Identification of a novel antiapoptotic protein that antagonizes ASK1 and CAD activities

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Diverse stimuli initiate the activation of apoptotic signaling pathways that often causes nuclear DNA fragmentation. Here, we report a new antiapoptotic protein, a caspase-activated DNase (CAD) inhibitor that interacts with ASK1 (CIIA). CIIA, by binding to apoptosis signal-regulating kinase 1 (ASK1), inhibits oligomerization-induced ASK1 activation. CIIA also associates with CAD and inhibits the nuclease activity of CAD without affecting caspase-3–mediated ICAD cleavage. Overexpressed CIIA reduces H2O2- and tumor necrosis factor-α–induced apoptosis. CIIA antisense oligonucleotides, which abolish expression of endogenous CIIA in murine L929 cells, block the inhibitory effect of CIIA on ASK1 activation, deoxyribonucleic acid fragmentation, and apoptosis. These findings suggest that CIIA is an endogenous antagonist of both ASK1- and CAD-mediated signaling.

Introduction

Apoptosis, or programmed cell death, is a fundamental cellular process for the self-elimination of unwanted or damaged cells. Apoptosis occurs during a variety of biological processes in multicellular organisms including development and pathogenesis of many diseases. Apoptotic cells undergo morphological changes such as chromosomal condensation, loss of mitochondrial membrane potential, and formation of apoptotic bodies. These morphological events follow biochemical events that include activation of caspases, changes in plasma membrane composition, and DNA fragmentation (Nagata, 1997; Green and Reed, 1998).

Caspases-3 and -7 have been shown to stimulate the fragmentation of chromosomal DNA through the activation of caspase-activated DNase (CAD), also known as caspase-acti-...
stimulated in response to a variety of cellular stresses, including UV light, osmotic and heat shock, DNA damaging agents, and proinflammatory cytokines (Derijard et al., 1994; Galcheva-Gargova et al., 1994; Han et al., 1994; Kiyrakis et al., 1994). The JNK–SAPK and p38 pathways have been shown to be associated with mechanisms of apoptotic cell death under certain conditions (Xia et al., 1995; Verheij et al., 1996; Minden and Karin, 1997; Ip and Davis, 1998).

Apoptosis signal-regulating kinase 1 (ASK1) is a MAPK kinase kinase that activates the JNK/SAPK and the p38 signaling cascades (Ichijo et al., 1997). ASK1 is shown to be involved in apoptosis, induced by tumor necrosis factor (TNF)-α, Fas, and cellular stresses (Ichijo et al., 1997). Here, we report the identification of an antiapoptotic protein that physically interacts with both ASK1 and CAD. This protein is named a CAD inhibitor that interacts with ASK1 (CIIA). CIIA inhibits stress- or TNF-α–induced ASK1 activation. Furthermore, CIIA inhibits CAD-mediated DNA fragmentation. Thus, our data suggest that CIIA functions as an endogenous antagonist of ASK1 and CAD activities.

Results

Isolation of a cDNA clone of CIIA

To better understand the mechanism underlying the regulation of ASK1 function, we searched initially for ASK1-binding proteins using the yeast two-hybrid screening method with a mouse adult brain cDNA library. From the two-hybrid study, we identified a new gene that encoded a 221-aa protein (Fig. 1 A). Surprisingly, the same gene was also identified in an independent yeast two-hybrid screening experiment using CAD as the bait. On the basis of the two-hybrid results, we named the protein encoded by this gene CIIA. We also found a human EST clone (GenBank/EMBL/DDBJ accession no. AA432040) by BLAST searches that has a high level of homology with the mouse CIIA gene. CIIA has no known conserved protein motif. A mouse multiple-tissue Northern blot analysis using a mouse CIIA cDNA probe revealed that a transcript of 1 kb was highly expressed in many adult tissues including heart, brain, liver, and kidney (Fig. 1 B). Immunoblot analysis using rabbit preimmune IgG or anti-CIIA antibody showed that CIIA protein was present in both the nucleus and cytoplasm in cultured cells (unpublished data).

