to a rare human disease of childhood characterized by proliferative lesions within the mandible and maxilla, suggesting that $3bp2$ could be implicated in osteoclast differentiation (14). However, the exact role of $3bp2$ in hematopoietic/lymphoid cell signaling remains ill-defined.

The 14-3-3 protein family constitutes a highly conserved group family of molecules present in high abundance in all eukaryotic cells (15, 16). They mediate signal transduction, cell cycle control, apoptosis, stress response, and malignant transformation by binding to phosphoserine-containing proteins such as c-Raf, Cdc25c, and BAD (17). 14-3-3 proteins are considered as scaffold molecules modulating protein-protein interactions, protein subcellular localization, and enzyme activities. Evidences of their involvement in lymphocyte signaling have been provided. For example, it has been shown that the binding of 14-3-3 to PKC prevents its translocation to the membrane, thereby decreasing its ability to activate the interleukin 2 promoter (18). In addition, the interaction of 14-3-3 with the catalytic subunit of the phosphatidylinositol 3-kinase and the Cbl protooncogene has been involved in the regulation of the Ras-dependent pathway leading to NFAT activation (19–21).

In this report, we have identified a novel interaction between 14-3-3 proteins and the adapter protein $3bp2$. By using a yeast two-hybrid system, pull-down assays and Western ligand blot analysis, we show that $3bp2$ specifically and directly associates in vitro and in vivo with 14-3-3. Our studies also provide ample evidence that $3bp2$ interacts with 14-3-3 via a serine phosphorylation-dependent mechanism and that PKC is one of the Ser/Thr kinases that phosphorylates $3bp2$ to facilitate this association. $3bp2$ depletion analysis revealed the presence of two 14-3-3 binding sites located to the central proline-rich domain of 3BP2 and containing two serine residues (Ser225 and Ser277) critical for 14-3-3/3BP2 interaction. Finally, gene repression analysis indicated below.

**Antibodies and Reagents**—Culture media, oligonucleotides, and enzymes were from Invitrogen. The chemicals were obtained from Sigma. Antibodies specific for Cbl, Raf-1, 14-3-3, and GST were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Myc (9E10) and anti-hemaggulinin (HA, 12CA5) monoclonal antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY) and Roche Diagnostics, respectively. The GST $3bp2$ SH2 domain fusion protein was previously described (9). Polyclonal antibodies against $3bp2$ were prepared following rabbit immunization with the GST $3bp2$ SH2 domain fusion protein. Alkaline phosphatase was from Roche Diagnostics, and purified catalytic subunit of rat brain PKC was obtained from Calbiochem. Plasmids—The yeast expression plasmids pACTII, and pC3A were previously described (22). The plasmids encoding 14-3-3 or 14-3-3 proteins were transfectected with the indicated plasmids.
RESULTS

Identification of 14-3-3 as 3BP2 Interacting Proteins—In an attempt to identify proteins able to bind to 3BP2, we employed the yeast two-hybrid interaction analysis. 3BP2 was fused in frame to the DNA-binding domain of LexA and used to screen a human lymphocyte library. 2.5 × 10⁶ transformants were screened. Of the 48 clones selected for sequence analysis, three 14-3-3 clones, one 14-3-3e clone, and one 14-3-3r clone were identified by comparison with the nucleotide data base at the National Library of Medicine using the BLAST algorithm (Fig. 1). For further experiments, we elected to use the 14-3-3r isoform, which is expressed abundantly in lymphoid cells.

