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The adaptor 3BP2 activates CD244-mediated cytotoxicity in PKC- and SAP-dependent mechanisms

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ABSTRACT

Natural killer (NK) cell cytotoxicity requires triggering of activation receptors over inhibitory receptors. CD244, a member of CD150 receptor family, positively regulates NK-mediated lyses by activating an intracellular multiproteic signaling network that involves the adaptors X-linked lymphoproliferative gene product SAP and 3BP2. However, the exact mechanisms used by 3BP2 to enhance CD244-mediated cytotoxicity are still not fully understood. Here using the human NK cell line YT-overexpressing 3BP2, we found that the adaptor increases CD244, PI3K, and Vav phosphorylation upon CD244 engagement. The use of enzymatic inhibitors revealed that 3BP2-dependent cytolysis enhancement was PKC-dependent and PI3K-ERK independent. Furthermore, 3BP2 overexpression enhanced PKC delta phosphorylation. SAP knockdown expression inhibited PKC delta activation, indicating that the activating role played by 3BP2 depends upon the presence of SAP. In conclusion, our data show that 3BP2 acts downstream of SAP, increases CD244 phosphorylation and links the receptor with PI3K, Vav, PLC gamma, and PKC downstream events in order to achieve maximum NK killing function.

1. Introduction

Molecular scaffolds composed of adapter proteins and enzymes, such as phospholipase C-γ (PLC-γ), phosphatidylinositol 3-kinase (PI3K), and Vav guanine nucleotide exchange factors, are assembled and activated at the plasma membrane by Src and/or Syk PTKs, thereby leading to the formation of large signaling complexes or "signalosomes". These scaffolds transduce signals to the cytoplasm, cytoskeleton, and nucleus in order to activate gene expression and metabolic changes involved in lymphocyte proliferation, differentiation, cytotoxicity and motility. SH3-binding protein 2 (3BP2) is a cytoplasmic adapter originally identified as a protein interacting with the SH3 domain of Abl PTK (Ren et al., 1993). 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. 3BP2 is preferentially expressed in hematopoietic tissues and regulates transcriptional activities via calcineurin- and Ras-dependent pathways in T lymphocytes (Deckert et al., 1998). A positive regulatory role of 3BP2 in B cell receptor (BCR) functionality (Foucault et al., 2005) has also been established. Moreover, in 3BP2-deficient mice optimal B cell activation and thymus-independent humoral responses are impaired (Chen et al., 2007; de la Fuente et al., 2006). 3BP2 also plays a key regulatory role in natural killer (NK) cells. 3BP2-dependent regulation of NK cell-mediated cytotoxicity depends upon PH, SH2, and proline-rich regions (Jevremovic et al., 2001). Moreover, phosphorylation of Tyr^{183} on 3BP2, which recruits Vav-1 and PLC-γ, is critical for the ability of 3BP2 to positively regulate NK cell-mediated killing (Jevremovic et al., 2001). Our group previously showed that 3BP2 was biochemically and functionally linked to the human CD244 receptor in NK cells (Saborit-Villarroya et al., 2005). CD244 (also known as 2B4) belongs to the CD150 family of immunoglobulin receptors. The receptors on this family are characterized by the presence on their cytoplasmic tail of the tyrosine consensus motif T-V/I-Y-x-x-V/I, which binds to the small adaptor protein SAP (SLAM-associated protein, also known as SH2D1a) (Sayos et al., 2000; Engel et al., 2003). SAP binds to

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src-kinase Fyn and increases the phosphorylation status and activation of these receptors (Chan et al., 2003; Chen et al., 2006). CD244 is expressed by all NK cells, basophils, and monocytes as well as a subset of $CD8^+$ T cells. Engagement of CD244 by Abs or its ligand CD48 induces natural cytotoxicity and IFN-γ production (Assarsson et al., 2004; Bhat et al., 2006; Kambayashi et al., 2001; Lee et al., 2003; Messmer et al., 2006). CD244 function is related to SAP expression (Endt et al., 2007; Vacca et al., 2006). In cells lacking SAP, CD244 is no longer phosphorylated following receptor ligation (Chen et al., 2004). SAP deficiency, which leads to X-linked proliferative syndrome, results in CD244-positive signaling impairment (Benoit et al., 2000; Nakajima et al., 2000; Tangye et al., 2000). It has also been reported that CD244 exerts an inhibitory action in XLP patients (Parolini et al., 2000). On the other hand, 3BP2 links CD244 to downstream events regulating the cytotoxic function of CD244 without affecting IFN-γ secretion (Sabori-Villarroya et al., 2005). To gain insights into 3BP2 functionality, we dissected CD244 pathway activation using different inhibitors. In addition, we searched for novel 3BP2 interacting proteins following CD244 triggering in the NK cell line YT. We found that 3BP2 associates with PI3K, Vav, and PLC-γ, after CD244 triggering occurs. Using specific enzymatic inhibitors, we found that 3BP2-dependent enhancement of cytotoxicity was dependent upon PKC activation, whereas PI3K and ERK inhibitors only partially affected CD224-mediated cytotoxicity and had no effect on 3BP2-dependent enhancement. Finally, 3BP2 overexpression not only enhanced PKC delta (PKC-δ) phosphorylation, but SAP expression was also found to be crucial for this activation to occur, thus indicating that SAP plays a role upstream of 3BP2.