CIIA physically interacts with ASK1 and CAD

In our initial studies, NIH 3T3 cells stably expressing HA-tagged CIIA (HA-CIIA) were subjected to glycerol gradient fractionation. After cell lysates were subjected to centrifugation at 1,000 g, the resulting soluble fraction was subjected to glycerol gradient centrifugation. Immunoblot analysis of
the glycerol gradient fractions revealed that the majority of ASK1 and CIIA were present in the same fractions (Fig. 2, fractions 10–16). CIIA and CAD also resided in the same fractions (Fig. 2, fractions 10–17). These fractionation results suggest that CIIA is in close proximity to ASK1 and to CAD. Next, we transiently transfected NIH 3T3 cells with CIIA plus CAD or CIIA plus ASK1 constructs and examined subcellular distribution of the proteins by immunofluorescence microscopy. Double-labeled immunostaining data revealed that ectopic CIIA was located in both the nucleus and the cytoplasm and that its distribution was overlapped with that of CAD or ASK1 (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200303003/DC1).

To test whether CIIA interacted directly with ASK1, we performed an in vitro binding study using recombinant GST-fused CIIA variants and in vitro–translated 35S-labeled ASK1 (Fig. 3 A). Both full-length CIIA and CIIA-AN associated with ASK1, whereas CIIA-ΔC and CIIA-CEN failed to bind to ASK1 (Fig. 3 A). In separate in vitro binding experiments, GST-CIIA interacted with in vitro–translated 35S-labeled full-length ASK1, ASK1-ΔC, and ASK1-NT, but not with ASK1-AN (Fig. 3 B). Thus, these data suggest that CIIA binds the NH2-terminal half region of ASK1. Other ASK1-interacting proteins such as TRAF2, GMT1, and Daxx have been also shown to bind the NH2-terminal region of ASK1 (Chang et al., 1998; Liu et al., 2000; Cho et al., 2001). Therefore, we examined whether CIIA could affect the binding of ASK1 with TRAF2, GMT1, or Daxx. A coimmunoprecipitation study revealed that CIIA inhibits the physical interaction of ASK1 with TRAF2, GMT1, or Daxx (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200303003/DC1).

Next, we tested a physical interaction between two endogenous CIIA and ASK1 proteins in intact cells by coimmunoprecipitation. Lysates of mouse embryonic fibroblasts (MEFs) or mouse brain tissue were subjected to immunoprecipitation using rabbit anti-CIIA antibody, or rabbit preimmune IgG. The resulting precipitates were subjected to SDS-PAGE and analyzed by immunoblotting (IB) with anti-ASK1 antibody. Immunoblotting of cell lysates (5% of total) with anti-ASK1 antibody was also shown. (D) 293T cells were transfected with expression vectors encoding ASK1-Flag and HA-CIIA as indicated. After 48 h of transfection, the cells were untreated or treated with 500 μM H2O2 for 1 h. Cell lysates were subjected to immunoprecipitation with anti-HA antibody, and the resulting immunoprecipitates were subjected to immunoblot analysis with anti-Flag antibody. Cell lysates were also subjected to immunoblot analysis with the indicated antibodies. (E) L929 cells were untreated or treated with 500 μM H2O2 for indicated time periods. Cell lysates were subjected to immunoprecipitation and the resulting immunoprecipitates were analyzed by immunoblotting as in C. Cell lysates (5% of total) were also subjected to immunoblot analysis with anti-ASK1 or anti-CIIA antibody.
precipitation using anti-CIIA antibody, and the resulting immunoprecipitates were analyzed by immunoblotting with anti-ASK1 antibody. Immunoblot data revealed that CIIA physically associates with ASK1 in MEFs and cells from mouse brain (Fig. 3 C). Physical association between CIIA and ASK1 was also confirmed by coimmunoprecipitation in 293T cells transfected with plasmids encoding HA-tagged CIIA and Flag-tagged ASK1 (ASK1-Flag; Fig. 3 D). Interestingly, the interaction between ectopic CIIA and ASK1 was increased by H$_2$O$_2$ treatment. Subsequently, we examined a time course of the H$_2$O$_2$ action on the physical association of endogenous CIIA and ASK1 proteins in L929 cells (Fig. 3 E). Coimmunoprecipitation results indicated that H$_2$O$_2$-induced enhancement of the interaction between the two endogenous proteins was maximal at 1 h.

