Determinants of the Nuclear Localization of the Heterodimeric DNA Fragmentation Factor (ICAD/CAD)

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Abstract. Programmed cell death or apoptosis leads to the activation of the caspase-activated DNase (CAD), which degrades chromosomal DNA into nucleosomal fragments. Biochemical studies revealed that CAD forms an inactive heterodimer with the inhibitor of caspase-activated DNase (ICAD), or its alternatively spliced variant, ICAD-S, in the cytoplasm. It was initially proposed that proteolytic cleavage of ICAD by activated caspases causes the dissociation of the ICAD/CAD heterodimer and the translocation of active CAD into the nucleus in apoptotic cells. Here, we show that endogenous and heterologously expressed ICAD and CAD reside predominantly in the nucleus in nonapoptotic cells. Deletional mutagenesis and GFP fusion proteins identified a bipartite nuclear localization signal (NLS) in ICAD and verified the function of the NLS in CAD. The two NLSs have an additive effect on the nuclear targeting of the CAD–ICAD complex, whereas ICAD-S, lacking its NLS, appears to have a modulatory role in the nuclear localization of CAD. Staurosporine-induced apoptosis evoked the proteolysis and disappearance of endogenous and exogenous ICAD from the nuclei of HeLa cells, as monitored by immunoblotting and immunofluorescence microscopy. Similar phenomenon was observed in the caspase-3–deficient MCF7 cells upon expressing procaspase-3 transiently. We conclude that a complex mechanism, involving the recognition of the NLSs of both ICAD and CAD, accounts for the constitutive accumulation of CAD in the nucleus, where caspase-3–dependent regulation of CAD activity takes place.

Key words: apoptosis • chromosomal DNA degradation • caspase-activated DNase • nuclear targeting • caspase-3

Introduction

A apoptosis is an essential process that controls cell numbers during development and participates in the elimination of cells that have undergone irreparable genomic damage (Jacobson et al., 1997; Nagata, 1997). A apoptotic cells show distinctive morphological perturbations, including membrane blebbing, cytoskeletal changes, chromatin condensation, and DNA fragmentation (Jacobson et al., 1997; Wyllie, 1980). The pathways and the nucleases involved in the progressive destruction of the genomic DNA have remained elusive until the recent cloning of the heterodimeric DNA fragmentation factor (ICAD/CAD or DFF; Liu et al., 1997; E nari et al., 1998).

The DNA fragmentation factor consists of two subunits, the 40-kD caspase-3–activated DNase (CAD1 or DFF40) and the 45-kD inhibitor of CAD (ICAD or DFF45; Liu et al., 1997, 1998; E nari et al., 1998; Sakahira et al., 1998). Biochemical isolation of the ICAD/CAD complex from the cytosolic fraction led to the cloning of the mouse and human orthologues of ICAD and CAD (Liu et al., 1997, 1998; E nari et al., 1998; H alenbeck et al., 1998). While the NH2-terminal CID E -N domain, identified in both ICAD and CAD, appears to account for heterodimerization (Inohara et al., 1998), a putative nuclear localization signal (NLS) was identified at the COOH terminus of CAD

Abbreviations used in this paper: CAD, caspase-3–activated DNase; DFF, DNA fragmentation factor; EGFP, enhanced green fluorescent protein; GST, glutathione-S-transferase; hCAD-L, full-length human ICAD; hICAD-S, alternatively spliced variant of hICAD; ICAD, inhibitor of caspase-activated DNase; NLS, nuclear localization signal.

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Heterodimerization of ICAD/CAD is obligatory not only to prevent chromosomal DNA degradation in growing cells, but also to ensure co- or posttranslational folding of CAD in the cytoplasm (Liu et al., 1997, 1998; Enari et al., 1998; H alenbeck et al., 1998; Samejima et al., 1998). According to this early model, activation of the apoptotic signaling cascade leads to the proteolysis of ICAD by effector caspases, permitting the dissociation of the ICAD–CAD complex, and the subsequent nuclear uptake of activated CAD (Enari et al., 1998). Besides the full-length ICAD (or ICAD-L), alternatively spliced variants, ICAD-S, lacking the last 66 and 62 amino acid residues at the COOH terminus have been identified in mice and humans, respectively (G u et al., 1999; Sakahira et al., 1999). These ICAD variants can also dimerize with CAD (G u et al., 1999), but their function is obscure.

While compelling evidence has characterized the endonuclease activity of mouse CAD (mCAD) and its human orthologue (hCAD; Enari et al., 1998; H alenbeck et al., 1998; Inohara et al., 1999; J anicke et al., 1998; Liu et al., 1998; M ukae et al., 1998; T ang and K idd, 1998), neither the colocalization of endogenous ICAD and CAD in the cytoplasm nor the nuclear translocation of activated CAD has been demonstrated. However, immunolocalization data, obtained on cells overexpressing epitope-tagged hCAD or hICAD, suggested that both ICAD and CAD are nuclear (Liu et al., 1998). In addition, a fusion protein, consisting of mICAD and the green fluorescent protein (GFP), has been found to associate with the nuclei of a variety of transfected cells. Based on these observations, it was proposed that the nuclear import of GFP-ICAD was accomplished by association with either CAD or polypeptides actively targeted into the nucleus (Samejima and Earnshaw, 1998). The heterodimerization of ICAD and CAD was not directly ensured in these experiments, thus, the controversy regarding the subcellular localization of the ICAD–CAD complex has remained largely unresolved.

