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Regulation of NK Cell-Mediated Cytotoxicity by the Adaptor Protein 3BP2

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Stimulation of lymphocytes through multichain immune recognition receptors activates multiple signaling pathways. Adaptor proteins play an important role in integrating these pathways by their ability to simultaneously bind multiple signaling components. Recently, the 3BP2 adaptor protein has been shown to positively regulate the transcriptional activity of T cells. However, the mechanisms by which signaling components are involved in this regulation remain unclear, as does a potential role for 3BP2 in the regulation of other cellular functions. Here we describe a positive regulatory role for 3BP2 in NK cell-mediated cytotoxicity. We also identify p95SLP-76 and phospholipase C-γ isomers as binding partners of 3BP2. Our results show that tyrosine-183 of 3BP2 is specifically involved in this interaction and that this residue critically influences 3BP2-dependent function. Therefore, 3BP2 regulates NK cell-mediated cytotoxicity by mobilizing key downstream signaling effectors.


Natural killer cells are CD16+CD56+, TCR−, and surface Ig− lymphocytes with multiple functions in the immune response, including the secretion of regulatory cytokines and the ability to directly kill certain malignant or virus-infected cells (1). One type of NK cell-mediated cytotoxicity is Ab-dependent cell-mediated cytotoxicity (ADCC), in which Fc receptors on an NK cell recognize an Ab-coated target cell. As a result of this interaction, the NK cell activates proximal Src (2, 3) and Syk (4, 5) family protein tyrosine kinases (PTKs), which in turn stimulate downstream effectors such as phospholipase C (PLC)-γ (6, 7) and the Rho family of GTP-binding proteins (8, 9). Subsequent reorganization of the cytoskeleton and activation of multiple downstream signaling elements results in the polarization of NK cytoxic granules and secretion of their contents (including perforin and granzyme B) onto the target cell. The general FcR structure and the signaling cascade that leads to this cytotoxic response are in many ways similar to receptors and cascades involved in the activation of B and T cells through sIg and TCR, respectively. This similarity led to the classification of surface Ig, TCR, and FcR into the family of so-called multichain immune recognition receptors (MIRR) (10). Another type of NK cell-mediated cytotoxicity is initiated by direct MHC-nonrestricted recognition of target cells.

This is often called natural cytotoxicity because no sensitization of NK cells is necessary and no memory develops. Although multiple potential triggering molecules have recently been identified on NK cells (reviewed in Ref. 11), the relationship of these and their ligands to the development of natural cytotoxicity remains incompletely characterized. However, available data indicate that signaling pathways used by natural cytotoxicity receptors are largely similar to those used by the FcR.

Adaptor proteins play a critical role in the regulation of multiple cellular functions such as proliferation, transcriptional regulation, and reorganization of the cytoskeleton (reviewed in Ref. 12). Adaptor proteins have no catalytic activity, but due to the presence of multiple phosphorylation sites and/or modular structure, they are able to physically bridge other signaling components. This brings downstream signaling effectors in close proximity to the upstream activators, which results in signal propagation and amplification. Activation of lymphocytes through MIRR uses multiple adaptor proteins. For example, Grb2, via its interaction with the guanine nucleotide exchange factor (GEF) Sos, links the GTP-binding protein Ras to upstream signaling complexes (reviewed in Ref. 13). Another adaptor protein Src homology (SH) 2 domain-containing leukocyte protein of 76 kDa (SLP-76) (14) and its homologue B cell linker protein (BLNK)/SLP-65 (15) are involved in the activation of PLC-γ, an enzyme necessary for receptor-initiated increases in the concentration of intracellular calcium (reviewed in Ref. 16). The calcium signal regulates multiple other enzymes, transcription factors, and cytoskeletal proteins. The transmembrane adaptor linker for activation of T cells (LAT) is present in specific glycosphingolipid microdomains (lipid rafts) (17) that coalesce during MIRR cross-linking. Phosphorylated LAT mobilizes PLC-γ and Grb2 into the signaling complex (18). We have previously described roles for LAT and SLP-76 during the development of NK cell-mediated cytotoxicity (19, 20).

The adaptor protein 3BP2 has been identified as a protein that interacts with the SH3 domain of the PTK Abl (21). Human 3BP2 is a 561-aa protein containing an N-terminal pleckstrin homology (PH) domain, an SH3-binding proline-rich region, and a C-terminal SH2 domain. Recently, the SH2 domain of 3BP2 has been shown to bind to the PTK Syk and the adaptor protein LAT in T...
cells (22). In addition, 3BP2 overexpression in T cells leads to increased NFAT- and AP-1-dependent transcription that uses the calcium-dependent phosphatase calcineurin and the G protein Ras. This increase requires functional PH and SH2 domains of 3BP2. However, it remains unclear which downstream molecules are targets for 3BP2 as does the mechanism of their mobilization. The potential role of 3BP2 in other cellular functions, such as cell-mediated cytotoxicity, also remains to be determined. In this paper, we show that 3BP2 is biochemically and functionally linked to the activating receptors on NK cells. 3BP2-dependent regulation of NK cell-mediated cytotoxicity depends on the PH, SH2, and proline-rich regions of 3BP2. We also identify PLC-γ and p95SH2 (Vav) molecules as binding partners of 3BP2. Our data suggest that the tyrosine-183 of 3BP2 and the SH2 domains of Vav and PLC-γ are specifically involved in this interaction. Importantly, mutation of the tyrosine-183 site abrogates the ability of 3BP2 to positively regulate NK cell-mediated killing. Based on our results, we conclude that 3BP2 has a key regulatory role during the development of NK cell-mediated cytotoxicity.