Next, we examined in vitro binding between CIIA and CAD using recombinant GST-CIIA variants and CIIA-CEN (Fig. 4 A). In comparison, $^{35}$S-labeled ICAD-L did not interact with CIIA in vitro. We also conducted in vitro binding studies using $^{35}$S-labeled CIIA and GST-fused CAD variants. $^{35}$S-Labeled CIIA bound to CAD, CAD-NT, and CAD-ΔC, but not to CAD-ΔN (Fig. 4 B). Interestingly, CAD-ΔN lacks amino acid residues 1–83, which is homologous to a NH$_2$-terminal domain of CIDE proteins (the CIDE-N domain; Inohara et al., 1998). It was reported previously that the NH$_2$-terminal domain of CAD is involved in binding to ICAD (Inohara et al., 1999). Therefore, we examined whether in vitro–translated $^{35}$S-labeled CIIA can form a tertiary complex with the CAD–ICAD complex. $^{35}$S-Labeled CIIA bound to the recombinant CAD–ICAD complex, but not to ICAD (Fig. 4 C).

Next, we examined the physical interaction between CIIA and CAD in 293T cells expressing HA-CIIA and CAD-Flag (Fig. 4 D). Immunoblot analysis of the HA immunoprecipitates revealed a physical association of CIIA with CAD in the cotransfected cells. We also examined whether ICAD...
could inhibit the interaction between CAD and CIIA. Coexpression of ICAD-L did not interfere with the interaction between HA-CIIA and CAD-Flag (Fig. 4 D). In fact, coexpression of ICAD-L resulted in an increase in the amount of CAD-Flag coimmunoprecipitated with HA-CIIA, presumably because of an increase in the expression of functional CAD-Flag. ICAD-L has been shown not only to inhibit CAD activity but also to enhance the expression of functional CAD (Enari et al., 1998; Sakahira et al., 1999). Physical association of two endogenous CIIA and CAD proteins was also confirmed in MEFs and cells from mouse brain by coimmunoprecipitation (Fig. 4 E). Next, we examined a possible effect of H$_2$O$_2$ on the physical interaction between endogenous CIIA and CAD in L929 cells. L929 cells were unexposed or exposed to H$_2$O$_2$ for various times, and cell lysates were analyzed by coimmunoprecipitation. Physical interaction between CIIA and CAD was maximal after 30 min of an exposure of L929 cells to H$_2$O$_2$ (Fig. 4 F).

**CIIA inhibits the kinase activity of ASK1**

Next, we investigated whether CIIA could affect the kinase activity of ASK1. Ectopic ASK1 was stimulated by an exposure of the transfected cells to UV light or H$_2$O$_2$ or by coexpression of TRAF2 (Fig. 5 A). Expression of CIIA suppressed UV-, H$_2$O$_2$-, and TRAF2-stimulated ASK1 activities in the cells. In comparison, CIIA neither bound to nor inhibited MAPK/extracellular signal–regulated kinase kinase 1 (MEKK1), another MAPK kinase kinase that stimulates the JNK–SAPK pathway (Figs. S3 and S4, available at http://www.jcb.org/cgi/content/full/jcb.200303003/DC1).

