Role of the adaptor protein CIKS in the activation of the IKK complex

Claudio Mauro, a Pasquale Vito, b Stefano Mellone, c Francesco Pacifico, c Alain Chariot, d Silvestro Formisano, a and Antonio Leonardi a, *

a Dipartimento di Biologia e Patologia Cellulare e Molecolare, ‘Federico II’ University of Naples, Via Pansini 5, Naples 80131, Italy
b Dipartimento di Scienze Biologiche e Ambientali, Università degli Studi del Sannio, Benevento, Italy
c Istituto di Endocrinologia e Oncologia Sperimentale, CNR, Naples, Italy
d Laboratory of Medical Chemistry and Human Genetics, Center for Cellular and Molecular Therapy, C.H.U. Sart-Tilman, 4000 Liège, Belgium

Received 29 July 2003

Abstract

Nuclear factor κB (NF-κB) plays a pivotal role in numerous cellular processes, including stress response, inflammation, and protection from apoptosis. Therefore, the activity of NF-κB needs to be tightly regulated. We have previously identified a novel gene, named CIKS (connection to IκB-kinase and SAPK), able to bind the regulatory sub-unit NEMO/IKKc and to activate NF-κB. Here, we demonstrate that CIKS forms homo-oligomers, interacts with NEMO/IKKc, and is recruited to the IKK-complex upon cell stimulation. In addition, we identified the regions of CIKS responsible for these functions. We found that the ability of CIKS to oligomerize, and to be recruited to the IKK-complex is not sufficient to activate NF-κB. In fact, a deletion mutant of CIKS able to oligomerize, to interact with NEMO/IKKc, and to be recruited to the IKK-complex does not activate NF-κB, suggesting that CIKS needs a second level of regulation to efficiently activate NF-κB.

© 2003 Elsevier Inc. All rights reserved.

Keywords: NF-κB; CIKS/Act1; NEMO/IKKc; Signal transduction

The NF-κB proteins are an evolutionary conserved family of transcription factors that regulate the expression of a variety of cellular genes involved in control of apoptosis, immune and inflammatory responses [1,2]. In most cell types NF-κB is sequestered in the cytoplasm, bound to inhibitors, collectively called IκBs [3]. Various stimuli, including cytokines, pathogens, and pathogen-related factors, lead to phosphorylation of IκB proteins on specific serine residues (Ser 32 and 36 for IκBα). This phosphorylation marks IκBs for ubiquitination by the SCF E3 ligase and subsequent degradation through a proteosome-dependent pathway [4]. The IκB inhibitors are phosphorylated by kinases residing in a large molecular weight complex (700–900 kDa) called the IκB kinase-complex (IKK-complex). IKK-complexes are composed of two catalytic sub-units, IKKα and β [5–9], and a regulatory sub-unit called NEMO/IKKγ [10,11]. In addition to the IκB proteins, it has been recently demonstrated that IKKs phosphorylate and regulate the processing of the precursors of p50 and p52 sub-units, respectively, p105/NF-κB1 and p100/NF-κB2 [12–14]. Beside these functions, IKKs may also modulate the transcriptional activity of NF-κB proteins, phosphorylating the transactivation domain of RelA [15].

NEMO/IKKγ was originally identified in a genetic complementation assay, as a factor able to restore NF-κB activation in cells unresponsive to a variety of stimuli that normally induce the NF-κB pathway [10]. NEMO/IKKγ was also identified in biochemical studies as a component of the high molecular weight IKK-complex [11]. NEMO/IKKγ contains a leucine zipper and two coiled–coil domains. These motifs are important for its oligomerization, which is critical for activating the IKK kinase activity [16] and for recruitment of upstream signalling mediators. Different proteins have been demonstrated to interact with NEMO/IKKγ. These include a kinase known as RIP [17], the cellular protein A20 [17], the viral transactivator tax [18–20], the cellular protein CIKS [21], and the adaptor protein TANK [22].
Thus NEMO/IKKγ can interact with a variety of different regulatory proteins that are important in regulating the activation of the NF-κB pathway in response to different stimuli. Despite all these evidences the functions and the mechanisms of NEMO/IKKγ activation remain to be determined. Particularly, it is still unclear how the IKKs are activated following different stimuli. It has been proposed that oligomerization of NEMO/IKKγ is sufficient to activate the kinase activity of the complex. For example, in response to TNF stimulation, RIP may trigger oligomerization of the IKK-complex through oligomerization of NEMO/IKKγ [16]. However, other mechanisms may exist to activate the kinase activity of the IKK-complex, such as direct phosphorylation of the IKKx/β by other kinases.