Materials and Methods

Reagents, cells, and Abs

Unless otherwise indicated, all chemicals were obtained from Sigma (St. Louis, MO). Human NK clones were cloned and passed as previously described (23). P. Cresswell (Yale University, New Haven, CT) and P. Farham (Stanford University, Palo Alto, CA) kindly provided the HLA class I-deficient C1R and 721.221 cell lines, respectively. Lysates from fibroblastic GMK47 and glioma Mo59K cell lines were obtained from L. Karnitz (Mayo Clinic, Rochester, MN). Anti-phosphotyrosine murine mAb 4G10 was purchased from Upstate Biotechnology (Lake Placid, NY), anti-Flag murine mAb FLAG-M2 from Sigma, and goat anti-mouse IgG F(ab')2 from ICN Pharmaceuticals (Aurora, OH). Anti-FcγRIII murine mAb 3G8 and anti-EE murine mAb were purified from ascites by affinity chromatography over protein A-agarose. Rabbit polyclonal antisera to 3BP2 were obtained from Cocalico Biologicals (Reamstown, PA) following immunization of rabbits with keyhole limpet hemocyanin-conjugated 3BP2 peptide 165–190 (SPYTPDNEYEHDDDESDYLDPEPSE), 345–370 (PTSEPPPVPANKP KFLKIAEEDPPRE), and 425–450 (SFSFEDNKPRQPSQADTGGDDSDYEPYE). An additional anti-3BP2 Ab obtained by immunizing rabbits with keyhole limpet hemocyanin-conjugated 3BP2 peptide 175–195 (biotin-NH2-HDDESDYLDPEPSE) was obtained from goat anti-3BP2 Ab obtained by immunizing rabbits with protein A-agarose. Rabbit polyclonal antisera to 3BP2 were obtained from Dr. A. Altman (La Jolla Institute for Allergy and Immunology, San Diego, CA). Anti-Vav-1 (9), Vav-2 (24), PLC-γ1 (6), PLC-γ2 (6), and LAT (19) Abs have been previously described.

DNA constructs and recombinant vaccinia virus

The 3BP2 sequence was amplified from cDNA prepared from NK cells using the following primers: 5'-CGGGGAGCAGCCATCGCCCGCGT GGA-3' and 5'-GGACTGAGATCTCCTAGGCCCAGTGTAGCC-3'. The amplified 3BP2 cDNA from reverse-transcribed NK RNA. The DNA sequence obtained matched the full-length 3BP2 sequence which of the bands corresponds to the full-length 3BP2, we amplified the 3BP2 cDNA from reverse-transcribed NK RNA. The 3BP2 cDNA was subcloned into pCR2 vector and EE-tagged at the 3'-end. The fragment was then subcloned into pSHN11 vaccinia recombination substrate. Using the recombinant vaccinia virus recombination substrate. Using the recombinant vaccinia virus recombination substrate.

Cell stimulation and immunoblot analysis

Cells were stimulated as previously described (25) and lysed in buffer containing 20 mM Tris-HCl, 40 mM EDTA, 50 mM NaF, 30 mM Na3VO4, 0.1% BSA, 1 mM NaVO3, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 1% Triton X-100 (pH 7.4). For immunoprecipitation experiments, buffer containing 50 mM Tris-HCl, 10 mM EDTA, and 1% Nonidet P-40 (pH 8.0) was used. Cell lysates were subjected to immunoprecipitation for 1–2 h with indicated Abs bound to protein A-Sepharose (rabbit Ab) or anti-mouse IgG-agarose (mouse Ab). After 1–2 h of affinity binding, beads were washed and bound proteins eluted with 4X sample buffer. These fractions were then separated by SDS-PAGE, transferred to Immobilon-P membrane (Millipore, Bedford, MA), and probed with specific Abs. For affinity purification of proteins specifically binding to phosphorytine-183 of 3BP2, phosphorylated and nonphosphorylated 3BP2 peptides 175–195 (biotin-NH2-HDDESDYLDPEPSE) were synthesized. As a control, the peptide encompassing phosphotyrosine-153 (biotin-NH2-SSSDTDFS(p)YGAVERVDPDSC-LCOOH) was used. These peptides were bound to streptavidin-agarose and incubated with cell lysates (Nonidet P-40-containing buffer), after which beads were washed and treated as described. In dephosphorylation experiments, beads were washed twice with RIPA buffer (0.1% SDS and 1% deoxycholate acid (pH 7.4)) and once with dephosphorylation buffer (New England Biolabs, Beverly, MA) after immunoprecipitation. Beads were then resuspended in 50 μl of dephosphorylation buffer containing 200 U of calf intestinal alkaline phosphatase (CIP; New England Biolabs) and incubated at 37°C for 3 h. The reaction was stopped with 1 μl of RIPA buffer, and the proteins were eluted using 4X sample buffer. Samples were then treated as described. In Far Western experiments, biotinylated peptides were used to probe proteins on the membrane. For the detection of primary Abs or biotinylated peptides on the membrane, protein A-HRP (Amersham, Little Chalfont, U.K.), anti-mouse-HRP (Amersham) or streptavidin-HRP (Pierce, Rockford, IL) were used together with the ECL detection system (Amersham).