Homo-oligomerization of ASK1 is one mechanism for ASK1 activation (Gotoh and Cooper, 1998; Liu et al., 2000). Therefore, we examined whether CIIA interferes with ASK1 oligomerization (Fig. 5 B). 293T cells were transfected with ASK1-Flag and ASK1-HA constructs in the absence or presence of the CIIA construct. Coimmunoprecipitation analysis indicated that ASK1-HA was found to be associated with ASK1-Flag in the transfected cells. The ASK1 homo-oligomerization was inhibited by coexpression of CIIA. These results suggest that CIIA may inhibit ASK1 activation, at least in part, by the suppression of ASK1 homo-oligomerization.

Next, we examined the effect of CIIA on the signaling events downstream of ASK1. ASK1 activation can induce stimulation of JNK, which in turn enhances the transcription-stimulating activity of c-Jun (Ichijo et al., 1997; Ip and Davis, 1998). Overexpressed ASK1-induced JNK1 activation and this ASK1-dependent JNK1 activation was blocked by CIIA expression in transfected 293T cells (Fig. 5 C). CIIA
also inhibited the ASK1-induced stimulation of the transcription-stimulating activity of c-Jun (Fig. 5D). CIIA did not interact physically with JNK1 or c-Jun (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200303003/DC1).

CIIA inhibits the DNase activity of CAD

Next, we investigated whether CIIA could modulate the DNase activity of CAD. An in vitro CAD assay showed that treatment of a recombinant CAD–ICAD-L complex with caspase-3 resulted in the stimulation of the nuclease activity of CAD, whereas GST alone or GST-ICAD-L did not. Caspase-3-dependent CAD activation was also inhibited by CIIA-DN, but not by CIIA-DN or CIIA-CEN (Fig. 6B). To further understand the mechanism underlying the inhibitory action of CIIA on CAD activation, we examined whether CIIA could block a caspase-3–catalyzed cleavage of ICAD. In vitro cleavage results indicated that CIIA did not inhibit a cleavage of ICAD by caspase-3 (Fig. 6C). Next, recombinant CAD–ICAD complex was pretreated with caspase-3 and examined for the nuclease activity in the absence or presence of CIIA. CIIA inhibited DNA fragmentation mediated by caspase-3–pretreated CAD–ICAD complex (Fig. 6D). Collectively, these results suggest that CIIA blocks the CAD-dependent DNA fragmentation through an inhibition of the DNase activity of CAD.

Next, the effect of CIIA on CAD-mediated DNA fragmentation was examined in 293T cells. The cells were transfected with various combinations of plasmids encoding CAD-Flag, ICAD-L, a constitutively active caspase-3, and HA-CIIA (Fig. 6E). Expression of CAD and ICAD-L with the caspase-3 enhanced DNA fragmentation in the transfected cells. Coexpression of CIIA inhibited the caspase-3/CAD-dependent DNA fragmentation. Together, our data suggest that CIIA suppresses the DNase activity of CAD.

CIIA reduces TNF-α- and H2O2-induced apoptosis

To further assess the function of CIIA, HA-CIIA construct was stably transfected into 293T cells, in which a level of endogenous CIIA was quite low (unpublished data). Expression of HA-CIIA did not affect endogenous levels of ASK1, CAD, or ICAD in 293T cells (unpublished data). TNF-α treatment of 293-neo control cells resulted in ASK1 activation, however, TNF-α–induced ASK1 activation was impaired in the cells expressing HA-CIIA (293-CIIA cells; Fig. 7A). CIIA overexpression also inhibited TNF-α–induced activation of JNK1, a downstream kinase of ASK1. Furthermore, TNF-α–induced DNA fragmentation was lowered in 293-CIIA cells, compared with that of 293-neo cells (Fig. 7B). ASK1 plays a crucial role in the mechanisms of TNF-α– and stress-induced apoptosis through the activation of stress-activated MAPKs, including JNK/SAPK (Ichijo et al., 1997), and the mitochondria-dependent activation of caspases (Hatai et al., 2000). Therefore, we examined the effect of CIIA on apoptotic cell death induced by TNF-α or H2O2 (Fig. 7C). 293-CIIA cells were more resistant to both...
TNF-α– and H$_2$O$_2$-induced apoptosis, compared with 293-neo cells. CIA also protected 293-CIIA cells against apoptosis induced by Daxx, an activator of ASK1 (Chang et al., 1998; unpublished data).