To better understand the subcellular compartmentalization of the apoptotic DNase, our objectives were to establish the cellular distribution and to elucidate the targeting determinants of the CAD–ICAD complex. Immunolocalization of the endogenous ICAD with two polycistronic antibod-
ies provides the direct evidence for the constitutive nuclear targeting of ICAD. Using dele
tional mutagenesis and fusion proteins, we have identified an NLS at the COOH terminus of ICAD and confirmed the function of the NLS of CAD. The two NLSs appear to contribute in an additive manner to the nuclear targeting of the ICAD–CAD heterodimer, implying that the caspase-3-dependent activation of CAD takes place in the nucleus.

Materials and Methods

Cell Lines and Transfection

HeLa, COS-1, MCF7, HT29, PANC, MDCK, CHO, and BHK-21 cells were grown in α-modified Eagle’s or DMEM medium supplemented with 10% FCS at 37°C under an atmosphere of 5% CO2. Transfections were performed with the calcium phosphate precipitation method, Effectene (QIAGEN), or FuGene (Roche) on 60–70% confluent cells. Cells were harvested after 48 h of transfection.

To generate HeLa cells stably expressing hICAD-C-myc, cells were transfected with the pcDNA3-hICAD-C-myc expression vector as calcium phosphate precipitates, and selected in the presence of 0.5 mg/ml Geneticin (GIBCO BRL). Clones were screened by indirect immu
nstaining using the mouse monoclonal anti-myc (9E10, Covance Research Products Inc.) antibody.

Plasmid Constructions

The cDNA of human ICAD was isolated by PCR cloning, using a cDNA library prepared from Caco-2 cells (American Type Culture Collection, accession number HB T37) as a template. The cDNAs encoding the mCAD and hCAD were provided by Dr. S. Nagata (O saka University Medical School, Osaka, Japan) and Dr. R. H alenbeck (Chiron Corp., Em
ergyville, CA), respectively. The membrane-targeted EGFP construct, en
coding EGFP and the ras farnesylation site (EGFP-F) and the cDNA of procaspase-3 were the gift of Dr. W. J iang (J iang, 1998) and Dr. V. D ixit, respectively. Both hICAD and mCAD were subcloned into the expres
sion plasmid pcDNA 3 or into a modified version, incorporating in-frame fusions of the coding sequences for HA, myc or flag epitopes, at either the COOH or the NH terminus. Deletion mutants ICAD 265-333 (ISACD-S), ICAD 306-333 (ICAD-L), mCAD 329-344 (ICADmNLS), and hCAD 329-338 (ICADhNLS) were generated by PCR mutagenesis. cDNA of fusion proteins, comprising EGFP (Cormack et al., 1996), and the NLS of hICAD (EGFP-ICAD 326-333) or mCAD (EGFP-CAD 326-344), were obtained by inserting the corresponding cDNA fragments (amino acids 306–331 from ICAD and 326–343 from CAD) into the EcoRI and Apal sites of the pEGFP-C1 (CLONTECH Laboratories, Inc.). The plasmid encoding GST-hICAD fusion protein was constructed by insertion of the full-length coding region of hICAD into the EcoRI and XhoI sites of pEGX-4T1 plasmid. A II constructs were verified by dyeoxy chain termi
nation DNA sequencing.

Bacterial Expression of hICAD and mCAD

To generate polyclonal anti-ICAD antibody, the full-length coding region of hICAD was fused in-frame with GST in the pGEX-4T1 vector and transformed in HB 101 bacteria. Production of the fusion protein was induced with 0.1 mM isopropyl β-D-thiogalactopyranoside. The bacteria suspension was lysed by sonication in 0.5 M NaCl, 20 mM Hepes, 10% glycerol, 0.1 mM EDTA, and 1 mM DTT, pH 7.5. GST-hICAD was purified from the soluble fraction using glutathione Sepharose 4B (Sigma Chemi
cal Co.), eluted with sonication buffer supplemented with 10 mM reduced glutathione, and further purified with SDS-PAGE. Gel slices, containing GST-hICAD were crushed for immunization of rabbits. Recombinant hICAD, hICAD-His, and mCAD-His, were expressed in BL21 (DE3) cells using the pET15b (Novagen) expression metal purified and purified according to the supplier’s recommendations using affinity metal chromato
graphy.

Polyclonal Antibody Production

Purified GST-hICAD fusion protein was sent to Harlam Bioproducts for Science for inoculation into rabbits. Immunization was achieved with four boost of injections (0.5 mg protein/rabbit). The specificity of the rabbit antibod-
ies was determined by comparing the activity of the immune and pre
immune serum. For immunoblotting and immunofluorescence, the anti
body was used at 1:1,000–1:3,000 dilution, respectively.

