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CaMKII targets Bcl10 in T-cell receptor induced activation of NF-κB

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ABSTRACT

Recognition of antigen by T- or B-cell receptors leads to formation of an immunological synapse and initiation of signalling events that collaborate to determine the nature of the adaptive immune response. Activation of NF-κB transcription factors has a key role in regulation of numerous genes with important functions in immune responses and inflammation and is of great importance for lymphocyte activation and differentiation. The activation of NF-κB depends on changes in intracellular Ca^{2+} levels, and both calmodulin (CaM) and a CaM-dependent kinase, CaMKII, help regulate NF-κB activation after T-cell receptor (TCR) stimulation, but the mechanisms are not well characterized. Here we have analyzed the functional role of CaMKII in the signalling pathway from the TCR to activation of IKK, the kinase that phosphorylates the NF-κB inhibitor IκB. We show that CaMKII is recruited to the immunological synapse where it interacts with and phosphorylates the signalling adaptor protein Bcl10. Furthermore, phosphorylation of the CARD domain of Bcl10 by CaMKII regulates the interactions within the important Carma1, Bcl10, Malt1 signalling complex and the essential signal induced ubiquitinations of Bcl10 and IKKγ. We propose a novel mechanism whereby Ca^{2+} signals can be integrated at the immunological synapse through CaMKII-dependent phosphorylation of Bcl10.

1. Introduction

In the adaptive immune system, foreign antigen is recognized by T- and B-lymphocytes through their antigen receptors. The early steps of antigen receptor activation show many similarities between T- and B-cells and result in a series of events whereby several proteins are recruited and activated in a highly ordered membrane-bound complex, the immunological synapse (Gonzalez et al., 2007; Jun and Goodnow, 2003). Formation of the immunological synapse is important for the subsequent activation of signalling pathways that together, by activating different subsets of transcription factors, determine the overall cellular response to the antigen. Activation of transcription factors of the NF-κB family is very important for the triggering of immunogenic responses rather than tolerance or apoptosis (Gonzalez et al., 2007; Jun and Goodnow, 2003; Schulze-Luehrmann and Ghosh, 2006).

Stimulation of the T-cell receptor (TCR) and co-receptors leads to activation of phospholipase C-γ1 (PLC-γ1), which produces the secondary messengers diacylglycerol (DAG) and inositol 1,4,5-triphospate (IP_3) (Weil and Israel, 2006). DAG activates members of the protein kinase C (PKC) family. T-cell receptor induced activation of NF-κB depends on PKC and downstream signalling events, which are currently under intense investigation. Much of the studies are focused on the complex of the molecular scaffolding proteins Carma1, Bcl10, and Malt1, often referred to as the CBM complex (Schulze-Luehrmann and Ghosh, 2006; Weil and Israel, 2006). These proteins are crucial for the organization of an immunological synapse and subsequent NF-κB activation (Schulze-Luehrmann and Ghosh, 2006; Thome, 2004). Carma1 is constitutively present in specialized parts of the membrane termed lipid rafts where signalling to NF-κB occurs, but further enriched after TCR engagement. PKCθ is rapidly recruited to the lipid rafts after TCR stimulation (Hayashi and Altman, 2007; Kabouridis, 2006; Weil and Israel, 2006) where it phosphorylates key residues in the linker region of Carma1 (Matsumoto et al., 2005; Rueda and Thome, 2005; Sommer et al., 2005), resulting in the release of its caspase recruitment domain (CARD). Carma1 then recruits the Bcl10–Malt1 complex through interaction between the CARD domains of Carma1 and Bcl10 (Rawlings et al., 2006; Schulze-Luehrmann and Ghosh, 2006) and the nearby coiled-coil domain of Carma1 (McCully and Pomerantz, 2008). Oligomerization of the CBM complex and other associated proteins subsequently leads to the recruitment and activation of the IκB kinase (IKK) (McCully and

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

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Pomerantz, 2008; Rawlings et al., 2006; Schulze-Luehrmann and Ghosh, 2006). Activated IKK phosphorylates the NF-κB inhibitor IκB, leading to its degradation, thereby releasing NF-κB so that it can enter the nucleus and act on its target genes. The molecular mechanisms behind the recruitment and activation of IKK by the CBM complex is not fully understood, but the integrity of the CBM complex is known to be tightly regulated by phosphorylations, ubiquitinations, and possibly other modifications (Thome and Weil, 2007). Furthermore, the CBM complex is subjected to negative feedback regulation through phosphorylations of Bcl10 by IKK, which leads to disruption of the interaction between Bcl10 and Malt1 and to ubiquitin-mediated degradation of Bcl10 (Hinz and Scheidereit, 2007; Lobry et al., 2007; Wegener et al., 2006; Zeng et al., 2007).