To further investigate the inhibitory effect of CIA on apoptosis, we transiently transfected 293T cells with expression vectors for full-length CIA, CIA-ΔN, and CIA-ΔC, and measured TNF-α– and H$_2$O$_2$-induced apoptosis as well

Figure 7. CIA inhibits apoptosis. (A) CIA inhibits TNF-α–stimulated ASK1 activity in 293-CIIA cells. 293T cells were stably transfected with pcDNA3 empty vector or pcDNA3-HA-CIIA, yielding 293-neo control or 293-CIIA cells, respectively. Heterogeneous populations of the stably transfected cells were used to avoid clonal variations. The cells were untreated or treated with 20 ng/ml TNF-α for 20 min. Cell lysates were subjected to immunoprecipitation with anti-ASK1 or anti-JNK1 antibodies, and the resulting immunoprecipitates were assayed for the kinase activities of endogenous ASK1 or JNK1 by immunocomplex kinase assay. Cell lysates were also examined by immunoblot analysis with anti-ASK1, anti-JNK1, or anti-HA antibody. (B) CIA decreases TNF-α–induced DNA fragmentation in 293-CIIA cells. 293-neo and 293-CIIA cells were treated with 20 ng/ml TNF-α plus 1 μg/ml actinomycin D for the indicated time periods. Chromosomal DNA was obtained from the cells and examined for DNA fragmentation. (C) CIA suppresses TNF-α– and H$_2$O$_2$–induced apoptosis. 293-neo and 293-CIIA cells were exposed to 20 ng/ml TNF-α plus 1 μg/ml actinomycin D (TNF-α/ActD) or 500 μM H$_2$O$_2$, incubated overnight, and assayed for apoptotic cell death by flow cytometry with annexin V staining. Data are mean ± SEM from three experiments. (D and E) 293T cells were transiently transfected for 50 h with plasmids encoding full-length CIA, CIA-ΔN, or CIA-ΔC. The cells were untreated or treated with 20 ng/ml TNF-α plus 1 μg/ml actinomycin D (TNF-α/ActD) or 500 μM H$_2$O$_2$, further incubated overnight, and assayed for apoptosis by DAPI staining. The data are presented as the mean ± SEM. The statistical significance is designated by an asterisk. P < 0.01 versus control (mock transfection).
as ASK1 and JNK1 activation in the transfected cells. CIIA and CIIA-ΔN inhibited TNF-α- and H₂O₂-stimulated apoptosis in the transfected cells (Fig. 7 F). In contrast, the effect of CIIA-ΔC was marginal. Together, these results suggest that the inhibition of ASK1 activity by CIIA is crucial for the antiapoptotic function of CIIA.

**CIIA antisense oligonucleotides prevent CIIA from inhibiting ASK1 activation and DNA fragmentation**

To test the role of endogenous CIIA protein in stress-induced ASK1 activation and DNA fragmentation, CIIA antisense oligonucleotides were transfected into L929 cells (Fig. 8 A). Among the antisense oligonucleotides used, CIIA-AS3 most effectively blocked the expression of endogenous CIIA in L929 cells. Inhibition of CIIA expression by CIIA-AS3 resulted in an increase in the UV-stimulated activity of endogenous ASK1 in L929 cells. Transfection of L929 cells with antisense oligonucleotides CIIA-AS3 (AS3) or its complementary sense oligonucleotides (Sense3). After 48 h of transfection, the cells were untreated (−) or treated (+) with 80 J/m² UV light, 20 ng/ml TNF-α plus 10 μg/ml cycloheximide (TNF/CHX), or 500 μM H₂O₂ and incubated overnight. The cells were then examined for (C) DNA fragmentation or for (D) apoptosis by DAPI staining. Data are the mean of triplicate determinations ± SEM. The data represent results from three independent experiments.