Immunofluorescence Microscopy

Fluorescence staining of transfected and nontransfected cells was carried out on glass coverslips after fixing (4% paraformaldehyde for 20 min) and permeabilizing (0.2% Triton X-100 in PBS for 5 min) the cells as previ
ously described (L echardeur et al., 1999). Primary antibodies were as follows: affinity-purified polyclonal goat anti-ICAD (K-17), anti-myc (monoclonal and polycional; Santa Cruz Biotechnology, Inc.), anti-HA (monoclonal 16B12; Covance Research Products Inc., and polycional, Santa Cruz Biotechnology, Inc., and anti-Flag (M2-monoclonal; Sigma Chemical Co.). Secondary anti-mouse and anti-rabbit antibodies were conjugated to fluorescein or rhodamine (jackson ImmunoRes earch Labo-
immunoﬂuorescence signal between the FITC and rhodamine ﬁlter sets was detected. Immunofluorescence micrographs were captured on a Zeiss Axioplan inverted ﬂuorescence microscope (63×/1.4 Planachromat objective) and a Contax camera as described previously (Lechardeur et al., 1999). Color slides (1600 ASA Kodachrome) were scanned by a Polaroid slide scanner, and the images were manipulated with Adobe Photoshop 5.0 software on a Power Macintosh. Exposure times and background subtraction were held constant for the documentation of the time course of ICAD distribution. Laser confocal ﬂuorescence microscopy and image processing was performed using the Zeiss LSM 510 imaging system with Plan neofluor 40×/1.3 objective.

Immunoblotting and Coinmunoprecipitation

For immunoblotting, cells were washed with ice-cold PBS and lysed in RIPA buffer (150 mM NaCl, 20 mM Tris-HCl, 1% Triton X-100, 0.5% SDS, and 0.5% sodium deoxycholate, pH 8.0) containing 10 μg/ml of leupentin and pepstatin, 10 mM iodoacetamide, and 1 mM PMSF for 20 min at 4°C. Nuclei and unbroken cells were removed by centrifugation (15,000 g for 15 min at 4°C). Soluble proteins were denatured in Lämmli sample buffer, separated with SDS-PAGE, and transferred to a nitrocellulose membrane. Immunoblotting, using anti-HA, anti-myc, or anti-ICAD primary antibodies and enhanced chemiluminescence Western blot kit (Amersham), were performed as previously described (Lukacs et al., 1994). The CAD-N-HA/ICAD-C-myc heterodimer was isolated with immunoprecipitation, using anti-HA antibody from cell lysates prepared in TNT buffer (0.2% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl, pH 7.2). The immunoprecipitate was separated with SDS-PAGE, transferred to nitrocellulose, and detected with anti-myc antibody.

Detection of Apoptosis

TUNEL assay was performed as previously described (Lechardeur et al., 1999). A annexin V staining and determination of caspase-3 activity of Hela cell extract were carried out with the A poa lert annexin V and caspase-3 fluorescent assay kits (CLONTECH Laboratories, Inc.), respectively.

Online Supplemental Materials

Supplemental Figure 1. Exogenous hICAD is conﬁned to the nucleus at a low level of expression. Hela cells were transiently transfected with hICAD-C-myc and immunostained with anti-myc and polyclonal anti-ICAD antibodies. (Available at http://www.jcb.org/cgi/content/full/150/2/321/DC1.)

Supplemental Figure 2. Expression level of ICAD-S and ICAD-L. (a and b) The expression level of ICAD-S relative to ICAD-L was determined with quantitative immunoblotting. (c) The weak cytosolic immunostaining of endogenous ICAD is eliminated in HeLa cells expressing exogenous mCAD-N-HA, presumably because of the nuclear exclusion of the hICAD-S/mICAD complex. (Available at http://www.jcb.org/cgi/content/full/150/2/321/DC1.)

Supplemental Figure 3. The NLSs of both hCAD and hICAD contribute to the nuclear accumulation of the ICAD-C–CAD complex. Coimmunoprecipitation of full-length or truncated hCAD-N-HA and hICAD-C-myc was carried out as depicted on Fig. 7 a. (Available at http://www.jcb.org/cgi/content/full/150/2/321/DC1.)

Supplemental Figure 4. Distribution of endogenous and exogenous hICAD in HeLa cells after biochemical fractionation. The ICAD content in size with the alternatively spliced variant, ICAD-D (Sa-kahira et al., 1998, 1999; G u et al., 1999), and was not de-tected with a polyclonal anti-ICAD antibody, speciﬁc for the COOH terminus of ICAD-D (data not shown). A comparable pattern of ICAD expression was revealed with the afﬁnity-puriﬁed goat polyclonal K–17 anti-ICAD antibody (Fig. 1 b). Thus, the rabbit and goat anti-hICAD immu-ne serum recognizes human ICAD, but cannot recog-nize hamster (CHO and BHK), mouse (10T), and dog (MC D K) orthologues (Fig. 1 b).

Results

Localization of ICAD in Nonapoptotic Cells

The subcellular localization of endogenous ICAD was examined using a polyclonal anti-hICAD antibody, developed by immunizing rabbits with puriﬁed GST-hICAD fusion protein. Western blot analysis showed that the rabbit anti-hICAD immune serum, but not the preimmune serum, recognizes recombinant hICAD with an apparent molecular mass of ~45 kD (Fig. 1 a), corresponding to the predicted molecular mass of hICAD (Liu et al., 1997). No immunocross-reactivity was observed with recombinant mCAD-D-(His)6 (apparent molecular mass ~40 kD), despite the presence of the conserved CIDE-N domains in both CAD and ICAD (I nohara et al., 1998) (Fig. 1 a).

