Molecular Immunology 46 (2009) 991–998

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

# C-mip interacts physically with RelA and inhibits nuclear factor kappa B activity

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ARTICLE INFO

*Article history:*
Received 22 July 2008
Received in revised form
16 September 2008
Accepted 18 September 2008
Available online 18 November 2008

*Keywords:*
NF-κB
RelA
T cell signaling
C-mip

ABSTRACT

The fine regulation of NF-κB activity is crucial for both resting and stimulated cells and relies on complex balance between multiple activators and inhibitors. We report here that c-mip, a recently identified pleckstrin homology (PH) and leucine-rich repeat (LRR)-domain-containing protein, inactivates GSKβ and interacts with RelA, a key member of the NF-κB family. We show that c-mip inhibits the degradation of I-κBα and impedes the dissociation of the NF-κB/I-κBα complexes. C-mip acts downstream signaling of classical NF-κB pathway and may represent one of the missing links in the control of NF-κB activity.



## 1. Introduction

The NF-κB family of transcription factors plays a central role in many processes of life through the regulation of genes involved in immunity, inflammation, cell proliferation, differentiation and apoptosis. The immune response leading to NF-κB activation can be triggered by several distinct signaling pathways, through the action of various receptors including the Toll-like receptors of the innate immune system and the antigen-specific T and B cell receptors (TCR and BCR) of the adaptive immune system. The TNF receptor superfamily represents a third major pathway that activates NF-κB. In mammalian cells, the NF-κB family consists of five members, NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB and cRel. NF-κB1 and NF-κB2 are synthesized as precursors of 105 and 100 kDa, that undergo posttranslational processing to yield their mature forms, p50 and p52, respectively (Karin and Ben-Neriah, 2000). The hallmark of NF-κB family is the presence in all members of a Rel-homology domain (RHD) at the N-terminus, responsible for DNA binding, dimerization and association with the I-κB inhibitory proteins. Among the NF-κB proteins, only RelA, RelB and c-Rel have a C-terminus transactivation domain, allowing their homo- or heterodimers to function as transcriptional activators, whereas homodimers of p50 and p52, which lack this domain, function as repressors (Ghosh and Karin, 2002).

The activity of NF-κB is tightly regulated by the I-κB inhibitory proteins. In resting cells, I-κB protein binds to nuclear localization site of the RHD, preventing nuclear translocation of the NF-κB protein, and thereby its transcriptional activity. Upon activation, the I-κB protein is phosphorylated, which allows its ubuquitination and subsequent degradation by proteasome. The phosphorylation of I-κB depends on the activation of the I-κB kinase (IKK) complex, which consists of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ/Nemo (Hayden and Ghosh, 2004). The IKK complex is at the crossroads of two major signaling pathways leading to NF-κB activation. The classical pathway involving the phosphorylation of I-κB protein, mainly I-κBα, by IKKβ and Nemo, is broadly recruited by proinflammatory cytokines, innate and adaptive immune receptors. The alternative (non-canonical) pathway depends strictly upon the phosphorylation and activation of IKKα by NF-κB-induced kinase (NIK). This pathway is independent of IKKβ and IKKγ and relies on the processing of p100 by NIK and IKKα. The alternative pathway is activated by specific inducers such as lymphotoxin β, B cell activating factor (BAFF), the CD40 ligand, the TNF ligand-like weak inducer of apoptosis (Tweak) and

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[1] D.S. is a recipient of Avenir Program (Institut National de la Santé et de la Recherche Médicale).

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doi:10.1016/j.molimm.2008.09.034

Rankl, but is not affected by most of the classical NF-κB inducers. These findings may explain why the classical NF-κB activation is rapidly recruited upon challenge by pathogens or DNA damage and is not influenced by inhibitors of protein synthesis, in contrast to the alternative pathway.

The inhibition of NF-κB activation can occur through several mechanisms, including inhibition of the initial stimulating signal (such as the binding of ligand to its receptor), blockage of a given step of the signaling cascade, interference with the nuclear translocation of the NF-κB dimer and inhibition of its nuclear activity.

In the course of our studies of the molecular mechanisms underlying the immunopathogenesis of idiopathic nephrotic syndrome, we recently isolated a new gene, c-mip (for c-maf inducing protein) (Sahali et al., 2002). The natural isoform, c-mip, encodes an 86-kDa protein. The predicted structure of c-mip includes an N-terminal region containing a pleckstrin homology domain (PH), a middle region characterized by the presence of several interacting docking sites, including a 14-3-3 module, a PKC domain, an Erk domain, and an SH3 domain similar to the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K) and a C-terminal region containing a leucine-rich repeat (LRR) domain. The basal expression level of the transcript is very low in adult tissues and the protein is not detectable in physiological conditions. C-mip is initially identified in the T lymphocytes of patients with minimal change nephrotic syndrome. Here, we report that c-mip inhibits NF-κB activation by interfering with the processing of I-κBα, as well as the nuclear translocation of RelA.