Cytotoxicity assays

The 51 Cr release assays measuring direct NK cell-mediated cytotoxicity or reverse ADCC were performed as previously described (23). Briefly, NK cells were infected with vaccinia virus-expressing 3BP2 constructs and then incubated with 51 Cr labeled target cells for 3–4 h at 161, 81, 41, and 2:1 E:T ratios. Released 51 Cr in the supernatant was measured in a gamma counter. For reverse ADCC, Fc-receptor positive P815 murine cells were used as targets in the conjugation with murine Abs specific for 3BP2. The conjugate was used to detect the presence of human NK cells (3G8; 0.15 μg/ml). No killing of P815 was observed in the absence of stimulating Ab. All results are expressed as LUs/106 effector cells.

Results

Ubiqutously expressed 3BP2 has different isoforms

Controversial reports regarding the expression of 3BP2 in different tissues (22, 26) prompted us to determine the expression pattern of 3BP2 in human cell lines representing different tissue types. We obtained four different rabbit anti-human 3BP2-specific Abs and tested several human cell lines, including hematopoietic, fibroblastic, and neuronal cells for the expression of 3BP2. As shown in Fig. 1A, 3BP2-specific Ab recognizes a 75-kDa band in all of the cell lines tested. Interestingly, in most of the cell lines, we observed a doublet with each part running very close to the other, whereas some cell lines (Jurkat and P116 (data not shown)) had a triplet when membranes were probed with anti-3BP2 Abs. This doublet/triplet pattern was also observed using three other Abs made to different regions of 3BP2 (data not shown). To determine which of the bands corresponds to the full-length 3BP2, we amplified the 3BP2 cDNA from reverse-transcribed NK RNA. The DNA sequence obtained matched the full-length 3BP2 sequence from the GenBank. The 3BP2 cDNA was then subcloned into a vaccinia virus recombination construct. Using the recombinant 3BP2 vector and EE-tagged at the 3'-end. The fragment was then subcloned into pSHN11 vaccinia recombination substrate.
vaccinia virus, 3BP2 was then overexpressed in NK and Jurkat T cells. As shown in Fig. 1B, the recombinant full-length 3BP2 corresponds to the upper band of the doublet or the middle band of the triplet. We then wanted to test whether these different isoforms may exist due to different posttranscriptional modification of 3BP2. Jurkat T cells were stimulated with the phosphatase inhibitor pervanadate, and 3BP2 and Lck proteins were immunoprecipitated using specific rabbit antisera. Pervanadate treatment inhibited phosphorylation. We stimulated NK cells with anti-FcR Ab and immunoprecipitated endogenous 3BP2 from the lysates. As shown in Fig. 2A, 3BP2 is tyrosine-phosphorylated upon stimulation of NK cells through the FcR. In a separate experiment, we found that this phosphorylation peaks at 1 min and rapidly decreases to baseline levels within 10 min (Fig. 2B), resembling the kinetics of other tyrosine-phosphorylated proteins (e.g., ζ-chain-associated protein of 70 kDa (ZAP-70), Syk, LAT, Vav, and PLC-γ) upon FcR cross-linking. We also tested whether 3BP2 is coupled to activating receptors in NK cells that mediate natural cytotoxicity. Because 3BP2 is ubiquitously expressed (including target cells we use for stimulation), we used vaccinia virus to overexpress EE-tagged 3BP2 in NK cells to specifically immunoprecipitate 3BP2 from NK cells. Following the infection with the 3BP2-encoding recombinant vaccinia virus, NK cells were incubated with NK-sensitive B lymphoblastoid cell line C1R. As shown in Fig. 2C, 3BP2 becomes tyrosine-phosphorylated following a 5-min incubation of NK cells with C1R targets. Similar results were obtained using the other NK-sensitive B lymphoblastoid cell line 721.221 (data not shown). In contrast, incubation of NK cell clones with the NK-resistant cell line P815 did not induce tyrosine phosphorylation of 3BP2 (data not shown). These data together suggest that 3BP2 is coupled to activating receptors on NK cells.

**3BP2 coimmunoprecipitates other signaling molecules following FcR cross-linking on NK cells**

The SH2 domain of 3BP2 has been shown to bind several tyrosine-phosphorylated proteins, including LAT and ZAP-70, upon TCR cross-linking of Jurkat T cells (22). In attempt to get a better understanding of the role of 3BP2 in NK cell activation, we analyzed 3BP2 immunoprecipitates from FcR-stimulated NK cells for associated signaling molecules. To examine coassociation of 3BP2 with other signaling molecules upon stimulation of NK cells through the FcR, we used a lysis buffer containing Nonidet P-40 instead of Triton X-100, because Nonidet P-40 preserves more...
protein-protein interactions. Fig. 3 shows that, upon FcR-cross-linking of NK cells, 3BP2 associates with several tyrosine-phosphorylated molecules of 35, 70, 90, 110 and 150 kDa. A similar set of tyrosine-phosphorylated proteins was associated with endogenous and overexpressed 3BP2 after FcR stimulation (Fig. 3A, compare left and right panels). Preclearing of 3BP2 immunoprecipitates with specific Abs showed that the 35- and 90-kDa bands correspond to LAT and Vav-1 (Fig. 3B). Therefore, these data indicate that FcR stimulation of NK cells leads to the formation of a complex in which 3BP2 is associated with a number of signaling molecules involved in the regulation of the NK cell cytotoxic response.