![Figure 8](image_url)
sense oligonucleotides (Fig. 8D). Collectively, these data suggest that CIIA functions as a natural antagonist against ASK1-mediated signaling and DNA fragmentation.

Discussion

Here, we identified a new protein, CIIA, which functions as an antagonist of both ASK1 and CAD activities. ASK1 has been shown to function in apoptosis triggered by a variety of cellular stresses and proinflammatory cytokines (Ichijo et al., 1997; Chang et al., 1998; Chen et al., 1999). Oligomerization of ASK1 is shown to be associated with the mechanism for ASK1 activation (Gotoh and Cooper, 1998; Nishitoh et al., 1998; Hoeflich et al., 1999; Liu et al., 2000). Our results suggest that CIIA, by binding ASK1, interferes with ASK1 oligomerization and blocks ASK1 activation. Interestingly, other ASK1 inhibitors, including thioredoxin and GST, appear to associate with CAD regardless of the presence of ICAD-L. Our data show that CIIA associates with the CIDE-N domain of CAD. Interestingly, CIIA does not associate with ICAD-L even though ICAD-L also contains a CIDE-N domain. Furthermore, CIIA can associate with CAD regardless of the presence of ICAD-L. The physical association of CIIA with CAD leads to the inhibition of the nuclease activity of CAD.

Upon exposure of cells to a variety of apoptotic stimuli, CIIA appears to suppress ASK1 activation and CAD-mediated DNA fragmentation. In this regard, it was reported recently that ASK1 activation enhances cytochrome c release from the mitochondria into the cytoplasm, as well as the subsequent activation of caspase-9 and downstream caspases (Hatai et al., 2000). The activated caspase cascade may stimulate downstream apoptotic pathways including CAD-mediated DNA pathways. Thus, CIIA may antagonize the ASK1-mediated apoptotic pathway with high efficiency by inhibiting ASK1 activation at an early stage of apoptosis and induction of ASK1-induced DNA fragmentation at a later stage.

On the basis of our findings, we propose that CIIA functions as a natural inhibitor of both ASK1 and CAD. The dual function of CIIA may constitute an integral part of the mechanism by which the apoptotic pathways in the cytoplasm and in the nucleus are controlled. In addition, while the present paper was in preparation, a human counterpart of mouse CIIA was reported. This human protein, named hVPS28, interacts with the Tsg101 protein and appears to be involved in endosomal sorting (Bishop and Woodman, 2001). Tsg101, originally discovered as a tumor susceptibility gene, has been implicated in transcriptional regulation and neoplastic transformation (Li and Cohen, 1996). Therefore, it is tempting to propose that CIIA may be a multifunctional regulator that associates with intracellular signaling networks for apoptosis, cellular stress, and tumorigenesis.

Materials and methods

Antibodies

Rabbit anti-ASK1 and anti-hexahistidine (His) pAbs were purchased from Santa Cruz Biotechnology. Rabbit anti-CAD and anti-ICAD pAbs were purchased from Oncogene Research Products. Affinity-purified rabbit anti-CIIA pAb was produced from rabbits immunized with His-CIIA protein. Mouse anti-HA, anti-Flag, anti-Myc, and anti-H7 mAbs were purchased from Roche Molecular Biochemicals, Stratagene, Cell Signaling Biotechnology, and Novagen, respectively.