Two major polypeptides were recognized by the anti-hICAD immuno serum, but not by the preimmune serum, in whole cell lysates of HeLa, COS-1, MCF7, HTE, and PA NC cells, all of human or primate origin (Fig. 1 b). The slower migrating band, with an apparent molecular mass of ~45 kD, corresponds to the predicted mass of the full-length hICAD (ICAD-L). The less abundant polypeptide, with an apparent molecular mass of ~36 kD, is consistent in size with the alternatively spliced variant, hICAD-D (Sa-kahira et al., 1998, 1999; G u et al., 1999), and was not de-tected with a polyclonal anti-ICAD antibody, speciﬁc for the COOH terminus of ICAD-D (data not shown). A comparable pattern of ICAD expression was revealed with the afﬁnity-puriﬁed goat polyclonal K–17 anti-ICAD antibody (Fig. 1 b). Thus, the rabbit and goat anti-hICAD immu-ne serum recognizes human ICAD, but cannot recog-nize hamster (CHO and BHK), mouse (10T), and dog (MC D K) orthologues (Fig. 1 b).

The subcellular distribution of endogenous hICAD was established with indirect immunofluorescence microscopy in five cell lines, including HeLa, HTE, PANC, COS-1, and MCF7, using two independent anti-ICAD antibodies.

Figure 1. Characterization of the anti-hICAD antibody. (a) The polyclonal rabbit anti-ICAD immune serum recognizes recombinant hICAD on immunoblot. Full-length hICAD and mCAD-D-(His), were expressed in BL21(D3) cells, and the bacterial lysates (~10 μg protein/lane) were separated with SDS-PAGE, transferred to nitrocellulose, and polypeptides were visualized by enhanced chemiluminescence using rabbit polyclonal anti-hICAD antiserum (α-ICAD), mouse monoclonal anti-His (α-His) primary antibody, or preimmune serum with the corresponding HRP-conjugated secondary antibody. Lysate obtained from sham-transformed bacteria were used as negative controls. (b) Western blot analysis of endogenous hICAD expression. Equal amounts of protein (50 μg) of the indicated cell lysate were separated with SDS-PAGE and subjected to immunoblotting as described in Materials and Methods. Two prominent immunoreactive polypeptides, with an apparent molecular mass of ~45 and ~36 kD, corresponding to the full-length hICAD (ICAD-L) and the alternatively spliced variant ICAD-D, respectively, are recognized with the rabbit (α-ICAD) and goat (K-17) anti-hICAD antibodies but not with the rabbit preimmune serum (preim).
The representative fluorescence micrographs of HeLa and HTE cells show that hICAD resides predominantly within the nucleus and is excluded from the nucleolus, with a consistently weak fluorescence signal in cytoplasm (Fig. 2, a and b). No nuclear staining was detectable with the rabbit preimmune serum or after the adsorption of the specific antibody to recombinant ICAD-His$_6$ (Fig. 2 a). Importantly, the strong nuclear staining of ICAD could be observed with the affinity-purified goat polyclonal anti-ICAD antibody, as illustrated in the HeLa cells (Fig. 2 c). These data indicate that hICAD constitutively accumulates in the nucleus in a variety of cells with the maintenance of a reduced level in the cytoplasm.

The Nuclear Localization Signal of ICAD

The nuclear import of ICAD could be achieved passively in association with CAD or another carrier molecule. Alternatively, an unrecognized NLS residing in ICAD could mediate nuclear uptake. Primary sequence analysis of the mouse and human ICAD revealed two series of basic residues separated by a 10-amino acid spacer at the extreme COOH terminus, which is reminiscent of the consensus sequence of the bipartite NLS of nucleoplasm (Fig. 3 a; Dingwall et al., 1988; Robbins et al., 1991). Although the alignment to consensus of the bipartite NLS was less striking for hICAD, compared with its mouse counterpart, the functional significance of the NLS was tested using a fusion protein, comprising the enhanced green fluorescent protein (EGFP) and the last 26-amino acid residues of hICAD (EGFP-ICAD$_{306-331}$). The transiently expressed EGFP-ICAD$_{306-331}$ was clearly nuclear (Fig. 3 b, bottom), in contrast to the EGFP, which was found uniformly dis-
tributed throughout the cells (Fig. 3 b, top). Thus, the COOH-terminal tail of hICAD is sufficient to confer nuclear import capacity to EGFP, and may be implicated in the nuclear localization of ICAD. During the revision of this manuscript similar results were reported by Samejina and Earnshaw (Samejina and Earnshaw, 2000).

Overexpressed hICAD consistently appeared largely nuclear, with a low level of cytosolic expression in different expression systems (HeLa, COS-1, and BHK), regardless of the epitope (c-myc, flag, or HA [influenza hemagglutinin]) introduced at the COOH or NH\(_2\) termini (Fig. 3 c, top and data not shown). Several lines of evidence indicate that NLS-dependent active uptake accounts for the nuclear localization of overexpressed hICAD. First, the nuclear accumulation of hICAD was clearly compromised without NLS. Strong cytosolic immunostaining was detected for hICAD\(\Delta\text{NLS-C-myc}\), a recombinant version of hICAD prepared by deleting the last 26-amino acid residues, comprising the NLS consensus (Fig. 3 c, bottom). Further, prominent cytosolic retention was observed for the epitope-tagged ICAD-S, representing the alternatively spliced isoform, which lacks the last 66 amino acid residues (Fig. 3 c, bottom right), suggesting that the nuclear import of exogenous hICAD cannot be attributed to association with CAD. Secondly, both stably and transiently transfected hICAD-C-myc was predominantly nuclear at expression levels, which were comparable to that of endogenous ICAD in HeLa cells (Fig. 4 a, left, and Figure S1 [available at http://www.jcb.org/cgi/content/full/150/2/321/D1]). Finally, depletion of the cellular ATP content by metabolic inhibitors dissipated the nucleocytoplasmic gradient of hICAD-C-myc (Fig. 4 a, middle), supporting the hypothesis that nuclear accumulation of hICAD involves active transport. Irreversible deterioration of the nuclear envelope could not account for this phenomenon, since hICAD-C-myc relocalized in the nucleus upon the recovery of the cellular ATP content (Fig. 4 a, right). These observations are consistent with the activity of a previously unrecognized NLS at the COOH terminus of hICAD. Furthermore, they also suggest that the weak cytosolic immunostaining, obtained with the polyclonal anti-ICAD antibodies (Fig. 2), is, most likely, because of the presence of low levels of monomeric ICAD-S, confined to the cytoplasm (Figure S2, a and b [available at http://www.jcb.org/cgi/content/full/150/2/321/D1]). Supporting this notion, the cytosolic immunostaining of ICAD-S was virtually abolished upon transient expression of CAD-N-HA, with a concomitant increase in the nuclear immunostaining of ICAD (Figure S2, c).