**SH2 domain of 3BP2 is necessary for its optimal tyrosine phosphorylation and coupling to LAT**

3BP2 has three known functional domains: a PH, a proline-rich, and an SH2 domain. We were interested in determining which one of these domains targets 3BP2 to the activating signaling complex. For that purpose, we designed three mutants of 3BP2: PH and SH2 deletion mutants (PH- and SH2-), respectively, and a proline-rich region mutant in which 14 prolines (7 from each of the 2 close proline-rich sequences) were mutated into alanine (P-A). All of the constructs were EE-tagged at the C terminus. NK cells were infected with vaccinia viruses encoding various mutants of 3BP2, stimulated through FcR, and different 3BP2 mutants were immunoprecipitated from the lysate using the anti-EE Ab. The immunoprecipitates were resolved by SDS-PAGE, transferred to a membrane, and probed with anti-phosphotyrosine (P-Tyr) or anti-EE mAb.

**FIGURE 2.** 3BP2 is tyrosine phosphorylated following the cross-linking of activating receptors on NK cells. A, Human NK cells (20 × 10⁶/sample) were stimulated with anti-FcR mAb 3G8 (1 μg/ml) for 1 min. Following the stimulation, lysates were incubated with protein A-bound anti-3BP2 serum or normal rabbit serum (as a control). Eluates were separated by SDS-PAGE, transferred to a nylon membrane, and probed with either anti-phosphotyrosine mAb 4G10 (upper panel) or anti-3BP2 serum (lower panel). B, The same as in A, except that NK cells were stimulated with anti-FcR mAb for indicated times. C, Human NK (10 × 10⁶/sample) cells were infected with EE-tagged wild-type 3BP2 (at 20:1 MOI for 6 h) and incubated with C1R target cells at 37°C for 5 min. 3BP2 was immunoprecipitated from the lysates using anti-EE mAb. Eluates were subjected to SDS-PAGE, transferred to the membrane, and probed with anti-phosphotyrosine (P-Tyr) or anti-EE mAb.

**FIGURE 3.** 3BP2 associates with multiple tyrosine-phosphorylated proteins upon stimulation of NK cells through the FcR. A, Left panel, Human NK cells (20 × 10⁶/sample) were stimulated, lysed in Nonidet P-40-containing buffer, and subjected to immunoprecipitation, SDS-PAGE, and blotting as in Fig. 2A. Right panel, NK cells (10 × 10⁶/sample) were infected with the control vaccinia virus (WR) at 20:1 MOI for 6 h or with the virus encoding the EE-tagged wild-type 3BP2. Infected cells were stimulated with anti-FcR mAb 3G8 (1 μg/ml) for 1 min and lysed in Nonidet P-40-containing buffer. Lysates were subjected to anti-EE immunoprecipitation (IP). Eluates were separated by SDS-PAGE, transferred to a membrane, and probed with anti-phosphotyrosine (P-Tyr) or anti-EE mAb (right panel). The large arrows identify 3BP2 or 3BP2.EE, and the small arrows identify associated proteins. B, Uninfected NK cells (20 × 10⁶/sample) were stimulated with anti-FcR mAb 3G8 for 1 min, and Nonidet P-40 lysates were precleared with anti-LAT or anti-Vav-1 Ab (lane 3) or anti-Vav-1 Ab (lane 4). After preclearing, lysates were incubated with the control rabbit serum or with anti-3BP2 serum. Eluates were subjected to SDS-PAGE, transferred to a membrane, and probed with anti-phosphotyrosine (P-Tyr) or anti-3BP2 serum.
found that the SH2 mutant does not associate with LAT, whereas all of the other mutants do (Fig. 4B). Therefore, the SH2 domain of 3BP2 is required for optimal tyrosine phosphorylation of 3BP2 following FcR cross-linking and for its ability to associate with the transmembrane adaptor protein LAT.

3BP2 regulates NK cell-mediated cytotoxicity

We have shown so far that 3BP2 takes part in the biochemical signaling initiated from the activating receptors on the surface of NK cells. To determine whether 3BP2 plays a functional role in the regulation of NK cell-mediated cytotoxicity, human NK cell clones were infected with the vaccinia virus (WR) or viruses encoding the EE-tagged wild-type or mutant 3BP2 proteins. Cells were then stimulated with anti-FcR mAb 3G8 (1 μg/ml) for 1 min and lysed in Triton X-100 (A) or in Nonidet P-40-containing buffer (B). Lysates were then subjected to immunoprecipitation using anti-EE mAb. Eluates were subjected to SDS-PAGE, transferred to a nylon membrane, and probed with either anti-phosphotyrosine (P-Tyr) or anti-EE mAb.

**FIGURE 4.** The SH2 domain is necessary for optimal 3BP2 phosphorylation and association with tyrosine-phosphorylated LAT. Human NK cells (10 × 10^6/sample) were infected at 20:1 MOI for 6 h with the control vaccinia virus (WR) or viruses encoding the EE-tagged wild-type or mutant 3BP2 proteins. Cells were then stimulated with anti-FcR mAb 3G8 (1 μg/ml) for 1 min and lysed in Triton X-100 (A) or in Nonidet P-40-containing buffer (B). Lysates were then subjected to immunoprecipitation using anti-EE mAb. Eluates were subjected to SDS-PAGE, transferred to a nylon membrane, and probed with either anti-phosphotyrosine (P-Tyr) or anti-EE mAb.

