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Interaction of calmodulin with Bcl10 modulates NF-κB activation

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ABSTRACT

Calcium signals resulting from antigen receptor activation are important in determining the responses of a T or B lymphocyte to an antigen. Calmodulin (CaM), a multi-functional sensor of intracellular calcium (Ca^{2+}) signals in cells, is required in the pathway from the T cell receptor (TCR) to activation of the key transcription factor NF-κB. Here we searched for a partner in direct interaction with CaM in the pathway, and found that CaM interacts specifically with the signaling adaptor Bcl10. The binding is Ca^{2+} dependent and of high affinity, with a K_d of approximately 160 nM. Proximity of CaM and Bcl10 *in vivo* is induced by increases in the intracellular Ca^{2+} level. The interaction is localized to the CARD domain of Bcl10, which interacts with the CARD domain of the upstream signaling partner Carma1. Binding of CaM to Bcl10 is shown to inhibit the ability of Bcl10 to interact with Carma1, an interaction that is required for signaling from the TCR to NF-κB. Furthermore, a mutant of Bcl10 with reduced binding to CaM shows increased activation of an NF-κB reporter, which is further enhanced by activating stimuli. We propose a novel mechanism whereby the Ca^{2+} sensor CaM regulates T cell responses to antigens by binding to Bcl10, thereby modulating its interaction with Carma1 and subsequent activation of NF-κB.

1. Introduction

Antigen receptor activation results in the recruitment and activation of a number of signaling mediators and lipid metabolizing enzymes, building up an immunological synapse at the T cell receptor (TCR) or B cell receptor (BCR) (Schulze-Luehrmann and Ghosh, 2006; Weil and Israel, 2004). The immunological synapse triggers downstream signaling pathways, leading to activation of transcription factors such as the NF-κB family, which is important for the regulation of activation, proliferation, and differentiation of T and B lymphocytes (Caamano and Hunter, 2002). In a resting cell, NF-κB dimers mainly reside in the cytoplasm, sequestered by inhibitory IκB proteins. Most signals to NF-κB converge on the activation of the IκB kinase (IKK), resulting in signal-induced phosphorylation and degradation of IκB, which allows NF-κB to enter the nucleus and activate gene transcription (Hayden and Ghosh, 2008).

T cell receptor-induced activation of NF-κB is dependent on the activation of protein kinase C (PKC)θ and the subsequent recruitment and activation of a signaling complex containing the proteins Carma1, Bcl10, and Malt1, which is often referred to as the CBM complex. The formation of this complex is crucial for T cell activation, since deletion of any of the proteins impairs the NF-κB responses (Thome, 2004). The molecular mechanisms by which these proteins promote IKK and NF-κB activation are not fully understood, however. Carma 1 and Bcl10 belong to the Caspase recruitment domain (CARD) subfamily of the large Death domain family of proteins. The CARD domains and other domains found in this family are highly conserved and are characterized by their homotypic interactions with proteins within the same subfamily. They have been highly implicated in cell signaling to apoptosis and immunity (Bouchier-Hayes and Martin, 2002; Park et al., 2007). Bcl10 and Carma1 interact with each other through their CARD motifs, and this interaction is crucial for NF-κB signaling after TCR stimulation, since deletion of the CARD domain of Carma1 or mutation of the CARD domain of Bcl10 prevents NF-κB activation (Rawlings et al., 2006).

In addition to activation of PKCθ-dependent pathways, triggering of the TCR also leads to production of inositol-1,4,5-triphosphate (IP_3), initiating the release of Ca^{2+} from intracellular stores. Depletion of intracellular reservoirs of Ca^{2+} activates the influx of Ca^{2+} through store-operated calcium release-activated Ca^{2+} (CRAC) channels in the plasma membrane, resulting in elevated levels of intracellular Ca^{2+}. Depending on the pattern and strength of these Ca^{2+} signals, signaling molecules and transcrip-

Abbreviations: CaM, calmodulin; TCR, T cell receptor; BCR, B cell receptor; CBM, Carma1, Bcl10, Malt1 complex; IKK, IκB kinase; PKC, protein kinase C; CARD, caspase recruitment domain; IP_3, inositol-1,4,5-triphosphate; CRAC, calcium release-activated Ca^{2+} channel; CaMK, calmodulin dependent kinase; PMA, phorbol-12-myristate-13-acetate.

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tion factors with different requirements for Ca^{2+} will be activated (Feske, 2007; Quintana et al., 2005). The activation of NF-κB transcription factors after TCR engagement is sensitive to such changes in intracellular Ca^{2+}, but the mechanisms are not well characterized. The main transducer of Ca^{2+} signals in cells is the Ca^{2+} binding protein calmodulin (CaM). CaM belongs to the EF-hand family of Ca^{2+} binding proteins and changes conformation upon Ca^{2+} binding, allowing it to bind to a new set of targets. CaM is involved in the regulation of numerous important processes such as proliferation, signaling, and differentiation (Chin and Means, 2000; Yamniuk and Vogel, 2004). In the present study we investigated the role of CaM in TCR-induced activation of NF-κB, and found that CaM interacts specifically with Bcl10. We present a novel mechanism by which CaM through interaction with Bcl10 can modulate the binding of Bcl10 to Carma1 and thereby NF-κB activation.