**The ICAD/CAD Heterodimer Is Nuclear in Nonapoptotic Cells**

It has been established previously that co- or posttranslational heterodimerization of CAD and ICAD is necessary for the expression of CAD. The possible impact of heterodimerization on the subcellular targeting of ICAD was assessed by transient coexpression of epitope-tagged human ICAD and mouse CAD.
Three lines of evidence indicated that epitope-tagged human ICAD, a highly conserved orthologue of mouse ICAD (Liu et al., 1997; Sakahira et al., 1998), dimerizes efficiently with mouse CAD. First, physical interaction between mCAD and hICAD was demonstrated by coimmunoprecipitation of the hICAD-C-myc/mCAD-N-HA complex with anti-HA antibody and subsequent immunoblotting the precipitate with anti-myc antibody (Fig. 5 a). Second, the expression level of mCAD-N-HA was substantially higher (more than sixfold) in the presence of exogenous hICAD-C-myc, as demonstrated by quantitative immunoblot analysis (Fig. 5 b, lanes 2 and 3). As a corollary, the level of expression of the exogenous hICAD-C-myc was significantly higher than that of the endogenous form, which was detected by the rabbit anti-hICAD antibody and subsequent immunoblotting the precipitate with anti-myc antibody (Fig. 5 b, lanes 2 and 3). Finally, the release of hICAD-C-myc, from the nucleus into the cytosol, was virtually undetectable from the nuclei that expressed both hICAD-C-myc and mCAD-N-HA (Fig. 4 b), but not hICAD-C-myc alone (Fig. 4 a). This may be explained by the diffusional barrier of the nuclear pore complex for polypeptides >45 kD, such as the hICAD-C-myc complex (molecular mass ∼85 kD; Dingwall and Laskey, 1991). Collectively, these experiments indicate that human ICAD can substitute its mouse counterpart as a chaperone and as an inhibitor of mCAD, as reported for the heterodimerization of monkey or avian ICAD with mCAD (Enari et al., 1998; Samejima et al., 1998). Since similar data were obtained with coexpressing human CAD and ICAD, we confirmed that expression of both mCAD and hICAD is strictly limited by the abundance of the endogenous ICAD and increased level of CAD through heterologous expression can be achieved by introducing and binding of exogenous ICAD.

The subcellular distribution of hICAD in the ICAD-CAD complex was investigated by immunofluorescence staining of HeLa cells, transiently cotransfected with epitope-tagged hICAD and mCAD. The nearly exclusive nuclear expression of heterologous hICAD and mCAD was independent of the epitope (Fig. 6 a), which is consistent with the localization of endogenous hICAD (Fig. 2). Similar subcellular distribution was observed in COS-1 and BHK cells (data not shown). A association or binding to nuclear constituents is unlikely to play a major role in the nuclear retention of ICAD/CAD. In contrast to the nuclear proteins, which are resistant to detergent extraction because of their association with the nuclear skeleton or chromosomal DNA (e.g., lamina-associated polypeptide 2α, (Lim and Li, 1996)), a low concentration of Triton X-100 was sufficient to remove both mCAD-N-HA and hICAD-C-myc from the nuclei (unpublished observation). As a corollary, monomeric hICAD appears to move freely in both directions across the nuclear pore complex.
distributed to activation of apoptosis, since neither increased chromosomal DNA fragmentation nor phosphatidylserine translocation into the outer leaflet of the plasma membrane was detectable by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay or annexin V staining, respectively. Finally, identical localization was demonstrated for the epitope-tagged hICAD/hCAD heterodimer (Fig. 6 b), supporting our hypothesis that nuclear targeting of I C A D / C A D is an inherent characteristic of the complex, rather than the consequence of heterologous expression, and dimerization I C A D does not impair its nuclear accumulation.