**FIGURE 5.** 3BP2 regulates NK cell-mediated cytotoxicity. NK cells were infected as in Fig. 4. A, Infected cells were incubated with 51 Cr-labeled P815 cells and anti-FcR Ab (reverse ADCC assay) for 3 h. Results are expressed as LU/10^6 effector cells. The levels of recombinant proteins were determined by Western blot analysis of 3BP2 immunoprecipitates using anti-3BP2 serum. B, Infected cells were incubated with 51 Cr-labeled C1R and 721.221 target cells (natural cytotoxicity assay). The levels of recombinant proteins were the same as in A because aliquots were derived from the same vaccinia-infected samples.

Phosphorylated tyrosine-183 of 3BP2 binds PLC-γ and Vav

3BP2 binding of phosphorylated LAT requires its SH2 domain (22, Fig. 4B). However, the mechanism by which 3BP2 associates with the GEF Vav-1 remains unknown. Vav-1 acts downstream of PTKs and known adaptor proteins, and its SH2 domain is required for coupling to the upstream regulators (reviewed in Ref. 28). We speculated that the association of Vav-1 with 3BP2 may be direct and that tyrosine residue(s) of 3BP2 may be involved in this interaction. Supporting this contention, tyrosine-183 of 3BP2 is part of the sequence YLEP, previously described to be a consensus binding site for the Vav-1 SH2 domain (29, 30). To test whether Vav-1 might interact with phosphorylated tyrosine-183, we made synthetic 3BP2 peptides containing the phosphorylated or nonphosphorylated tyrosine-183. Lysates of nonstimulated or pervanadate-stimulated NK cells were incubated with the biotinylated peptides bound to streptavidin-agarose beads. Affinity purified fractions were then subjected to SDS-PAGE, transferred to a nylon membrane, and probed with the anti-phosphotyrosine Ab. As shown in Fig. 6A, two major tyrosine-phosphorylated bands of ~90 and 150 kDa were found to associate with the phosphorylated, but not the nonphosphorylated, peptide. The same two tyrosine-phosphorylated bands were detected if, instead of pervanadate, NK cells were stimulated with anti-FcR mAb (data not shown). Reblotting the membrane with Vav-1- and PLC-γ-specific antisera revealed bands overlapping with phosphorylated p90 and p150, respectively. When the peptide containing phosphorylated tyrosine-153 was used for affinity purification of NK cell lysates after...
FcR stimulation, no specific phosphorylated proteins were detected (Fig. 6B). This supports the specificity of the interaction between phosphotyrosine-183 of 3BP2 and Vav-1 or PLC-γ proteins. To test more specifically which isoforms of Vav and PLC-γ bind to phospho-Y183 peptide, the experiment was repeated using Vav-1-, Vav-2-, PLC-γ1-, and PLC-γ2-specific immunoprecipitates as controls. Fig. 6C and D, clearly shows that all four molecules can specifically bind to the phosphorylated, but not to the nonphosphorylated Y183 peptide. Finally, to determine whether this interaction between the phosphotyrosine-183 of 3BP2 and the Vav and PLC-γ molecules is truly direct, we infected Jurkat T cells with vaccinia viruses expressing FLAG-tagged wild-type or SH2 domain mutants of Vav-1 and Vav-2 (F.Vav-1.wt, F.Vav-1.R696A, F.Vav-2.wt, and F.Vav-2.R698A) or with the vaccinia viruses expressing the wild-type PLC-γ1 and PLC-γ2 proteins. Vav and PLC-γ isoforms were then immunoprecipitated using anti-FLAG mAb M2, anti-PLC-γ1, or anti-PLC-γ2 serum. Eluates were subjected to SDS-PAGE, transferred to a membrane, and probed with the biotinylated peptide containing phosphorylated tyrosine-183, which was then visualized by streptavidin-HRP (Far Western assay). After stripping the peptide, the membrane was probed with anti-FLAG mAb.

FIGURE 6. Phosphotyrosine-183 of 3BP2 selectively binds Vav and PLC-γ molecules. A, Human NK cells (10^6/sample) were left unstimulated or stimulated with pervanadate for 5 min. Lysates were incubated with empty streptavidin-agarose beads (−) or beads with biotinylated peptides encompassing 3BP2 tyrosine-183 that was phosphorylated (183-P) or nonphosphorylated (183). Eluates were subjected to SDS-PAGE, transferred to a membrane, and probed with anti-phosphotyrosine (P-Tyr) mAb, anti-PLC-γ1, anti-PLC-γ2, or anti-Vav-1 serum. B, The same as in A, except that cells were stimulated with anti-FcR mAb, and the negative control was phosphorylated peptide encompassing the tyrosine-153 of 3BP2. C, Lysates of unstimulated NK cells were incubated with beads bound to phosphorylated or nonphosphorylated 183 peptides as well as with protein A-Sepharose bound to anti-PLC-γ1 or anti-PLC-γ2 serum. Eluates were subjected to SDS-PAGE, transferred to a membrane, and probed with anti-PLC-γ1 or anti-PLC-γ2 serum. D, The same as in C, except that anti-Vav-1 and Vav-2 sera were used. E, Jurkat T cells (3 × 10^6/sample) were infected at MOI 10:1 for 2 h with the control vaccinia virus (WR, lane 1) or with viruses encoding FLAG-tagged wild-type Vav-1 and Vav-2 (lanes 2 and 4), SH2 domain mutants of Vav-1 and Vav-2 (lanes 3 and 5), or nontagged PLC-γ1 (lane 6) or PLC-γ2 (lane 7). After lysis of infected cells, proteins were immunoprecipitated using anti-FLAG mAb M2, anti-PLC-γ1, or anti-PLC-γ2 serum. Eluates were subjected to SDS-PAGE, transferred to a membrane, and probed with the biotinylated peptide containing phosphorylated tyrosine-183, which was then visualized by streptavidin-HRP (Far Western assay). After stripping the peptide, the membrane was probed with anti-FLAG mAb.
Tyrosine-183 is phosphorylated and binds Vav and PLC-γ during activation of NK cells through natural cytotoxicity receptors