DNA constructs

ASK1 deletion mutants, ASK1-ΔN, ASK1-ΔC, and ASK1-NT, were generated by PCR, and subcloned into pcDNA3 vector (Invitrogen; Cho et al., 2001). Daxx(498–740) was a gift from S.H. Kim (Sungkyunkwan University, Su- won, Korea). JNK1, TRAF2, and caspase-3 cDNA clones were from R.J. Davis (University of Massachusetts, Worcester, MA). D.G. Goeddel Tulark Inc., South San Francisco, CA), and Dr. Y.K. Chung (Kwangju Institute of Science and Technology, Kwangju, Korea), respectively. CAD and ICAD-L cDNAs were obtained by RT-PCR (Park et al., 2000). The cDNA of the mouse CIIA gene was obtained from the screening of a mouse adult brain cDNA library (CLONTECH Laboratories, Inc.) and 5′-RACE. Human CIIA cDNA was obtained from human fetal brain total RNA (CLONTECH Laboratories, Inc.) by RT-PCR using primers CIIA-H1 (5′-CCACAGCCTAGAGCATGTTACATG-3′) and CIIA-H2 (5′-CGCCGGTACCACTGACGAAAGCC-3′), whose nucleotide sequences were determined from the human CIIA EST clones (zw 80e08.s1; zw 80e08.r1).

Yeast two-hybrid screening

Yeast two-hybrid screening was performed according to the manufacturer’s protocol (CLONTECH Laboratories, Inc.). In brief, a full-length cDNA of either ASK1 or CAD was inserted adjacent to the LexA DNA-binding domain in the pLexA bait vector. About 2 × 10⁶ clones of a mouse adult brain cDNA library (CLONTECH Laboratories, Inc.) were screened using Saccharomyces cerevisiae EGY48[p8op-lacZ]. Positive clones were rescued from yeast cotransformants using Escherichia coli K87 cells, and the cDNA inserts in the rescued plasmids were sequenced.

Isolation of the mouse CIIA cDNA

A 714-bp fragment of CIIA cDNA that had been isolated from the yeast two-hybrid screening using ASK1 as the bait was used as a probe to isolate cDNA clones from a mouse brain Lambda ZAPII cDNA library (Stratagene). Plate hybridization was performed at 42°C for 12 h in 5× SSPE, 0.1% SDS, 5× Denhardt’s solution, 50% formamide, and 100 μg/ml denatured salmon sperm DNA. Positive CIIA clones were in vivo excised, recovered in a pBluescript KS(-) plasmid, and sequenced. To obtain the 5′ region of mouse CIIA, 5′-RACE was performed using mouse brain total RNA (CLONTECH Laboratories, Inc.) and a 5′-RACE kit (Roche Molecular Biochemicals). The gene-specific antisense primer sequences used for 5′-RACE were 5′-GGGCCAGCGGAGAAGATGATACACATAGG-3′ and 5′-GGATGTCTCAACGGGGATCCGCTAC-3′.

Northern blot analysis

A 714-bp fragment of mouse CIIA cDNA excised by BamH1 and Accl was labeled with α-32P ATP by a random priming method and hybridized with a mouse multiple-tissue mRNA blot (CLONTECH Laboratories, Inc.)

Cell culture, transfection, and apoptotic cell death

293T, L929, and HeLa cells were cultured in DME supplemented with 10% FBS. DNA transfections were performed with the LipofectAMINE® (GIBCO BRL, GenePorter 2 (Gene Therapy Systems, Inc.), calcium phosphate, or electroporation method. Apoptotic cell death was measured by flow cytometry (Facs®Calibur; Becton Dickinson) with annexin V staining or by DAPI staining. For annexin V staining, cultured cells were resuspended in binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl₂) and stained with FITC–annexin V and propidium iodide. Apoptotic cells (Annexin V–FITC positive, propidium iodide negative) were analyzed by flow cytometry (Facs®Calibur; Becton Dickinson). For DAPI staining, cultured cells were transfected with pEGFP (CLONTECH Laboratories, Inc.) and expression vectors for the indicated proteins. After transfection, the cells were washed twice with PBS solution. Next,
the cells were fixed with 0.25% glutaraldehyde, permeabilized with 0.1% Triton X-100, and stained with DAPI. The DAPI-stained nuclei in GFP-positive cells were examined for apoptotic morphology by fluorescence microscopy. The percentage of GFP-expressing cells that were apoptotic was determined from three independent dishes.