2. Materials and methods

2.1. Cells and reagents

3BP2-EGFP and SH2-3BP2-EGFP YT transfectants were generated as previously described (Sabori-Villarroya et al., 2005). YT cells (human NK cell line), 3BP2-EGFP YT cells, SH2-3BP2-EGFP YT cells, P815 (murine mastocytoma cell line) cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 10 mM Hepes, 50 U/ml penicillin and 50 μg/ml streptomycin from Gibco (Life Technologies, Gaithersburg, MD). Peroxidase-conjugated anti-mouse IgG was purchased from Dako (Denmark), while Fab'_2 goat anti-mouse (GAM) and Actin antibody were obtained from Sigma–Aldrich (St. Louis, MO). Horseradish peroxidase-conjugated anti-phosphotyrosine monoclonal antibody cocktail was acquired from Zymed (San Francisco, CA). Monoclonal antibodies against CD244 [clone 69] and 3BP2 [clone 256.5.1] have been previously described (Sabori-Villarroya et al., 2005). Monoclonal antibodies against human CD84 [CD84 clone 2.15] (Martin et al., 2001) and p-selectin [clone KO 2.3] (Massaguer et al., 2000), with the same isotype as the CD244 antibody (IgG1), were used as IgG controls. Rabbit anti-human CD244 was a gift from Dr. E. Long (National Institute of Allergy and Infectious Diseases, Rockville, MD). Mouse anti-SAP was a gift from Dr. C. Terhorst (Beth Israel Deaconess Medical Center, Boston, MA). Anti-Vav 1 monoclonal antibody, anti-PLCγ 1 antibody, and anti-phospho-Akt antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit peroxidase was obtained from Bio-Rad (Hercules, CA). Anti-phospho-ERK1/2, Anti-phospho-PKC-δ and anti-total PKC-δ antibodies were acquired from Cell Signaling Technology (Beverly, MA), Anti-total ERK1/2 monoclonal antibody and anti-phosphotyrosine for immunoprecipitation were obtained from Zymed (San Francisco, CA). Anti-p85α antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Wortmannin and Bisindolylmaleimide I/ Gö6850 were acquired from Calbiochem (San Diego, CA). The MEK inhibitor U0126 was obtained from Cell Signaling Technology (Beverly, MA).

2.2. Cell activation, immunoprecipitation and immunoblotting

YT, 3BP2-EGFP YT or SH2-3BP2-EGFP YT cells were activated with anti-CD244 antibody (5 μg/ml) and cross-linking (Fab'_2 goat anti-mouse, 10 μg/ml), when indicated, for various time periods. Cells were starved overnight (RPMI with 0.1% FCS) in order to reduce basal phosphorylation levels. Inhibitors or vehicles, when present, were added immediately before cellular activation.

Immunoprecipitations and immunoblottings with the indicated antibodies were carried out as previously described (Sayos et al., 2001).

2.3. Cell-mediated cytotoxicity assay

P815 FcR^+ target cells were labeled by incubating 1×10^6 cells with 100 μCi of $Na_2{}^{51}CrO_4$ (Amersham Bioscience, Buchinghamshire, UK) for 90 min at 37 °C under 5% CO_2 in air. Target cells were subsequently washed twice with medium and kept for 1h in culture in order to reduce the background produced by the spontaneous liberation of ^{51}Cr after cell labeling, and then washed twice again. 1×10^4 labeled target cells (100 μl) were incubated with the effector YT cell suspension or 3BP2-EGFP YT cells (100 μl) in the presence of anti-CD244 or isotype control IgG (200 ng/ml) and in the presence of the inhibitor or vehicle. Effector YT or 3BP2-EGFP-YT cells were resuspended at 1, 5, 10, 20, or 40 times the number of labeled target cells. Cells were then incubated for 4 h at 37 °C and 100 μl of the supernatant was removed from each well for γ-radiation counting. The maximum ^{51}Cr release was assessed by incubating P815 FcR^+ cells in the presence of 5% Tx-100 in the culture media. Specific lysis was calculated as previously described (Sabori-Villarroya et al., 2005).

2.4. RNA interference assays

Synthetic siRNA oligonucleotides were synthesized and purified using a Sure Silencing™ siRNA Kit (Ambion Inc, Cambridgeshire, UK). The DNA template for the synthesis of siRNA against human SAP that renders the best results (more than 90% inhibition) was as follows: 5′-AATACAATGCCTTGATCTGGCCCTGTCTC-3′ (sense) and 5′-AAGCCAGATCAAGGCATTGTACCTGTCTC-3′ (antisense). DNA templates for the siRNA control were included in the kit. For siRNA transfections 5×10^7 YT cells or 3BP2-YT cells were transfected with 5 μg of each siRNA (control and SAP) using nucleofector (Amaxa Inc, Gaithersburg, MD). Twenty-four hours after transfection, cells were starved with 1% fetal calf serum overnight. The following day, cells were activated as described above in order to analyze PKC-δ phosphorylation. The ratio between phosphorylated PKC-δ and total PKC-δ was assessed by quantifying the intensity of the bands in the blot using Image Gauge program.

3. Results

3.1. 3BP2 overexpression increases protein phosphorylation following CD244 ligation

As we reported previously, the adaptor 3BP2 increases cytotoxicity after CD244 triggering in YT cells (Sabori-Villarroya et al., 2005). 3BP2 comprises an N-terminal pleckstrin homology (PH) domain, an SH3-binding proline-rich region and a C-terminal SH2 domain (Fig. 1A). In an attempt to identify the effector proteins related to 3BP2 signaling during CD244 activation, we triggered CD244 on YT