Previously, we reported on the identification of a NEMO/IKKγ-interacting protein identified in a yeast two-hybrid screen using NEMO/IKKγ as bait, which we called CIKS [21] (a.k.a. Act-1) [23]. CIKS does not have any known enzymatic activity and contains a helix–loop–helix motif at the amino terminus and a coiled–coil at the carboxyl terminus. Forced expression of CIKS activates NF-κB and JNK/SAPK pathways, suggesting that CIKS may act as an adaptor protein, linking upstream signalling pathways to the NF-κB and JNK/SAPK pathways. Recently, Qian et al. [24] reported that CIKS may be involved in CD40 signalling, at least in epithelial cells. Kanamori et al. [25] reported a potential involvement of CIKS in the IL-1/Toll pathway, by virtue of its ability to interact with TRAF6.

In this study, we used CIKS as a model to investigate the molecular mechanism by which the IKK-complex is activated. Particularly, we demonstrated that CIKS forms oligomers, interacts with NEMO/IKKγ, and is recruited to the IKK-complex after cell stimulation. In addition we identified the regions of CIKS responsible for these functions. However, a deletion mutant of CIKS able to oligomerize, to interact with NEMO/IKKγ, and to be recruited to the IKK-complex does not activate NF-κB, suggesting that CIKS needs a second level of regulation to efficiently activate NF-κB.

**Materials and methods**

**Cell culture and biological reagents.** HeLa, HEK293 were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1% (v/v) penicillin/streptomycin, and 1% glutamine.

We used two anti-CIKS polyclonal antibodies: one was generated in rabbits and was directed against a recombinant peptide encompassing amino acids 190–382 of human CIKS; the other was from Santa Cruz Biotechnologies. The mouse monoclonal IgM to human CD40 (clone 14G7) was from Caltag. Anti-FLAG and anti-FLAG agarose gel were from Sigma. All other antibodies were from Santa Cruz Biotechnologies.

Full-length NEMO/IKKγ and full-length CIKS have been previously described [21]. The HA-CIKS ΔC300 (aa 1–274), HA-CIKS ΔN300 (aa 300–574), and HA-CIKS ΔN87 (aa 87–574) were generated by PCR. For yeast experiments CIKS full-length and different CIKS deletion mutants were cloned in-frame with the Gal4 DNA binding domain of the vector pGBK7T (Clontech) or with the Gal4 activation domain of the vector pGAD7 (Clontech).

**Transfection, immunoprecipitation, and luciferase assays.** LipofectAMINE-mediated transfections were performed according to the manufacturer’s instructions (Life Technologies). For immunoprecipitation of transfected proteins, HeLa cells (2×10⁶) or HEK293 (3×10⁶) were transiently transfected and 24 h after transfection cells were lysed in Triton X-100 lysis buffer [20 mM Hepes, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM NaVO₄, 10 mM β-glycerophosphate, 5 mM NaF, and “Complete Protease Inhibitor” mixture (Roche Molecular Biochemicals)]. After an additional 15 min on ice, cell extracts were centrifuged for 20 min at 14,000 rpm at 4°C and supernatants were immunoprecipitated by using anti-HA or anti-FLAG antibodies bound to agarose beads. The immunoprecipitates were washed five times with Triton X-100 lysis buffer and subjected to SDS-PAGE.