Constitutive Interaction between 14-3-3 and 3BP2 in Resting Cells—Biochemical interaction between 3BP2 and 14-3-3 was next tested by co-immunoprecipitation assay. COS-1 cells were transiently transfected with plasmids encoding Myc-tagged 3BP2, HA-tagged 14-3-3r, or empty vectors. Lysates from unstimulated cells were incubated with anti-HA antibody, resolved by SDS-PAGE, and immunoblotted with anti-Myc antibody. As shown in Fig. 2A, 3BP2 co-immunoprecipitated with 14-3-3, indicating that there is a constitutive complex in resting COS-1 cells between 3BP2 and 14-3-3. This result was confirmed by an in vitro pull-down assay using GST-14-3-3r (Fig. 2B). Lysates of COS-1 cells transfected with Myc-tagged 3BP2 were incubated with recombinant GST or GST-14-3-3r fusion protein. 3BP2 bound to immobilized GST-14-3-3r fusion protein but not to GST protein when lysate proteins were eluted from immobilized GST proteins and immunoblotted with anti-Myc antibody. To further demonstrate that 3BP2 interacts with 14-3-3, we immunoprecipitated endogenous 3BP2 (or Raf-1 as control) from U937 cells (Fig. 2C). Western blot with anti-14-3-3 antibody showed that 3BP2 co-immunoprecipitated with a significant fraction of endogenous 14-3-3 proteins in resting monocytic U937 cells, reaching 0.5–1% of total 14-3-3r, as judged by Western blot analysis of one-tenth of total cell lysate. Binding of 14-3-3r to Raf-1 is also showed for comparison (Fig. 2C).

To further localize the association of 3BP2 and 14-3-3, we used double immunofluorescence staining and confocal microscopy and analyzed COS-1 cells co-transfected with Myc-tagged 3BP2 and HA-tagged 14-3-3r. 3BP2 was mainly localized in the cytoplasm with a small fraction constitutively associated with the membrane (Fig. 2D and Ref. 9). 14-3-3 staining was found diffusely in the cytoplasm, partially overlapping with 3BP2 staining (Fig. 2D, middle and right panels). This suggests that
membranes were incubated with GST-14-3-3 antibody and Protein G-Sepharose. Immunoprecipitates were resolved
min at 37 °C. Infected with 3BP2-Myc vector were stimulated or not with PMA for 15 min. A
GST-14-3-3-antibody was used to precipitate 3BP2 in lysates from unstimulated and activated COS-1 cells and treated these
membranes with anti-Myc antibody and protein G-Sepharose. Immunoprecipitates were resolved by SDS-7.5% PAGE and
transferred onto nitrocellulose membrane. The membranes were incubated with GST-14-3-3-antibody and ECL. The membrane was then
stripped and reprobed with anti-Myc antibody. C. COS-1 cells were transfected with 3BP2-Myc expression vector and with CDNA encoding
constitutively active Ras. The lysates were analyzed with pull-down assay using GST-14-3-3-antibody and anti-Myc immunoblotting like above. D. COS-1 cells
were transfected with HA-3BP2 vector were either stimulated or unstimulated with forskolin (10 μM) and isobutylmethylxanthine (100 μM) for 15
min at 37 °C. The lysates were immunoprecipitated with anti-HA antibody and subjected to Western ligand blot with GST-14-3-3-
fusion protein, and binding was detected with anti-GST and anti-3BP2 antibodies (Fig. 4). Surprisingly, all of the
Ser/Thr kinases tested increased the interaction between 3BP2 and 14-3-3, and binding was
detected with anti-Myc antibody (Fig. 4). Stripping and reprobing the membrane with
anti-HA antibody showed that similar amounts of 3BP2 were present in all samples. These results suggest that phosphorylation
of 3BP2 was required for its association with 14-3-3.

PMA stimulation increases both 3BP2/14-3-3 interaction and PKC activation. To determine whether PKC directly phosphor-
ylates 3BP2, 3BP2 immunoprecipitates from transfected COS-1 cells were used in in vitro kinase assay with a purified
catalytic domain of rat brain PKC. 3BP2 phosphorylation was not detectable in the absence of PKC. When PKC was added, a
band of phosphorylated 3BP2 was clearly visible (Fig. 4B). Probing the membrane with anti-3BP2 antibody showed that similar
amounts of 3BP2 were immunoprecipitated in all of the samples (bottom panel). These results support the notion that
PKC directly phosphorylates 3BP2 in vitro.