The IP_3 produced after TCR engagement binds to calcium (Ca^{2+}) channels and results in elevated levels of intracellular Ca^{2+} within seconds after TCR activation. The strength and duration of these Ca^{2+} signals are, together with the other signalling events, important determinants of the outcome of receptor activation (Feske, 2007; Quintana et al., 2005). Although the NF-κB activity is known to be regulated by different patterns of Ca^{2+} signals, such as those created by TCR activation (Dolmetsch et al., 1997, 1998; Quintana et al., 2005), the mechanisms are not well characterized. Ca^{2+} signals in cells are transmitted predominantly by the Ca^{2+} sensor protein calmodulin (CaM) (Chin and Means, 2000; Yamniuk and Vogel, 2004). Signalling that increases the intracellular Ca^{2+} concentration leads to binding of Ca^{2+} to CaM, which changes its structure and allows it to interact with a new range of target proteins. We have previously found that CaM participates in the regulation of NF-κB both by the direct interaction with NF-κB proteins and Bcl10 and through the activation of a CaM-dependent kinase, CaMKII (Antonsson et al., 2003; Edin et al., 2010; Hughes et al., 1998, 2001). Here we analyze the functional role of CaMKII in the pathway from the TCR to NF-κB activation and present a novel mechanism whereby Ca^{2+} signals can be integrated at the immunological synapse through CaMKII-dependent phosphorylation of Bcl10.

2. Material and methods

2.1. Inducers, inhibitors, and antibodies

Unless otherwise indicated, drugs and antibodies were added to cells at final concentrations of 25 ng/ml PMA (Sigma), 1 μg/ml ionomycin (Calbiochem), 10 μg/ml W7 (Sigma), 20 μM KN93 (Calbiochem), 10 μg/ml α-CD3 (OKT3), and 5 μg/ml α-CD28 (BD Pharmingen). Antibodies against PKCθ (C-18), Bcl10 (331.3 and H-197), ERK2 (C-14) (which also detects ERK1), IKKα/β (H-470), IKKγ (FL-419), and IκBα (C-15) and normal rabbit and mouse IgG were from Santa Cruz. Antibodies against phospho-p44/p42 MAP kinase, phospho-JNK1/2, JNK (56G8), and phospho-IKKα/β (16A6) were from Cell Signalling Technology. α-Carma1 (AL220) was from Alexis. α-flagM_2, and α-FlagM_2 agarose were from Sigma. α-HA (12CA5) was from Roche. α-Mouse Cy5, α-goat Rhodamine Red, and α-rabbit FITC were from Jackson Immunoresearch Laboratories.

2.2. Plasmids and mutagenesis

Human wild-type, constitutively active (threonine 286 to aspartate mutated) and inactive $CaMKII_{\gamma B}$ cDNA of pSRα. BKS eukaryotic expression plasmid previously described (Nghiem et al., 1993) were subcloned to the expression vector pCDNA1/Amp using EcoRI. Expression plasmids for Bcl10, Carma1, and MALT1 were obtained by subcloning cDNA of mouse Bcl10 (IMAGE ID 4976147), mouse Carma1 (IMAGE ID 5318165), and human MALT1 (IMAGE ID 4765195) (Mammalian Gene Collection) from pOTB7 to pCDNA1/Amp using EcoRI/NotI, EcoRV/NotI and BamH1/XhoI, respectively. Expression plasmids for N-terminally flagged-MALT1 and mutated derivatives of Bcl10 and Carma1 were constructed using standard cloning and PCR techniques. The Carma1Δlinker mutant was constructed by deletion of amino acids 453–658. 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), the CMV-βgal normalization plasmid (Corneliussen et al., 1994), and the expression vector for HA-Ubiquitin (Lim et al., 2005) have been described previously.

2.3. 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 according to the manufacturer's instructions (Qiagen). The purified preparations were dialyzed against 20 mM Tris (pH 8.0), 100 mM NaCl, 0.05% Triton X-100, 10% glycerol, and 2 mM DTT.

2.4. Cell culture and transient transfections

Jurkat T-cells and the Jurkat derived T-cell line with Bcl10 downregulated through stable expression of shRNA against Bcl10 (Wu and Ashwell, 2008) were grown in RPMI supplemented with 5% fetal calf serum and antibiotics. Cells were transiently transfected with indicated amounts of expression plasmids by electroporation as described (Hughes et al., 1998). For reporter experiments, expression plasmids were transfected together with the NF-κB-dependent reporter and normalization plasmid (Hughes et al., 1998). After expression for the indicated times, cells were harvested or treated with drugs or antibodies for the indicated times and then harvested. Luciferase activity was measured with the Luciferase assay system (Promega).

2.5. In vitro binding and phosphorylation experiments

Binding of Bcl10 to purified Carma1 (amino acids 1–160) coupled to Sepharose (GE Healthcare) was as previously described (Edin et al., 2010). Analyzes of the western blots were with α-Bcl10 (H-197) or α-Carma1 (AL-220) antibodies and the SuperSignal Chemiluminescence Substrate (Pierce). Western blots were indicated, quantified using the Chemidoc™ XRS Gel documentation system and the Quantity One® software (BioRad). For *in vitro* phosphorylations, 1 ng of CaMKII and the indicated amounts of Bcl10 and/or Carma1 were used in a buffer containing 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM DTT, 1.2 μM CaM, 10 μM ATP, 1.5 μCi [γ-^{32}P]ATP and protease inhibitor cocktail tablet without EDTA (Roche). Reactions were incubated for the indicated times at 30 °C, stopped by addition of sample buffer, and separated by 10% SDS-PAGE. Incorporation of ^{32}P was measured using a phosphorimager, and Bcl10 and Carma1 were detected by western blot as above.

2.6. Immunoprecipitations

Harvested cells were resuspended in lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 2 mM $CaCl_2$, 1% Triton X-100, 10% glycerol, 10 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, and protease inhibitor cocktail