For immunoprecipitation of colon fractions, fractions were incubated overnight with 10 μl of anti-FLAG antibodies bound to agarose beads. The immunoprecipitates were washed five times with Triton X-100 lysis buffer and subjected to SDS-PAGE.

For luciferase assay, HeLa (2×10⁶) or HEK293 (4×10⁶) were seeded in 6-well (35 mm) plates. After 12 h cells were transfected with 0.5 μg of Ig-κB-luciferase reporter plasmid and various amounts of each expression plasmid. Total amount of transfected DNA was kept constant by supplementing empty expression plasmid as needed. Cell extracts were prepared 24 h after transfection and reporter gene activity was determined via the luciferase assay system (Promega). Expression of the pRSV-β-gal vector (0.2 μg) was used to normalize transfection efficiencies.

**Gel filtration of cellular extract.** HeLa cells were lysed in Triton X-100 lysis buffer. Lysates were incubated for 15 min on ice and clarified by centrifugation at 14,000 rpm for 15 min, 4°C. Supernatants were collected and recentrifuged for 1 h at 100,000g at 4°C. One milligram of the S-100 extracts (0.5 ml) was loaded onto a Superdex S200 HR (Amersham–Pharcmaica Biotech). Proteins were eluted from the column at the flow rate of 0.3 ml/min. Fractions (0.4 ml) were precipitated with 10% trichloroacetic acid, resuspended in Laemmli’s buffer, and analyzed by SDS-PAGE followed by Western blotting for IKKα, IKKβ, NEMO/IKKγ, and CIKS using appropriate antibodies.

**Kinase assay.** For IK kinase activation, endogenous IKKβ was immunoprecipitated from the fractions eluted from the column by using anti-IKKβ antibodies and the kinase activity was assayed by using GST-λκB (aa 1–54) as substrate.

**Results**

**CIKS forms oligomers**

We have previously identified a novel protein we called CIKS by using NEMO/IKKγ as bait in a yeast two-hybrid screening [21] (a.k.a. Act-1) [23]. The activities of CIKS loosely resemble those of TRAF proteins. In fact, similar to TRAF2, 5 or 6, overexpression of CIKS activates SAPK/JNK and IKK kinases [21,26–30]. The capacity of TRAFs to activate downstream pathways appears to be linked to the ability of these proteins to oligomerize [30]. In order to investigate if a similar molecular mechanism was involved in the ability of CIKS to activate NF-κB, we examined, in yeast, if CIKS was able to form oligomers. Yeast strain AH109
was transformed with various deletion mutants of CIKS fused to the GAL4-DNA-BD in the presence of full-length CIKS fused to the GAL4-AD (Fig. 1A). The ability of the two fusion proteins to interact was judged by growth of the yeast strain AH109 on selective media. It was possible to detect an interaction between CIKS-DNABD and CIKS-AD, suggesting that CIKS was able to oligomerize, at least in yeast. While any deletion at the amino terminus of CIKS did not affect its ability to oligomerize, deletion of the carboxyl-terminus coiled-coil domain disrupted the interaction.

We confirmed the results obtained in yeast, in transient transfection experiments. HeLa cells were transfected with FLAG-tagged CIKS together with HA-tagged CIKS or HA-tagged deletion mutants of CIKS, lacking the amino- or the carboxyl-terminus (CIKS ΔN300 and CIKS ΔC300). Cell extracts were immunoprecipitated by using anti-FLAG antibodies and the co-immunoprecipitated proteins were visualized by Western blot with anti-HA-antibodies (Fig. 1B, upper panel). Full-length HA-CIKS and HA-CIKS ΔN300 were co-immunoprecipitated with FLAG-CIKS (Fig. 1B upper panel, lanes 5 and 6) while HA-CIKS ΔC300 was not (Fig. 1B upper panel, lane 7), despite the higher level of expression in HeLa cells. We obtained similar results also in HEK293 cells (data not shown). These data strongly suggest that CIKS is able to form homo-oligomers and that this ability resides in the C-terminal region.