To further define the nature of the 3BP2/14-3-3 association, we evaluated the ability of different Ser/Thr kinases to modu-
late it. COS-1 cells were transfected with 3BP2-Myc expression vector and with cDNAs encoding for constitutively active forms of
PKA, Akt, and PKCβ. The lysates were incubated with GST-14-3-3-antibody and ECL. The lysates were immunoprecipitated with
anti-HA antibody, and Western ligand blot with GST-14-3-3-fusion protein and 3BP2 antibody, Raf-1, which associates directly with 14-3-3-proteins, was
used here as a positive control. More 3BP2 was associated with
GST-14-3-3-r in lysates from PMA or pervanadate-stimulated
cells than in unstimulated samples (Fig. 3A).

tively active form of Ras. As shown in Fig. 3C, activation of Ras mitogenic pathway led to increases 3BP2/14-3-3 interaction.
Next, COS-1 cells were transfected with HA-tagged 3BP2 con-
struct and either unstimulated or stimulated with PKA ago-
nists, forskolin, and isobutylmethylxanthine. The lysates were
immunoprecipitated with anti-HA antibody, and Western li-
gand blot was performed as described above. Fig. 3D shows that cell stimulation with forskolin and isobutylmethylxan-
thine increased the basal binding of 14-3-3-r to 3BP2. Alto-
gether, our results indicate that the constitutive and direct association between 14-3-3 and 3BP2 is reinforced when cells are
stimulated by agonists of PKC and PKA pathways.

3BP2 Phosphorylation Increases 14-3-3 Binding—To determine whether phosphorylation on serine residues was involved in
the interaction between 3BP2 and 14-3-3, we immunoprecip-
itated 3BP2 from resting COS-1 cells and treated these
immune complexes with 20 units/ml alkaline phosphatase.
The samples were then analyzed for binding of GST-14-3-3-r by
Western ligand blotting. Treatment of anti-HA precipitates
with alkaline phosphatase totally abrogated 3BP2/14-3-3 inter-
action (Fig. 4A). Stripping and reprobing the membrane with
anti-HA antibody showed that similar amounts of 3BP2 were
present in all samples. These results suggest that phosphory-
lation of 3BP2 was required for its association with 14-3-3.

Next, COS-1 cells were transfected by different 3BP2-cDNA
constructs and activated or not with PMA 15 min at 37 °C. A

a significant fraction of 3BP2 and 14-3-3 proteins co-localized in
COS-1 cells.

The Interaction between 14-3-3 and 3BP2 is Direct and
Increased by Agonists of PKC and PKA Pathways—Next, we used an
in vitro pull-down assay and a Western ligand blot technique to study the effect of mitogenic stimulation on 3BP2/14-
3-3 interaction. First, COS-1 cells were transfected with Myc-
tagged 3BP2, and stimulated with PMA or pervanadate
treatment. The cell lysates were incubated with recombinant
GST or GST-14-3-3-r fusion protein, and bound proteins, which
were recovered with glutathione-Sepharose beads, were sub-
jected to SDS-PAGE and immunoblotting with anti-Myc anti-
body. Raf-1, which associates directly with 14-3-3-proteins, was
used here as a positive control. More 3BP2 was associated with
GST-14-3-3-r in lysates from PMA or pervanadate-stimulated
cells than in unstimulated samples (Fig. 3A).

to determine whether the association between 3BP2 and
14-3-3-r is direct, we used the Western ligand blot technique.
COS-1 cells were transfected with Myc-tagged 3BP2, either
unstimulated or stimulated with PMA, and the lysates were
immunoprecipitated with anti-3BP2 antibody. Electrophoresed
precipitated 3BP2 was probed with a recombinant GST-14-3-3-
r fusion protein, and binding was detected with anti-GST mon-
oclonal antibody (Fig 3B). As expected, GST-14-3-3-r bound
immunoprecipitated 3BP2 from both unstimulated and activated
cells. Immunoblotting of the same membrane with anti-3BP2
antibody showed that a similar amount of 3BP2 proteins was
present in immunoprecipitates from resting and PMA-stimu-
lated cells.