We have shown that the tyrosine-183 of 3BP2 can bind to Vav and PLC-γ isoforms. We next wanted to test whether this residue mediates binding of 3BP2 to Vav and PLC-γ in vivo during natural cytotoxicity. For that purpose, NK cells were infected with the control vaccinia virus or with the vaccinia virus expressing the wild-type or the Y183F mutant of 3BP2. Due to asynchronous activation of NK cells, the signal that can be detected upon cell-cell stimulation is weak and does not allow us detection of associated molecules (data not shown). To overcome this, NK cells were also infected with the vaccinia viruses expressing PLC-γ2 or FLAG-Vav-2 proteins. PLC-γ2 has been shown to be the critical isoform for the regulation of NK cell cytotoxicity (31). Target cells were fixed to avoid their potential activation and were then incubated with infected NK cells. As shown in Fig. 7A, the activation-induced phosphorylation of the Y183F mutant is lower than the phosphorylation of the wild type 3BP2 (Fig. 7A, compare wt to Y183F), suggesting that this residue contributes to the phosphorylation of 3BP2 upon NK cell activation. Similar modest hypophosphorylation of the Y183F mutant is seen after FcR stimulation (data not shown). However, this residue is not the only tyrosine that is biochemically modified during NK cell activation, because the Y183F mutant is still tyrosine phosphorylated in response to NK cell stimulation with sensitive target cells or through the FcR. Phosphorylated bands corresponding to Vav and PLC-γ proteins (Fig. 7A), as well as Vav-2 (Fig. 7B), were associated with the wild-type 3BP2 upon stimulation of NK cells with sensitive target cells. This association was largely inhibited when the Y183F mutant was used. Therefore, tyrosine-183 mediates binding of 3BP2 to downstream signaling effectors Vav and PLC-γ during the development of NK cell-mediated natural cytotoxicity.

Tyrosine-183 is necessary for the enhancement of natural cytotoxicity by 3BP2

To test whether the tyrosine-183 of 3BP2 has a biologically relevant role, NK cells were infected with control vaccinia virus or recombinant virus encoding the wild-type 3BP2 or the Y183F mutant, and natural cytotoxicity toward C1R and 721.221 target cells was analyzed. As described before, the wild-type 3BP2 increases killing of both sensitive targets (Fig. 8, compare WR and 3BP2.wt). However, the Y183F mutation reversed this increase (Fig. 8, compare 3BP2.wt and Y183F). This indicates that the tyrosine-183 has a functional role in regulating natural cytotoxicity of NK cells. Interestingly, when tested in the reverse ADCC assay for FcR-mediated cytotoxicity, the Y183F mutant inhibited cytotoxicity of some NK clones, while there were also clones in which the mutation did not have any effect (data not shown). These data support the earlier observation of clonal heterogeneity in the signals generated during the development of FcR-mediated NK cytotoxicity (19).

Discussion

Activation of lymphocytes through MIRR initiates multiple and complex signaling cascades. In general, the proximal Src and Syk families of PTKs phosphorylate and activate a number of downstream effectors, which results in the divergence of signals regulating distinct cellular functions such as gene transcription, cellular proliferation, cell-mediated killing, etc. Tyrosine phosphorylation of proteins plays two key roles in early signaling: First, many enzymes change configuration into an active state once critical tyrosine residues are phosphorylated; and, second, phosphotyrosines act as binding sites for mobilization of SH2- or phosphotyrosine binding domain-containing proteins to their upstream regulators. This process of sequential building of signaling complexes

![Image](http://www.jimmunol.org/)

**FIGURE 7.** Tyrosine-183 binds Vav and PLC-γ isoforms during the development of natural cytotoxicity. A, NK cells (15 × 10⁶/sample) were infected for 5 h at a MOI of 15:1 with the control vaccinia virus (WR) or with viruses encoding the EE-tagged wild-type or Y183F mutant of 3BP2. All cells were also infected with the virus encoding PLC-γ2 (MOI, 5:1). Infected cells were then incubated with 721.221 target cells for indicated time. EE-immunoprecipitates from the Nonidet P-40 lysates were subjected to SDS-PAGE, transferred to a membrane, and probed with anti-phosphotyrosine (P-Tyr) or anti-EE mAb. B, The same as in A, except that instead of PLC-γ2, FLAG-tagged Vav-2 was overexpressed, and cells were stimulated with CIR targets.