CIKS is recruited to the IKK-complex

We previously demonstrated that CIKS interacts with NEMO/IKKγ and when overexpressed activates the transcription factor NF-κB via IKKs [21]. Given the ability of CIKS to interact with NEMO/IKKγ and to activate NF-κB, we investigated if CIKS was recruited to the IKK-complex. We reasoned that if forced expression of CIKS mimics the active form of the protein then, when overexpressed, CIKS might be recruited to the IKK-complex. To this purpose, HeLa cells were transiently transfected with an expression vector encoding FLAG-tagged CIKS and cellular extract was loaded onto a Superdex S200 HR Fast Protein Liquid Chromatography column. Fractions eluted from the column were analyzed for the presence of specific components of the IKK-complex and CIKS, using Western blot analysis (Fig. 2). The majority of endogenous IKKα, IKKβ, and NEMO/IKKγ were eluted in a peak centered around a volume of 9 ml (relative molecular mass higher than 660,000 Da), confirming that the IKK-complex is preconstituted in unstimulated cells. Analysis of chromatographic distribution of transfected CIKS showed that this protein was eluted in the same fractions containing the components of the IKK-complex and CIKS, using Western blot analysis (Fig. 2). The majority of endogenous IKKα, IKKβ, and NEMO/IKKγ were eluted in a peak centered around a volume of 9 ml (relative molecular mass higher than 660,000 Da), confirming that the IKK-complex is preconstituted in unstimulated cells. Analysis of chromatographic distribution of transfected CIKS showed that this protein was eluted in the same fractions containing the components of the IKK-complex. In addition, immunoprecipitating transfected CIKS from these high molecular weight fractions, it was possible to detect endogenous NEMO/IKKγ co-immunoprecipitating with transfected CIKS. To further demonstrate that the interaction of CIKS with the IKK-complex correlates with the functional activation of the complex, we immunoprecipitated endogenous IKKβ from the high molecular weight fractions and assayed its ability

---

**Fig. 1.** Mapping the oligomerization domain of CIKS. (A) Yeast-two hybrid experiment. The CIKS constructs cloned in-frame with the GAL4 DNA binding domain of the vector pGBK T7 are schematically illustrated. Plus and minus signs indicate growth or absence of growth of the transformed yeast on medium lacking tryptophan, leucine, histidine, and adenine in the presence of 15 mM of 3-aminotriazole. (B) Co-immunoprecipitation experiment. HeLa cells were transfected with the indicated expression vector. Total extracts were immunoprecipitated with anti-FLAG antibodies followed by Western blot analyses with anti-HA antibodies (top panel). The presence of the different constructs in the whole cell lysate is demonstrated by Western blot in the middle and bottom panels.
to phosphorylate GST-IκBα. It was possible to isolate a specific IκB kinase activity in high molecular weight fractions from CIKS transfected cells. These data demonstrated that transfected CIKS was recruited to the IKK-complex, via its ability to interact with NEMO/IKKγ and triggered activation of the kinase activity of the complex.

We then analyzed the chromatographic distribution of endogenous CIKS in untransfected cells (Fig. 3). Most of the protein was eluted in a peak centered around a volume of 15 ml, corresponding to a molecular mass of about 70,000 Da, very similar to its predicted molecular weight of 64,000 Da. Recently, it has been reported that CIKS may function as an adaptor molecule in CD40-mediated pathways in epithelial cells [24]. In order to investigate if also the functionally active form of endogenous CIKS was recruited to the IKK-complex, we analyzed the chromatographic distribution of endogenous CIKS in HeLa cells after CD40 triggering. To this purpose HeLa cells were treated with a stimulating anti-CD40 antibody for thirty minutes. After stimulation, cells were washed and the lysate was loaded onto a Superdex S200 HR Fast Protein Liquid Chromatography column. Part of endogenous CIKS was still eluted in a peak corresponding to a molecular mass of 70,000 Da. However, a fraction of CIKS moved to the high molecular weight fractions and was coeluted with components of the IKK-complex (Fig. 3), suggesting that following triggering of CD40, at least part of endogenous CIKS was recruited to the IKK-complex. These results suggested that endogenous CIKS was retained in the cytoplasm in a monomeric form in unstimulated cells, while after stimulation it was recruited to the IKK-complex.