A similar in vitro pull-down assay using a GST-14-3-3-r was
performed from unstimulated COS-1 cells transfected with
plasmids encoding for 3BP2-Myc without or with a constitu-

Fig. 3. The interaction between 14-3-3-r and 3BP2 is direct and
increased by agonists of PKC and PKA pathways. A. COS-1 cells were transfected with 3BP2-Myc expression vector. After 48 h, they were either left unstimulated or stimulated for 15 min at 37 °C with PMA (100 ng/ml) or pervanadate (10 μM). The lysates (0.5 × 10° cell equivalents) were precipitated with 10 μg of GST or GST-14-3-3-r and recovered with glutathione-Sepharose beads. The washed beads were subjected to SDS-7.5% PAGE, transferred onto nitrocellulose membrane, and immunoblotted with antibodies against Myc epitope or Raf, and visualized with ECL (left panel). Fractions of the same lysates (0.5 × 10° cell equivalents) were directly subjected to Western blot analyses with anti-Myc or anti-Raf antibodies (right panel). B. COS-1 cells transfected with 3BP2-Myc vector were stimulated or not with PMA for 15 min at 37 °C. The lysates were immunoprecipitated with anti-Myc antibody and protein G-Sepharose. Immunoprecipitates were resolved by SDS-5.5% PAGE and transferred onto nitrocellulose membrane. The membranes were incubated with GST-14-3-3-r, and binding was de-
tected with anti-GST antibody and ECL. The membrane was then
stripped and reprobed with anti-Myc antibody. C. COS-1 cells were
transfected with 3BP2-Myc expression vector and with CDNA encoding
constitutively active Ras. The lysates were analyzed with pull-down assay using GST-14-3-3-r and anti-Myc immunoblotting like above. D. COS-1 cells transfected with HA-3BP2 vector were either stimulated or unstimulated with forskolin (10 μM) and isobutylmethylxanthine (100 μM) for 15 min at 37 °C. The lysates were immunoprecipitated with anti-HA antibody and subjected to Western ligand blot with GST-14-3-3-
r. IP, immunoprecipitation; PVM, pervanadate.

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Western ligand blot experiment was then done using a GST-14-3-3 fusion protein. Immunoblotting with anti-HA antibody was used to normalize for expression of each 3BP2 construct. In resting and PMA-stimulated cells, the interaction was lost when the first or second PR domain of 3BP2 were deleted. The third PR domain, which does not contain any putative 14-3-3 binding site, was not required for this interaction (Fig. 5B). These results show that two PR domains in 3BP2 are critical for 14-3-3 binding.

Serine 277 in 3BP2 Is Critical for 14-3-3 Binding—14-3-3 isoforms have been shown to bind conserved motifs in a manner dependent on the phosphorylation of serine residues (24). We thus searched for serine residues matching predicted consensus sequences within the first and the second proline-rich do-