2. Materials and methods

2.1. Plasmids and mutagenesis

Expression vectors for Bcl10 and Carma1 were obtained by subcloning cDNA of mouse Bcl10, IMAGE ID 4976147, and mouse Carma1, IMAGE ID 5318165 (both from Mammalian Gene Collection), from pOTB7 to pCDNA1/Amp using EcoRI/NotI and EcoRV/NotI, respectively. Mutated derivatives of Bcl10 and Carma1 were constructed using standard cloning and PCR techniques. For expression in *E. coli*, Bcl10 and Carma1 cDNA were amplified by PCR and subcloned into the EcoRI/XhoI sites of the pET-20b + His expression vector (Onions et al., 1997) using the In Fusion™CF Liquid PCR cloning kit (Clontech). The NF-κB luciferase reporter plasmid (Hughes et al., 1998) and the hCMV-βgal normalization plasmid (Corneliussen et al., 1994) have been described previously. Co-transfection with a green fluorescent protein (GFP) expressing plasmid (Hughes et al., 2001) was used in transfections for proximity ligation assays.

2.2. Expression and purification of proteins

C-terminally $(His)_6$-tagged Bcl10 or Carma1 constructs were expressed in *E. coli* strain Rosetta BL21. Cells were lysed by sonication in 100 mM NaH_2PO_4, 10 mM Tris (pH 8.0), 8 M urea, and 10 mM imidazol, and Bcl10 and Carma1 derivatives were purified from the insoluble fraction by Ni-NTA agarose chromatography (Qiagen) according to the manufacturer's instructions. The purified preparations were dialyzed against 20 mM Tris (pH 8.0), 100 mM NaCl, 0.05% Triton X-100, 10% glycerol, and 1 mM dithiothreitol (DTT).

2.3. In vitro binding experiments

For CaM Sepharose binding experiments, 0.5 μg of purified proteins were incubated with 10 μl CaM Sepharose (GE Healthcare) in binding buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM DTT, protease inhibitor cocktail tablet without EDTA (Roche)) supplemented with either 1 mM $CaCl_2$ or 1 mM EGTA, with rotation overnight at 4 °C. Where indicated, 5 μM CaMKII peptide (amino acids 290–309) (Sigma) was added to the binding reaction. The CaM Sepharose was washed three times with binding buffer with 1% Triton X-100, and bound proteins were eluted with binding buffer supplemented with 2 mM EGTA. For binding of Bcl10 to Carma1, purified Carma1 (amino acids 1–160) was coupled to CNBr-activated Sepharose (GE Healthcare) according to the manufacturer's instructions. Bcl10 was preincubated in binding buffer with or without CaM (Upstate), as indicated, using rotation overnight at 4 °C before addition of 10 μl of Carma1 (1–160) Sepharose. Reactions were incubated for 4 additional hours at 4 °C before washing as above and elution by boiling in sample buffer. Samples were separated by 10% SDS-PAGE and analyzed for Bcl10 or Carma1 by Western blot using α-Bcl10 N-term (H-197) or α-Bcl10 C-term (331.3) (both from Santa Cruz), or α-Carma1 (AL-220) (Alexis) antibodies and the SuperSignal Chemiluminescence Substrate (Pierce). All binding experiments were performed at least three times. Where indicated, Western blots were quantified using the Chemidoc™ XRS gel documentation system and Quantity One® software (BioRad).

For spectrofluorimetric analysis, dansylated CaM (5-(dimethylamino)naphthalene-1-sulfonyl-CaM) was prepared using standard procedures (Kincaid et al., 1982), and 20 nM dansylated CaM and increasing concentrations of Bcl10 protein were allowed to equilibrate for 2 h in a buffer containing 20 mM Tris (pH 8.0), 100 mM NaCl, and 100 μM $CaCl_2$. The fluorescence emission was recorded at 490 nm following excitation at 340 nm with a SPEX FluoroMax-2 fluorescence spectrometer. Measurements were repeated three times for each solution. The concentration of bound Bcl10 was plotted against free Bcl10 and the data were fit by one-site-specific binding with Hill slope (GraphPad Prism version 5.00).

2.4. Cell culture and transient transfections

The Jurkat-derived T cell line with Bcl10 down-regulated is stably expressing an shRNA against Bcl10 (Wu and Ashwell, 2008). Jurkat T cells and this Jurkat derivative with down-regulated Bcl10 were maintained in RPMI supplemented with 5% FCS and antibiotics. Cells were transiently transfected with 5 μg of expression vector. The cells were stimulated 24 h after transfection. PMA (Sigma) was used at a concentration of 1 ng/ml and ionomycin (Calbiochem) at 1 μg/ml. For proximity ligation assays (PLA), cells were co-transfected with the GFP expression vector to identify successfully electroporated cells. Cells were stimulated with the indicated drugs for 10 min and harvested. For reporter experiments, cells were co-transfected with the NF-κB-dependent reporter and normalization plasmid by electroporation as described (Hughes et al., 1998). Cells were stimulated for 2 h and harvested. Luciferase activity was measured with the Luciferase assay system (Promega) and normalized to β-galactosidase from the co-transfected hCMV-βgal plasmid.

2.5. Proximity ligation assays

The harvested lymphocytes were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 and blocked with 5% FCS in PBS. Proximity ligation assays (PLA) (Jarvius et al., 2007; Söderberg et al., 2006, 2008, 2007) were performed with Duolink *in situ* PLA kits purchased from Olink Bioscience (http://www.olink.com/). In brief, cells were stained for intracellular proteins using a goat polyclonal antibody against CaM (N-19) and a mouse monoclonal antibody against Bcl10 (331.3) (both from Santa Cruz Biotechnology). Anti-goat IgG and anti-mouse IgG secondary antibodies conjugated with oligonucleotides (PLA probes) were subsequently used according to the manufacturer's protocol to generate fluorescence signals only when the two PLA probes were in close proximity (≤40 nm). The fluorescence signal from each detected pair of PLA probes was visualized as a distinct individual red dot (Jarvius et al., 2007; Söderberg et al., 2006, 2008, 2007). Nuclei were counterstained with Hoechst 33342 dye.