The Role of ICAD in the Nuclear Targeting of the ICAD–CAD Complex

Polypeptides >45 kD require an NLS and interactions with nuclear transport receptors to be targeted specifically into the nucleus (Kalderon et al., 1984; Newmeyer and Forbes, 1988; Dingwall and Laskey, 1991). It is possible that nuclear accumulation of the ICAD–CAD complex, with a molecular mass of ~85 kD, relies on either or both of the NLS motifs of its constituents. Given that the heterologously expressed ICAD–CAD complex is largely comprised of epitope-tagged components, the individual contribution of NLS of CAD and ICAD could be elucidated. The subcellular distribution of the ICAD–CAD complex was scored on the basis of the nucleocytoplasmic gradient of mCAD, since heterodimerization is obligatory for the expression of CAD (Enari et al., 1998; Halenbeck et al., 1998; Liu et al., 1998; Inohara et al., 1999). Data in the previous section and analysis of 400 transfected cells showed that hICAD–mCAD complex is essentially nuclear (Fig. 6 and Fig. 7, a and b, filled bars). In contrast, deletion of the ICAD NLS impeded the nuclear import of the complex, reflected by the cytosolic localization.
The NLSs of both mCAD and hICAD contribute to the nuclear accumulation of the ICAD–CAD complex. Coimmunolocalization of full-length or truncated mCAD–N-HA and hICAD–C-myc was carried out as on Fig. 6. (a) HeLa cells were cotransfected with the indicated expression constructs encoding for the full-length or truncated mCAD–N-HA and hICAD–C-myc and visualized by indirect immunostaining. Identical fields are shown for ICAD (red) and CAD (green) staining. (b and c) Estimation of the nucleocytoplasmic concentration gradient of the exogenous ICAD–CAD complex, based on the subcellular distribution of mCAD–N-HA or mCAD–NLS–N-HA in the presence of hICAD–C-myc or hICAD–S–C-myc. According to the nucleocytoplasmic concentration gradient of CAD–N-HA or CAD–NLS–N-HA, the distribution pattern of transfected cells was classified into four groups: (1) mCAD was exclusively nuclear (nuclear); (2) mCAD concentration was higher in the nucleus than in cytoplasm; (3) no significant concentration difference could be recognized between the nucleus and the cytoplasm; and (4) the concentration of mCAD in the cytoplasm was higher than in the nucleus. The composition of the exogenous ICAD–CAD complex is indicated on the right: wt, mCAD–N-HA; ΔNLS, mCAD–ΔNLS–N-HA; and S, hICAD–S–C-myc. For each transfection, >400 cells were scored in two to three independent experiments. Bar, 10 μm.

of mCAD–N-HA and hICAD–ΔNLS–C-myc heterodimer (Fig. 7a). Similar subcellular distribution of mCAD was observed in the presence of ICAD–S–C-myc (Fig. 7a), suggesting that the NLS of hICAD is necessary for the efficient nuclear accumulation of the CAD–ICAD complex. Furthermore, these results also imply that nuclear uptake of CAD may be influenced by the expression level of ICAD–S. This latter possibility was verified by determining the subcellular distribution of hICAD in complex with hICAD–S. Nuclear accumulation of mCAD was observed only in 52% of the expressors and showed substantial cytosolic staining in the rest of the transfecants upon coexpression with hICAD–S (Fig. 7c, empty bars).

The possibility that hICAD–ΔNLS or hICAD–S is unable to chaperone the folding of mCAD, leading to a nonnative, and perhaps mistargeted mCAD seems unlikely since the expression level of mCAD was augmented by either the full-length or truncated variants of hICAD more than...
six-fold, as measured by quantitative immunoblot analysis (Fig. 8 a, lanes 1–4). Similar distribution patterns were observed when human CAD-N-HA was coexpressed with the full-length or truncated hICADs (Figure S3 [available at http://www.jcb.org/cgi/content/full/150/2/321/D C1]), precluding the possibility that dimerization of hICAD with mCAD induced the exposure of a buried NLS. Hence, we conclude that the NLS of hICAD has an important role in the constitutive nuclear localization of the ICA D/CAD heterodimer.

The Role of CAD in the Nuclear Targeting of the ICAD–CAD Complex

To examine the possible role of CAD in the nuclear targeting of the ICAD–CAD complex, the function of its putative NLS was also evaluated. The COOH-terminal tail of mCAD, containing the positively charged amino acid cluster (326RRKKQPRKKRPARKR344), was fused in-frame to EGFP. The transiently expressed EGFP-CAD326-344 chimeric polypeptide was nuclear in HeLa cells, indicating that the COOH terminus of mCAD is sufficient to confer nuclear import capacity to EGFP (Fig. 8 b).

The significance of the NLS in the context of mCAD was demonstrated by immunolocalization of the COOH terminally truncated mCAD lacking its NLS (CADΔNLS). Deletion of the NLS disrupted the nuclear import of the CADΔNLS-N-HA as well as the coexpressed ICAD-C-myc (Fig. 7 a). In contrast to the exclusive nuclear localization of mCAD and hICAD (Figs. 6 and 7, a and b, filled bar), the complex comprising the CADΔNLS and hICAD remained cytosolic for >50% of the transfectants (Fig. 7 c, filled bar). The rest of the transfectants displayed incomplete nuclear accumulation with significant amount of cytosolic mCADΔNLS-NHA (Fig. 7 c). Importantly, deletion of the NLS in mCAD does not seem to interfere with its biosynthesis and folding, since the expression level of mCADΔNLS was comparable to that of mCAD, in the presence of either the full-length or the truncated hICADs (Fig. 8 a, lanes 6–8). Furthermore, deletion of the putative NLS of the human CAD caused a similar subcellular distribution of the ICAD–CADΔNLS complex (Figure S3).

Thus, perturbation of the nuclear localization of the ICA D/ CAD heterodimer by CADΔNLS provides direct evidence for the role of CAD NLS. Therefore, we conclude that NLSs of both ICAD and CAD contribute to the constitutive nuclear import of the complex. Our results also imply that translocation of activated CAD from the cytoplasm into the nucleus is not occurring specifically to achieve fragmentation of chromosomal DNA during apoptosis.