mains of 3BP2. Two serine residues, Ser^{225} and Ser^{277}, were identified within such consensus motifs in the first and the second proline-rich domain, respectively (Fig. 6A). These two residues were substituted with alanine residues using site-directed mutagenesis, and 14-3-3/3BP2 interaction was analyzed using the two-hybrid system. 3BP2 S225A mutation decreased the interaction, whereas S277A mutation abolished it (Fig. 6C). Immunoblotting with anti-3BP2 antibody was used to normalize for expression of each 3BP2 construct. Together, these results show that 3BP2 serine residues Ser^{225} and Ser^{277} are required for basal and stimulation-dependent association between 3BP2 and 14-3-3. To test whether serine residues Ser^{225} and Ser^{277} are important for PKC-dependent 3BP2 phosphorylation, COS-1 cells were transfected with plasmids encoding HA-tagged 3BP2 or S225A or S277A mutants, and an in vitro kinase assay was performed as described above. c-Cbl was used here as substrate to control PKC activity under our experimental conditions. Fig. 6D shows a reduced level of phosphorylation of 3BP2 when serine 225 or 277 was mutated to alanine. Apparently, the effect of mutation of serine 277 to alanine had a greater impact on 3BP2 phos-
Fig. 6. Serine 277 in 3BP2 is critical for 14-3-3 binding. A, identification of 14-3-3 consensus binding motifs in 3BP2. Shown is the alignment of PR1, PR2, and PR3 amino acid sequences of murine and human 3BP2. Two conserved binding sites for 14-3-3 on 3BP2 are boxed, and the putative phosphorylated serine residues indicated. B, yeast two-hybrid interactions. The indicated serine-to-alanine 3BP2 mutants were fused to the DNA-binding domain of LexA, whereas 14-3-3 constructs were fused to the Gal4 activation domain (GAD). Yeast were co-transformed with the indicated plasmid combinations, and the interactions were assayed using a β-galactosidase filter assay. +, no interaction; +, strong interaction. Similar results were obtained by assessing yeast growth on histidine-deficient medium. A LexA-lamin expression vector was used as a negative control. C, Jurkat TAg cells were transfected with 10 μg of empty pMT3 expression vector or the indicated 3BP2-cDNA constructs. The cells were unstimulated or stimulated with PMA (100 ng/ml) for 15 min at 37°C. 3BP2 immunoprecipitates were resolved by SDS-7.5% PAGE and transferred onto nitrocellulose membrane. The membranes were incubated with GST-14-3-3, and binding was detected with anti-GST antibody and ECL. The membrane was then stripped and reprobed with anti-3BP2 antibody. D, HA-tagged 3BP2 constructs or HA-tagged c-Cbl were expressed in COS-1 cells and immunoprecipitated with anti-HA and protein G-Sepharose beads. The immunoprecipitates were incubated with a purified catalytic subunit of rat PKC (0.1 unit/ml) and with 20 μCi of [γ-32P]ATP at 30°C for 1 h. The samples were run on SDS-PAGE (7.5% gel); the gel was then dried and exposed to film. After autoradiography, the membrane was subjected to immunoblot analysis using anti-HA antibody. IP, immunoprecipitation.

Phosphorylation by PKC, with an ∼50% decrease, an effect consistent with the decreased binding of 14-3-3 shown above. Together, this suggests that serines 225 and 277 are required for optimal 3BP2 phosphorylation by PKC.

14-3-3 Negatively Regulates 3BP2-induced NFAT Activation in Lymphoid Cells—3BP2 is a positive regulator of NFAT activity in T cells (9). The effect of 14-3-3 expression on 3BP2 activity was investigated in transfection assays using a NFAT reporter gene. Jurkat TAg cells were co-transfected with plasmids encoding NFAT reporter construct and wild-type 3BP2, 3BP2 S277A, 14-3-3, or a combination of these plasmids. As previously described, 3BP2 overexpression potentiated NFAT activity, whereas 14-3-3 had the opposite effect (Fig. 7A). The expression of the S277A mutant, which shows reduced binding to 14-3-3, led to a stronger activation of NFAT relative to wild-type 3BP2. On the other hand, 14-3-3 overexpression completely prevented NFAT activation induced by 3BP2, whereas it only reduced by 50% the activity of the S277A mutant. Immunoblot analysis with anti-3BP2 and anti-HA antibodies showed similar levels of protein expression. To ascertain this finding in another cell type, 3BP2 plasmids were transfected in Daudi B cells, together with the NFAT reporter. As shown in Fig. 7B, expression of 3BP2 S277A mutant in these cells led to a dramatic increase of NFAT activation, compared with the effect of wild-type 3BP2.

PMA stimulation increased 14-3-3 binding to 3BP2 as demonstrated in previous sections. We therefore questioned the effect of PMA on 3BP2-induced NFAT activation. Jurkat cells were electroporated with plasmids encoding for NFAT reporter and wild-type 3BP2. One hour after the transfection, the cells were washed and resuspended in culture medium containing or not 50 ng/ml of PMA. The luciferase activities were then determined 24 h after. Fig. 7C shows that PMA stimulation causes a 55% decrease of 3BP2-induced NFAT activation, whereas it did not affect 3BP2 expression. This suggests that the PKC phosphorylation might mediate 14-3-3 binding on 3BP2 and then inhibit its positive effect on NFAT activity. Together, these results suggest that 14-3-3 binding to 3BP2 following PMA stimulation negatively regulates its positive adapter function in lymphoid cells.