**FIGURE 8.** Tyrosine-183 is necessary for the function of 3BP2 during natural cytotoxicity. NK cells were infected for 6 h at a MOI of 20:1 with the control vaccinia virus (WR) or with viruses encoding the EE-tagged wild-type (wt) or Y183F mutant (Y183F) of 3BP2. Infected cells were tested in natural cytotoxicity assays against C1R and 721.221 target cells. Results are expressed in LU/10⁶ effector cells.
is facilitated by adaptor proteins, which simultaneously bind several other signaling proteins due to the presence of phosphorylation sites and/or other protein-protein interaction domains. A number of adaptor proteins have been discovered in the past several years. It is becoming increasingly obvious that these proteins play a very important role in the regulation of cellular functions. One of the best examples of the importance of these proteins is the finding that a subset of children with B cell immunodeficiency lack the functional adaptor protein BLNK/SLP-65 (32). The phenotype of these patients resembles that seen in X-linked immunodeficiency, caused by the absence of the PTK Btk. However, although many of the adaptor proteins have been cloned, their specific roles and the exact place in signaling cascades remain largely elusive.

3BP2 is a ubiquitously expressed cytoplasmic adaptor protein. Its SH2 domain directly binds to phosphotyrosines of the Syk family of PTKs and, possibly, to the transmembrane adaptor protein LAT (22). 3BP2 has been shown to participate in the regulation of the TCR-initiated activation of T cells, because overexpression of 3BP2 increases the NFAT- and AP-1-dependent transcription necessary for the production of IL-2. This positive regulatory function requires the GTPase Ras for the AP-1 activation and the calcium-activated phosphatase calcineurin for NFAT activation. However, the mechanism by which 3BP2 mediates its positive effect on downstream signaling molecules remains elusive. In this paper, we studied the role of 3BP2 in NK cell-mediated cytotoxicity. Stimulation of NK cells activates signaling pathways largely overlapping with those used by T cells. However, there are also differences in usage of specific signaling components (33–38). Here we have described that 3BP2 is an active component of the activation cascade that leads to NK cell-mediated cytotoxicity. 3BP2 is tyrosine-phosphorylated upon stimulation of NK cells through the FcR or receptors involved in the generation of natural cytotoxicity. The phosphorylation of 3BP2 depends on the presence of PTKs of the Src and Syk families, because 3BP2 does not undergo receptor-initiated tyrosine phosphorylation in somatic mutants of Jurkat T cells lacking Lck or ZAP-70 reconstituted with a functional FcγRIII receptor (D. Jevremovic and P. J. Leibson, unpublished observation). Three functional domains of 3BP2 have been described previously, including the PH domain, the proline-rich region, and the SH2 domain. Deletion of the SH2 domain largely diminishes receptor-initiated tyrosine phosphorylation of 3BP2, whereas deletion of the PH domain or mutation of the proline-rich region does not have an appreciable biochemical effect. This indicates that the SH2 domain of 3BP2 is involved in its targeting to the upstream regulators, as is the case with multiple other signaling proteins. However, when tested in the functional assays, all three domains of 3BP2 are necessary for its function in enhancing cell-mediated cytotoxicity (Fig. 5). The proline-rich region deletion mutant has previously been shown to have only a minor defect in regulating transcription in T cells (22). The observed difference supports an earlier notion that intracellular mechanisms regulating NK cell-mediated cytotoxicity and transcription in T cells are not always overlapping (38). Alternatively, deletion of the entire proline-rich region may result in a conformation different from that of a protein with multiple proline to alanine point mutations.

Adaptor proteins function through their ability to bind to other signaling proteins. Two of the tyrosine-phosphorylated proteins associated with 3BP2 upon stimulation of NK cells are Vav-1 and Vav-2. The Vav protooncogenes act as a GEF for the Rho family of GTP-binding proteins, which are involved in regulation of signals leading to cytoskeletal rearrangements, calcium response, and gene transcription (reviewed in Ref. 39). Vav-1 activates select members of the Rho family of GTPases and acts as a critical regulator of signaling pathways that lead to the activation of NK and T cells (9, 28). Recently, a Vav-1 homologue, Vav-2, has been identified (40–42). It has been shown that whereas Vav-1 acts as a GEF for Rac-1, Rac-2, and RhoG, Vav-2 has more affinity for the RhoA subfamily of G proteins (39). Both Vav-1 and Vav-2 require their SH2 domains to get tyrosine phosphorylated, which is the key step in the activation of their GEF function. Several proteins have been described as capable of mobilizing Vav-1 to the TCR-cross-linking-initiated signaling complex in T cells through the interaction between their phosphotyrosines and the Vav-1 SH2 domain. These proteins include the adaptor protein SLP-76 (43, 44) and PTKs of the Syk family (45, 46). However, SLP-76-deficient T cells phosphorylate Vav-1 upon TCR stimulation similarly to the wild-type cells (14). Also, although ZAP-70-deficient T cells lack inducible phosphorylation of Vav-1 (47), this fact may be explained by the absence of the activating kinase instead of the inability of Vav-1 to be docked to the signaling complex. Therefore, the mechanism by which the members of the Vav family of proteins are targeted to their upstream regulators remains unresolved.