**Immunocomplex kinase assays**

Cell lysates were subjected to immunoprecipitation using the proper antibody, and the resulting immunoprecipitates were assayed for the indicated protein kinase activities as described previously (Park et al., 2001). Phosphorylated substrates were resolved by SDS-PAGE, and phosphorylation was quantified using a phosphomager (model BAS2500, Fuji). GST-MKK6/K82A and GST-c-Jun[1–79] were used as substrates for ASK1 and JNK/SAPK.

**In vitro binding assay**

CIIA, ASK1, CAD, or their variants were in vitro translated in the presence of [35S]methionine using the TNT reticulocyte lysate system (Promega). The 35S-labeled proteins were incubated at 4°C for 3 h with GST-fused proteins immobilized on glutathione-Sepharose beads or with T7-tagged proteins immobilized on anti-T7 antibody/protein G-Sepharose beads in a buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM DTT, 0.1% NP-40, and 5 mg/ml BSA. The bound 35S-labeled proteins were eluted from the beads and analyzed by SDS-PAGE and autoradiography.

**Luciferase reporter assay of c-Jun-dependent transcription**

The transcription-stimulating activity of c-Jun was measured with the PathDetect luciferase reporter kit (Stratagene). 293T cells were transfected for 48 h with luciferase reporter plasmid pFR-Luc, pFA2-c-Jun, and pcDNA3-β-gal and the indicated combinations of plasmids for ASK1 and CIIA. The soluble fraction of the cell lysates was assayed for luciferase activity using a luciferase assay kit (Promega) and for β-galactosidase activity. The luciferase activities in the transfected cells were normalized with reference to the β-galactosidase activities in the same cells.

**CAD and DNA fragmentation assays**

A His-tagged CAD–ICAD-L complex was bacterially expressed using pET23b (Novagen) and purified with Ni2+-NTA-agarose (QIAGEN). 1 μg of the His-CAD–ICAD-L complex protein was incubated for 2 h at 37°C with 2 μg GST-CIA or its various deletion mutants in the absence or presence of 200 ng of recombinant caspase-3 in 50 μl of a nuclease reaction buffer containing 10 mM Hepes, pH 7.5, 1 mM EDTA, 5 mM MgCl2, 50 mM NaCl, 1 mg/ml BSA, and 0.1 mg/ml chromosomal DNA extracted from Jurkat cells (Halenbeck et al., 1998). CAD-mediated DNA fragmentation was analyzed by electrophoresis on a 2% agarose gel and staining with DAPI. The DAPI-stained nuclei in GFP-positive cells were counted using a confocal microscope. The percentage of GFP-expressing cells that were apoptotic was determined from three independent dishes.

**Glycerol gradient centrifugation**

NIH 3T3 cells stably expressing HA-CIIA were homogenized using a Dounce homogenizer in PBS solution containing 1 mM PMSF, 2 μg/ml leupeptin, and 2 μg/ml aprotinin. Cell extracts were subjected to centrifugation at 1,000 g for 10 min, and the resulting soluble fraction was layered on the top of linear 15–35% (wt/wt) glycerol gradient adjusted to 20 mM Tris-HCl, pH 6.7, 150 mM MgCl2, and 10 mM KCl. Centrifugation was performed at 39,000 rpm for 18 h at 4°C using a rotor (model SW40Ti; Beckman Coulter). 22 fractions of the soluble fraction were collected sequentially from the bottom and equal volumes were analyzed by SDS-PAGE and immunoblotting with the use of anti-HA, anti-ASK1, anti-CAD, and anti-ICAD antibodies.

**Online supplemental material**

Fig. S1 shows subcellular distribution of ectopic CIA, ASK1, and CAD in NIH 3T3 cells. Fig. S2 shows the effect of CIA on the binding of ASK1 with TRAF2, GSTM1, or Daxx. Fig. S3 shows immunoprecipitation data indicating that CIA does not bind JNK1, MEKK1, or c-Jun. Fig. S4 shows an effect of CIA on MEKK1 activity. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200303003/DC1.

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