DISCUSSION

In the present study, we found that the cytoplasmic adapter protein 3BP2 (also known as SH3BP2) specifically and directly associates in vitro and in vivo with 14-3-3 proteins. This interaction, which is constitutively detected in yeast and in mammalian cells, is enhanced by phorbol ester stimulation and co-expression of different serine/threonine kinases, including PKC. This interaction most likely requires the phosphorylation of 3BP2 by upstream kinases because we found that: (i) treatment of 3BP2 by alkaline phosphatase disrupts the 14-3-3/3BP2 interaction, (ii) in vitro 3BP2 is a substrate of purified PKC, and (iii) point mutation of two serine residues (Ser225 and Ser277) disrupts 14-3-3/3BP2 interaction and decreased PKC-dependent 3BP2 phosphorylation. Functionally, a 3BP2 mutant protein incapable of binding 14-3-3 was a more potent activator of NFAT transcriptional activities than the wild-type protein, suggesting that 14-3-3 binding to 3BP2 following phosphorylation might mediate 14-3-3 binding on 3BP2 and then inhibit its positive effect on NFAT activity. Together, these results suggest that 14-3-3 binding to 3BP2 following PMA stimulation negatively regulates its positive adapter function in lymphoid cells.

14-3-3 constitutes a highly conserved and ubiquitously expressed family of proteins. They function as dimeric scaffold proteins involved in the formation of complexes with serine phosphorylated proteins (15, 24). A consensus sequence that mediates phosphorylation-dependent association to 14-3-3 was previously described, 3BP2 overexpression potentiated NFAT transcriptional activities than the wild-type protein, suggesting that 14-3-3 binding to 3BP2 following phosphorylation might mediate 14-3-3 binding on 3BP2 and then inhibit its positive effect on NFAT activity. Together, these results suggest that 14-3-3 binding to 3BP2 following PMA stimulation negatively regulates its positive adapter function in lymphoid cells.

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which was initially found in Cbl (25), whereas the second sequence RXfP577XP represents an almost perfect match of the consensus 14-3-3 binding sequence found in several 14-3-3 binding proteins including Cdc25c, PKC, and Raf-1 (16). This second motif differs from the consensus only by the absence of a serine residue in position –2 relative to the putative phosphorylated serine residue. Nevertheless, Western ligand blot and yeast two-hybrid analysis of 3BP2 point mutations showed that a mutant of 3BP2 in which serine 277 was replaced by a nonphosphorylatable alanine (S277A) failed totally to bind 14-3-3, whereas 3BP2 Ser255 mutant only displayed a decreased ability to bind 14-3-3. Several 14-3-3 binding proteins such as Raf-1, c-Cbl, and BAD contain two 14-3-3 binding sequences separated by polypeptides fragments of various length, and tandem binding to adjacent 14-3-3 sites has been shown to facilitate the formation of high affinity dimeric complexes (24). Recently, it has been postulated that a first site called “gatekeeper” is indispensable for a stable 14-3-3 interaction, whereas a second site enhances the interaction but has too weak an affinity to bind 14-3-3 alone (24). Consistent with this model, our studies suggest that 3BP2 Ser277 residue might constitute the gatekeeper residue, whereas Ser255 might represent the “enhancer” residue, to facilitate 14-3-3 dimer fixation on 3BP2. Additional experiments are clearly needed to ascertain the mechanism of 14-3-3/3BP2 interaction. For example, it would be interesting to perform phosphoamino acid analysis on serine-mutated 3BP2 proteins.