The NLSs of ICAD and CAD Contribute to the Nuclear Uptake of ICAD–CAD Complex in an Additive Manner

To assess whether the NLS of each constituent participates in the nuclear targeting of the ICAD–CAD complex, the subcellular distribution of the heterodimer composed of CADΔNLS and ICADΔNLS was examined. In striking contrast to the nuclear colocalization of mCAD-N-HA and hICAD-C-myc (Fig. 9 a), the nuclear targeting of the complex lacking both NLSs was virtually abolished, as illustrated by the laser confocal fluorescent micrographs (Fig. 9 b). Comparable results were obtained with the mCADΔNLS-N-HA/hICAD-S-C-myc complex (Fig. 7 a). Nuclear accumulation of the mCADΔNLS-N-HA/hICAD-S-C-myc complex could not be recognized in 81% of the transfected cells, and appeared to be excluded from the nucleus in 19% of the expressors (Fig. 7 b, empty bar). Since the cytosolic expression of the ICAD/CAD was substantially more pronounced when both NLSs were absent, in contrast to single NLS deletion, we conclude that the NLSs of both ICAD and CAD are required for efficient nuclear import of the heterodimer.
Staurosporine-induced Apoptosis Is Associated with the Caspase-dependent Release of ICAD from the Nucleus

Immunolocalization of endogenous hICAD suggests that proteolytic activation of ICAD/CAD takes place in the nucleus, rather than in the cytoplasm as proposed previously (Enari et al., 1998). To test this prediction, the cellular distribution of endogenous and epitope-tagged hICAD was monitored upon staurosporine-induced apoptosis. According to the immunostaining, staurosporine evoked a time-dependent disappearance of hICAD from the nuclei of HeLa cells. The nuclear expression of the endogenous hICAD appears to be abolished in most of the cells after 3 h of incubation with staurosporine (Fig. 10 a). Nearly complete elimination of the fluorescence signal derived from the exogenous ICAD-C-myc was observed after 2 h of incubation with staurosporine (Fig. 10 b). The more pronounced diminution of the ICAD-C-myc could be attributed to an increased caspase susceptibility of the monomeric hICAD-C-myc, and/or the small size of c-myc epitope, as compared with multiple epitopes detected with the polyclonal anti-ICAD antibody.

In sharp contrast to HeLa cells, the nuclear expression of the endogenous hICAD was preserved upon staurosporine treatment of MCF7 cells, a cell line which lacks functional caspase-3 (Janicke et al., 1998; Fig. 10 c). Consistent with the immunostaining results, a significant decrease in the total cellular pool of hICAD (and ICAD-S) was documented with immunoblot analysis of HeLa, but not MCF7 cells, upon induction of apoptosis with staurosporine (Fig. 10, d–e). The effect of staurosporine on ICAD degradation was sensitive to DEVD-CHO, an inhibitor of caspases-3, -6, and -7 (data not shown).

The lack of a staurosporine effect on the degradation of ICAD in MCF7 cells could not be explained by the absence of the upstream apoptotic signaling cascade, since hICAD could be eliminated from the nuclei after the supplementation of the cells with procaspase-3. MCF7 cells were cotransfected with plasmids encoding procaspase-3 and membrane-targeted EGFP (EGFPF) at a molar ratio of 4:1. The inclusion of EGFPF facilitated the identification of procaspase-3 expressors. A titration of procaspase-3 with staurosporine caused the disappearance of endogenous ICAD from the nuclei of MCF7 cells expressing both EGFPF and caspase-3, but not EGFPF alone, as detected with immunostaining (Fig. 11). These in situ immunolocalization studies suggest that expression of caspase-3 is indispensable for proteolytic activation of ICAD/CAD, which takes place in the nucleus.

Discussion

Based on in vitro reconstitution of chromosomal DNA degradation, it was initially postulated that proteolytic cleavage of ICAD by caspase(s) would allow the dissociation of ICAD from CAD in the cytosol and unmasking the NLS of CAD, permitting the nuclear import of the active nuclease (Enari et al., 1998). However, recent reports of the nuclear localization of heterologously expressed epitope-tagged hICAD and GFP-mICAD fusion proteins were inconsistent with this activation mechanism, and sug-
Figure 10. The effect of staurosporine on the localization and expression of hICAD. The subcellular distribution of endogenous hICAD (a and c) and transiently overexpressed hICAD-C-myc (b) were visualized in HeLa (a and b) and MCF7 (c) cells before and after staurosporine (2 μM) treatment for 2 h at 37°C. Cells were fixed, permeabilized, and hICAD and hICAD-C-myc were immunostained with the rabbit polyclonal anti-hICAD (α-ICAD) and anti-myc antibodies, respectively. Chromosomal DNA was visualized with DAPI, and identical fields are shown for hICAD and DNA staining. (d and e) The effect of staurosporine on the expression level of hICAD. HeLa (d) and MCF7 (e) cells were incubated in the presence of 2 μM staurosporine for the indicated time. Cellular proteins were solubilized in RIPA buffer, and equal amounts of protein (50 μg) were analyzed by immunoblotting using polyclonal rabbit (α-ICAD) and goat (K-17) anti-hICAD antibodies. Bars: 10 μm.
gested that ICAD may play a direct role in the nuclear targeting of CAD in nonapoptotic cells (Liu et al., 1998; Samejima and Earnshaw, 1998). To resolve these conflicting observations and establish the mechanism of ICAD’s action, immunolocalization of endogenous ICAD was performed in growing and apoptotic cells. Our results offer direct evidence for the constitutive nuclear targeting of the ICAD–CAD complex in nonapoptotic cells and document the caspase-3–dependent proteolytic cleavage of ICAD in the nucleus.