Both the N-terminal and the C-terminal regions of CIKS are required for interaction with NEMO/IKKγ

We next investigated which region of CIKS was necessary for the interaction with NEMO/IKKγ. HeLa cells were transfected with an expression vector encoding FLAG-tagged NEMO/IKKγ. Cell extracts (S-100) were loaded onto a Superdex S200 FPLC column. (A) Elution profile of the column. Position of the molecular weight standards used to calibrate the column is indicated by arrows. Tg: thyroglobulin, MW 660,000 Da; BSA: bovine serum albumin, MW 67,000 Da; and Lys: lysozyme, MW 14,000. (B) Fractions isolated from the Superdex S-200 column were subjected to either Western blot or immunoprecipitation or kinase assay. For immunoprecipitation, fractions were incubated with anti-FLAG monoclonal antibodies (clone M2 from Sigma) bound to agarose beads. For kinase assay, fractions were incubated with anti-IKKβ antibodies (Santa Cruz #7607). Molecular weight markers are indicated at the top of the figure.
interact with NEMO/IKKγ, we investigated which of these properties was necessary to activate NF-κB. HeLa cells were transfected with an Ig-κB luciferase reporter plasmid in presence of CIKS full-length or different deletion mutants of CIKS (Fig. 5A). Full-length CIKS was able to strongly activate a κB-driven luciferase reporter plasmid, while the N-terminal deletion mutants ΔN87 and ΔN300 and the C-terminal deletion mutants ΔC300 did not. The incapacity of the ΔN300 and ΔC300 mutants to activate NF-κB might be explained by the inability of these mutants to form oligomers and to interact with NEMO/IKKγ. Interestingly, the ΔN87 deletion mutant that forms oligomers and interacts with NEMO/IKKγ does not activate NF-κB. The amount of each transfected plasmid was normalized given the different expression levels of these constructs (see Fig. 1B and data not shown).

Next we investigated the chromatographic distribution of these CIKS mutants. HeLa cells were transfected with the ΔN87, ΔN300, and ΔC300 deletion mutants. Cell extracts (S100) were loaded onto a Superdex S200 HR Fast Protein Liquid Chromatography column and fractions eluted from the column were analyzed by Western blot by using anti-FLAG antibodies (Fig. 5B). The C-terminal deletion mutant ΔC300 was eluted in a peak corresponding to a molecular mass of about 30,000 Da. The N-terminus deletion mutants ΔN87 and ΔN300 were eluted in peaks corresponding to molecular masses higher than 660,000 Da and of about 120,000 Da, respectively. The chromatographic distribution of these mutants reflected their ability to form oligomers and to interact with NEMO/IKKγ. In fact, the ΔC300 mutant that was not able to oligomerize and to interact with NEMO/IKKγ was eluted at a volume corresponding to its predicted molecular weight. In contrast, the ΔN87 mutant, still able to interact with NEMO/IKKγ and to oligomerize, was co-eluted with components of the IKK-complex. The ΔN300 mutant that retained the ability to oligomerize but not to interact with NEMO/IKKγ was eluted at a volume intermediate between the other two mutants.

Taken together these results suggest that the ability of CIKS to form oligomers and to be recruited to the IKK-complex resides in different regions of the protein and that both activities are necessary but not sufficient to correctly activate NF-κB.