The analysis of the amino acid sequence of 3BP2 revealed that the tyrosine-183 is a potentially good binding partner for the Vav-1 SH2 domain (29, 30). We synthesized a peptide encompassing phosphotyrosine-183 and in a series of experiments showed that this site can directly bind to the SH2 domains of both Vav-1 and Vav-2. However, based on direct binding experiments, it seems that the tyrosine-183 has significantly higher affinity for Vav-2. Although Vav-1 and Vav-2 share the same general structure and a significant homology throughout the sequence, only the SH2 domain of Vav-1 has a threonine at the βD5 position, which is the key position that determines specificity. This difference may potentially cause a different binding specificity of the two SH2 domains for phosphotyrosine-containing proteins. We have previously shown that both Vav-1 and Vav-2 overexpression increases cell-mediated cytotoxicity in NK and T lymphocytes (9, 24). However, Vav-1, but not Vav-2, selectively controls the NFAT/AP-1-dependent transcription in T cells (24), which indicates that the two isoforms do not have simply a redundant function. Also, whereas Vav-1 is expressed exclusively in hematopoietic cell types, Vav-2 has a more ubiquitous expression, similar to 3BP2. Our data indicate that both Vav-1 and Vav-2 can bind to the same sequence encompassing the phosphotyrosine-183 of 3BP2, but the Vav-2–3BP2 interaction seems more likely to mediate the positive regulatory role of 3BP2 in NK cells. This notion is supported by the fact that in vivo association with the 3BP2 (but not with the Y183F) mutant was more readily detected for Vav-2 than for Vav-1 (D. Jevremovic and P. J. Leibson, unpublished observation). Interestingly, the Y183F mutation does not completely abolish 3BP2-Vav association in cells. This residual binding may be explained by other potential means of direct 3BP2-Vav interaction, such as other 3BP2 tyrosines with the SH2 domain of Vav or the proline-rich region of 3BP2 with the SH2 domain of Vav or the proline-rich region of 3BP2 with the SH2 domain of Vav. Alternatively, Vav may be brought into the complex indirectly via its interaction with other signaling components.

PLC-γ is the critical mediator for the activation of intracellular calcium in lymphocytes. It cleaves membrane phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate and diacylglycerol. For its activation, PLC-γ has to be targeted to the membrane and tyrosine phosphorylated (16). In this paper, we showed that the tyrosine-183 of 3BP2 could bind to both PLC-γ isoforms expressed in NK cells. Although we could not specifically preclear with anti-PLC-γ2 Abs the 150 kDa-band associating with 3BP2 immunoprecipitates after FcR stimulation (data not shown), coexpression of PLC-γ2 with the 3BP2 molecules enabled us to detect this association in vivo after target cell stimulation. Supporting the
ability of 3BP2 to directly interact with PLC-γ are known structural features of the PLC-γ SH2 domains. PLC-γ isoforms have two tandem SH2 domains. Both SH2 domains in each isoform have a cysteine residue at the βD5 position. It has been shown that the C-terminal SH2 domain of both PLC-γ1 and PLC-γ2 preferentially binds to phosphotyrosines with a proline residue at the pY + 3 position. The tyrosine-183 of 3BP2 is in the sequence YLEG, which would allow this interaction. Targeting of PLC-γ to the plasma membrane can be a function of transmembrane signaling molecules, PTKs, products of phosphatidylinositol-3 kinase, and adaptor proteins (16). For example, LAT-18) or SLP-76-deficient T cells (14) as well as BLNK-deficient B cells (15) do not activate PLC-γ upon stimulation through TCR or BCR, respectively. Also, PTKs of the Syk and Tec families are necessary for PLC-γ activation (reviewed in Ref. 48). The multitude of different factors regulating PLC-γ translocation suggests a very tight control of this enzyme’s activity. It will be interesting to determine the specific role of 3BP2 in the context of all these other regulatory proteins; more specifically, it will be interesting to determine whether 3BP2-deficient cells would be capable of PLC-γ activation.

The tyrosine-183 of 3BP2 is necessary for the positive regulatory role of 3BP2 in natural cytotoxicity. This is likely due to its binding to Vav and PLC-γ molecules. However, relative affinities of PLC-γ and Vav for this site were not assessed. Because multiple molecules of the same protein are involved in signal transduction, it is possible that PLC-γ and Vav simultaneously bind to different 3BP2 molecules. Alternatively, PLC-γ or Vav may preferentially bind to this site in vivo. Although the role of 3BP2 in natural cytotoxicity requires the tyrosine-183, ADCC seems to be influenced by Y183F mutation only in a subset of NK clones. Clonal differences in response to overexpression of signaling proteins have been described before (19). There are many possible explanations for this clonal heterogeneity. Preliminary data suggest that the two different types of clones differ in the level of FcR-induced PLC-γ2 tyrosine phosphorylation upon Y183F mutant overexpression, whereas Vav-1 and Vav-2 phosphorylation is the same. However, this issue requires further investigation, specifically to determine whether the levels of FcR expression and/or levels of the endogenous 3BP2 protein and other signaling molecules can be correlated with the ability of the Y183F mutation to abolish the effect of the wild-type 3BP2 on ADCC.

Natural cytotoxicity is a unique feature of NK cells. Receptors involved in mediating cellular cytotoxicity against sensitive target cells are still poorly defined. Recently, a number of activating receptors has been discovered (11), and this will enable more precise analysis of signaling pathways involved during natural cytotoxicity. We found that overexpression of 3BP2, but not the deletion mutants or the Y183F mutant, increases cytotoxicity mediated by 2B4- and CD94-activating receptors (D. Jevremovic and P. J. Leibson, unpublished observation). Although direct stimulation of NK cells by sensitive target cells is poorly defined at the receptor level, it still has an important role in defining biochemical interactions. These experiments help understand interactions that actually occur in vivo in which multiple cell surface molecules are engaged at the same time.

It remains a major challenge to dissect in detail signaling pathways involving so many different components. In this paper, we have described how the 3BP2 adaptor protein connects Vav and PLC-γ isoforms to the upstream regulators during NK cell-mediated cytotoxicity. Explanation of the role of 3BP2 in other cell systems and in interactions with other molecules awaits future investigations.

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References