A number of observations indicate that the ICAD/CAD heterodimer resides, predominantly, in the nucleus of nonapoptotic cells. A panel of epitope-tagged ICAD and CAD variants was expressed in combinations or individually to demonstrate that they localize and colocalize in the nucleus. Nuclear localization was independent of the expression system or the epitope employed, and did not coincide with either DNA fragmentation or phosphatidylserine translocation (Figs. 4, 6, and 9). Moreover, inhibition of caspase activity with DEVD-CHO had no effect on the nuclear localization of exogenous ICAD–CAD complex in nonapoptotic cells and document the caspase-3–dependent proteolytic cleavage of ICAD in the nucleus.

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The role of the NLS of CAD was assessed in cells cotransfected with hICAD and truncated CAD. Incorporation of mCADΔNLS, or hCADΔNLS into the heterodimer partially disrupted its nuclear accumulation, similar to that observed in the presence of hICADΔNLS, implying that the NLS of CAD is also recognized by the
nuclear import machinery in the complex. Importantly, when the NLSs of both ICAD and CAD were deleted, the nuclear exclusion of mCAD ΔNLS/hICAD ΔS and mCAD ΔNLS/hCAD ΔNLS became obvious (Fig. 9). The more pronounced cytosolic accumulation of the double mutants relative to that of single NLS deletion suggests that the two NLSs have an additive effect on the nuclear targeting efficiency of the ICAD-CAD complex. A cumulative effect of multiple nuclear localization signals on the targeting of soluble polypeptides is not without precedent. A number of proteins harbor two or more NLSs (e.g., c-myc, Matx2, and p53), which are required to achieve complete nuclear localization. Multiple copies of NLS apparently ensure more efficient targeting than do single copies (Dworetzky et al., 1988; Jans and Hubner, 1996). The unique feature of ICAD/CAD targeting is that the two NLSs reside within distinct components of the complex.

In the light of the nuclear localization of the ICAD-CAD complex, it is reasonable to assume that the DNA fragmentation activity, which was isolated from the cytosol of apoptotic cells, was released from the nucleus during the preparation procedure, when ATP-dependent nuclear import was mitigated (Mitamura et al., 1998; Sabol et al., 1998). Perturbing the integrity of the nuclear envelope may have contributed to the cytosolic appearance of the ICAD-CAD complex during biochemical purification (Enari et al., 1998). Perturbation of the nuclear import may have contributed to the cytosolic appearance of the ICAD-CAD complex during biochemical purification (Enari et al., 1998; Mitamura et al., 1998; Sabol et al., 1998). This speculation is supported by the findings that comparable amounts of ICAD and ICAD-C-myc/CAD-N-HA were associated with the nuclear and cytosolic fractions of HeLa cells, regardless of the homogenization technique used (nitrogen cavitation or freeze-thaw method, Figure S4 [available at http://www.jcb.org/cgi/content/full/150/2/321/DC1]). Furthermore, rapid loss of the exogenous ICAD-CAD complex was detected from the nuclei of NIH3T3 cell during digitonin fractionation (Dr. Robert Halenbeck, personal communication). These observations are in sharp contrast with the exclusive nuclear localization of the ICAD-C-myC/CAD-N-HA complex, as demonstrated by immunostaining, but are in line with previous reports documenting the loss of nuclear proteins during subcellular fractionation (Liu et al., 1998; Pain et al., 1983).

**Activation of the ICAD/CAD Heterodimer Occurs in the Nucleus**

The constitutive nuclear accumulation of ICAD/CAD dimer implies that regulation of CAD activity, most likely, takes place in the nucleus. How nuclear ICAD/CAD is activated upon apoptosis is an important question in understanding the mechanism of chromosomal DNA degradation. Convincing in vivo and in vitro data demonstrate that activation of CAD requires the proteolytic cleavage of ICAD by the caspase cysteine proteases (Mitamura et al., 1998; Sabol et al., 1998; Samejima et al., 1998; Tang and Kidd, 1998; Sakahira et al., 1999). Our results provide some insights into this process in situ. Progressive disappearance of both the endogenous and the heterologously expressed hICAD was observed upon staurosporine-induced apoptosis from the nuclei of HeLa cells (Fig. 10). In contrast, staurosporine failed to promote ICAD degradation in caspase-3-deficient MC/7 cells, unless these cells were transiently transfected with the expression plasmid encoding procaspase-3 (Figs. 10 and 11), underlining the central role of caspase-3 in the regulation of CAD (Enari et al., 1998; Liu et al., 1998).

In conclusion, the experimental data presented in this study indicate that two independent NLSs, identified in ICAD and CAD, are necessary and sufficient to render highly efficient, constitutive nuclear targeting of the ICAD/
CAD heterodimer. These observations, together with the nuclear accumulation of endogenous hiCAD, and its redistribution on staurosporine-induced apoptosis in both HeLa and caspase-3 transfected MCF7 cells, imply that regulation of the apoptotic nuclease by the proteolytic cleavage of ICAD takes place in the nucleus.

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