Our studies do not identify the Ser/Thr kinases that directly phosphorylate 3BP2 to increase its association with 14-3-3 proteins. However, they provide ample evidence that 3BP2 interacts with 14-3-3 via a serine phosphorylation-dependent mechanism and that PKC is one of the Ser/Thr kinases phosphorylating 3BP2 to facilitate this association. The two 14-3-3 binding motifs identified by our experiments contain a basic arginine residue in position –3 relative to the putative phosphorylated serine residue. Such a sequence could represent a consensus phosphorylation motif for various Ser/Thr kinases, including PKA, PKC, or calmodulin-dependent protein kinase II (28). Indeed, co-expression of 3BP2 with constitutive active PKA and PKC in COS-1 cells enhanced 3BP2/14-3-3 interaction. Our observation that co-expression of active Akt (PKB) also led to a stronger interaction between 14-3-3 and 3BP2 is the likely result of an indirect phosphorylation event because 3BP2 does not contain a consensus phosphorylation sequence as previously defined (29). Moreover, the basal interaction between 3BP2 and 14-3-3 was reinforced following cell stimulation with PMA, a known PKC activator, and treatment of wild-type 3BP2 with alkaline phosphatase significantly reduced the amount of 3BP2 that binds to 14-3-3. In addition, 3BP2 was phosphorylated by purified rat brain PKC in vitro, and a reduced phosphorylation of 3BP2 by PKC was observed when serine 277 was mutated to alanine, indicating that this residue is a direct target for PKC at least in vitro. This effect is also consistent with a marked decreased binding of 14-3-3 to the 3BP2 S277A mutant. Finally, stimulation of Jurkat T cells with PKA agonists (forskolin and isobutylmethylxanthine) also increased the association between 14-3-3 and 3BP2, suggesting that 3BP2 can be directly or indirectly phosphorylated by different Ser/Thr kinases to induce its interaction with 14-3-3.

3BP2 was initially cloned as an Abl SH3-binding protein of unknown function (10). We next identified 3BP2 as a Syk kinase-interacting protein that plays a positive adapter func-
tion on NFAT and AP-1 transcriptional activation through the binding of its SH2 domain to the T cell signaling proteins (9). More recently, a positive regulatory function of 3BP2 downstream surface receptors coupled to cytoplasmic PTKs was also reported in NK (12) and mast cells (13). In addition, our studies have shown that 3BP2 is tyrosine-phosphorylated by several PTKs, including Syk and Fyn (data not shown). Thus, 3BP2 adapter functions appear to be linked to PTK-dependent signaling pathways through the capacity of its SH2 domain to interact with tyrosine-phosphorylated proteins and by being itself a PTK substrate. Our findings that 3BP2 also represents a target for serine/threonine kinases such as PKC and binds several 14-3-3 isoforms (namely 14-3-3e, ε, and θ isoforms) suggest that 3BP2 could integrate PTK- and PKC-dependent signaling pathways in various cell types, including lymphocytes. It is interesting to note that in T cells a similar function was attributed to c-Chi (25), and PLCγ (30), two signaling proteins found to interact with 3BP2 SH2 domain (9). However, the role of 3BP2 serine phosphorylation is presently unclear. Our studies show that, relative to wild-type 3BP2, the S277A mutant, which is incapable of binding 14-3-3, was a more potent activator of NFAT in both T and B cells. Also, under conditions promoting the association between 14-3-3 and 3BP2, PMA stimulation decreased 3BP2-induced NFAT activation in Jurkat T cells. Together, these results suggest that 14-3-3 may negatively regulates 3BP2 adapter function on the signaling pathways leading to NFAT transcriptional activities. In our model, activation of Ser/Thr kinases like PKC (or PKA) following receptor stimulation would lead to serine phosphorylation of 3BP2. This would allow the direct binding of 14-3-3 dimers on 3BP2 (Fig. 8). Subsequent events leading to inhibition of 3BP2 function in NFAT pathway by 14-3-3 remain elusive. 14-3-3 binding could modify 3BP2 subcellular localization in a manner similar to the regulation of PKC9 (18) or the Ras effector RIN1 (31). Alternatively, the binding of 14-3-3 to 3BP2 could prevent the formation of 3BP2-associated protein complexes, including proteins like Syk family PTKs (9), Vav1 (Ref. 12 and our unpublished observations), PLCγ (9, 12, 13), and LAT (9, 13), which are required for signal transduction.

Growing evidence supporting the importance of adapter proteins has been presented in numerous signal transduction cascades (3, 4). Together with our previous finding that 3BP2 regulates a Ras- and a calcineurin-dependent pathways, respectively, required for AP-1 and NFAT activation in T cells (9), the results reported in the present study indicate that 3BP2 may also play an important role as an integrator of multiple signal-