Discussion

In the present paper, we reported a functional characterization of the adaptor protein CIKS. We demonstrated that CIKS was able to form oligomers and once overexpressed was recruited to the IKK-complex through interaction with NEMO/IKKγ. In addition, we demonstrated that both transfected CIKS and endogenous CIKS after CD40 triggering were recruited to the IKK-complex. The ability of CIKS to form oligomers resided in the C-terminal region, while the ability to interact with NEMO/IKKγ was mediated by the central region of the protein. Both the capacities
to oligomerize and to interact with NEMO/IKKγ were necessary to activate NF-κB. In fact, deletions affecting one of these functions abolished the ability of CIKS to activate NF-κB. However, these functions were not sufficient for a correct activation of NF-κB. In fact a small N-terminus deletion mutant (ΔN87) still able to interact with NEMO/IKKγ and to form oligomers did not activate NF-κB. Our data suggest that in addition to oligomerization and interaction with NEMO/IKKγ, CIKS needs a second level of regulation to efficiently activate NF-κB.

CIKS has been proposed to interact with TRAF3 and by virtue of this interaction has been proposed to be involved in the CD40 signalling. Quian et al. [24] proposed a role for CIKS in the CD40 pathway. Based on this model CIKS is recruited to CD40 after receptor triggering. Our data extend this model, demonstrating that after CD40 triggering, CIKS connects the receptor and the IKK-complex, through its interaction with NEMO/IKKγ, mediating the activation of NF-κB. It remains to be determined whether the IKK-complex is recruited to the cytoplasmic domain of the receptor or, after interaction with CD40, CIKS dissociates from the receptor to interact with NEMO/IKKγ.

The functions of CIKS resemble those of TRAF proteins. These are adaptor molecules that mediate NF-κB activation by different receptors, such as members of the TNF and IL-1/Toll receptor family. Activation of NF-κB following TNF-receptor engagement is known to be initiated recruiting different adaptor molecules like TRADD, RIP, and TRAF on the trimerized cytoplasmic domain of the TNF receptor [31]. RIP and TRAF, in turn, transiently recruit and activate the IKK complex, via NEMO/IKKγ [17,32]. However, it is not yet clear how the IKK-complex is activated. Recently, it has been demonstrated that the enforced oligomerization of NEMO/IKKγ or any component of the IKK-complex is sufficient to activate the kinase activity of the complex. By virtue of its ability to form oligomers and to interact with NEMO/IKKγ after CD40 triggering, CIKS may function in a similar way to activate the IKK-complex. However, while the ability of CIKS to oligomerize and to be recruited to the complex is both necessary to activate NF-κB, these are not sufficient. In fact, a small deletion of the N-terminal domain of CIKS (CIKS ΔN87), that leaves unaltered the ability of CIKS to interact with NEMO/IKKγ, to be recruited to the IKK-complex and to form oligomers, abolished its ability to activate NF-κB. It is possible that the ΔN87 mutant is incapable of inducing conformational changes important for the activation of the kinase activity of the IKK-complex. However, it is tempting to speculate that the N-terminus of CIKS is interacting with some other adaptor molecule(s) or some kinase(s) that are, in turn, responsible for the activation of the IKK-complex. This hypothesis is currently under investigation. It has been reported previously that CIKS interacts with the protein kinase TAK1 [24] but the exact role of this kinase in CD40-mediated NF-κB activation needs to be further addressed. In addition, the importance of other MAP3 kinase kinase kinase such as NIK, MEKK1, and MEKK3 in the activation of the IKKs is still controversial.

In summary, our data provide evidences about the role of the adaptor protein CIKS in the activation of the IKK-complex. After CD40 triggering CIKS is recruited to the IKK-complex, through its ability to interact with NEMO/IKKγ and to form oligomers. However, these abilities are not sufficient to activate NF-κB, suggesting that CIKS needs a second level of regulation to efficiently activate NF-κB.

Acknowledgments

The authors thank R. Acquaviva for assistance with the FPLC and helpful discussion. This work was supported by grants from AIRC (Associazione Italiana Ricerca sul Cancro) to A.L. and Ministero della Universita’ e Ricerca Scientifica Grant 2001065217 to S.F.

References