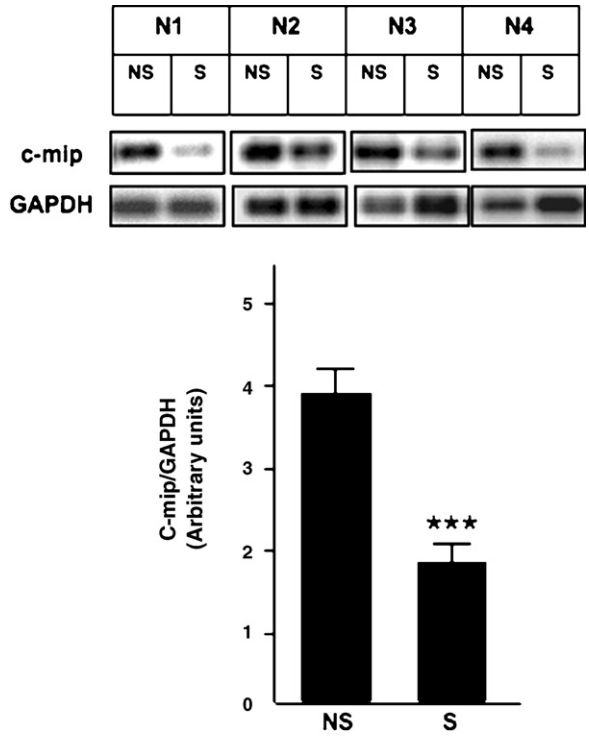


**Fig. 1.** Effect of ConA/PHA on c-mip expression n PBMC. PBMC were isolated from normal subjects and stimulated (S) or not (NS) with ConA and PHA for 72 h. The total RNA was then extracted and the expression os c-mip was analyzed by semi-quantitative RT-PCR. Statistical analyses were carried out on data from 3 independent experiments using the two-tailed *t*-test ($p < 0.0001$; unpaired *t*-test).

## 2. Results

### 2.1. Overexpression of c-mip blocks NF-κB activation

We first analyzed the expression of c-mip in peripheral blood mononuclear cells (PBMCs) following activation by mitogens. In resting PBMCs, the basal expression of c-mip is very lower and mainly restricted to some mature B cells and few T cells (data not shown). The basal level of the transcript was found profoundly reduced in PBMCs isolated from normal subjects and stimulated with Concanavalin A (ConA) and PhytohemagglutininA (PHA) during 72 h (Fig. 1). This result led us to suppose that c-mip is inhibited because it interferes with cell activation pathways. Since NF-κB plays a key role in the expression of genes that are essential for lymphocyte activation and the generation of the immune response, we sought to determine whether c-mip affects NF-κB activity. We co-transfected Jurkat cells with a c-mip expression plasmid and an NF-κB-Luc reporter and analyzed the luciferase activity driven by NF-κB enhancer element. Overexpression of c-mip inhibited constitutive NF-κB activity in Jurkat cells (80% decrease; $p < 0.0001$) (Fig. 2A). This effect was independent of the cell line used, since it also occurred in c-mip-overexpressing-PBMCs stimulated overnight with ConA/PHA (40% decrease; $p < 0.05$) (Fig. 2B).

The ConA and PHA mitogens mimic T cell activation by antigen receptors even in the absence of antigen presenting cells (APCs) and antigen (Gajewski et al., 1989), through the activation of several signaling pathways including NF-κB. The regulation of the NF-κB pathway occurs at different levels, including the activation of PKCθ, degradation of I-κB inhibitory proteins, and association with nuclear coactivators such as cAMP response element-binding protein (CREB)-binding protein (CBP) (Ghosh et al., 1998). We thus asked which step of the NF-κB pathway could be affected by c-mip. PKCθ is a potential target that controls NF-κB activation induced by proximal receptor signaling. We tested the possibility that c-mip inhibits NF-κB by interfering with PKCθ activation. The basal expression of phospho-PKCθ (pPKCθ), was easily detectable in Jurkat cells, as well as in PBMC and further increased following stimulation by ConA/PHA. Overexpression of c-mip did not alter the level of pPKCθ expression in Jurkat cells or in PBMCs, under either basal or stimulated conditions (data not shown). In addition, we pretreated c-mip-overexpressing-cell with Calphostin C, an inhibitor of PKC and then measured the NF-κB-driven luciferase

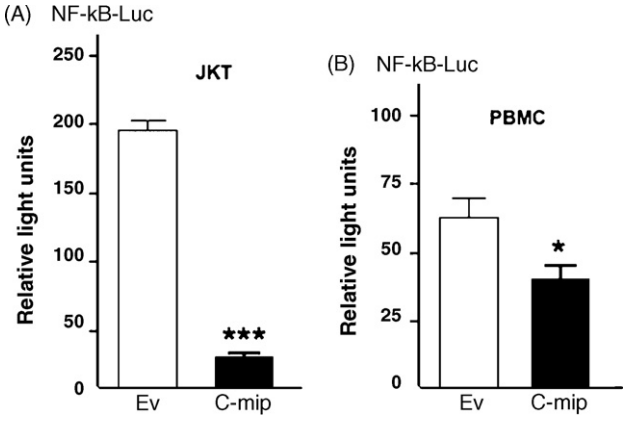


**Fig. 2.** C-mip blocks NF-κB activation. (A) Luciferase activity was measured in lysates from Jurkat cells transiently co-transfected with a c-mip expression plasmid or empty vector (Ev), and the NF-κB-Luc reporter plasmid expressing firefly luciferase, as described in Methods. Renilla luciferase expression by a phRL-null vector was used as an internal control for transfection. The data are presented as relative luciferase activity (firefly luciferase/renilla luciferase). (B) PBMCs of eight normal subjects were transiently co-transfected with c-mip expression plasmid or its empty vector (Ev), then stimulated overnight with ConA (5 μg/ml) and PHA (2 μg/ml). Statistical analyses were carried out on data from 5 independent experiments using the two-tailed *t*-test (Jurkat cells: $^{***}p < 0.0001$; unpaired *t*-test. PBMCs: $^{*}p < 0.05$; paired *t*-test).