TUCAN/CARDINAL and DRAL participate in a common pathway for modulation of NF-κB activation

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Abstract Proteins containing the caspase recruiting domain (CARD) have emerged as critical regulators of different signal transduction pathways, including those controlling apoptosis and activation of necrosis factor (NF)-κB transcription factor. TUCAN/CARDINAL is a recently identified CARD-containing protein involved in regulation of caspases and NF-κB activation. We find that TUCAN/CARDINAL associates with DRAL, a p53-responsive gene implicated in induction of apoptosis. We also show that, whereas TUCAN/CARDINAL exerts a suppressive effect on NF-κB activity, expression of DRAL results in enhancement of NF-κB activation. Thus, our observations suggest that DRAL and TUCAN/CARDINAL may participate in a regulatory mechanism that coordinates cellular responses controlled by NF-κB transcription factor. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Caspase recruitment domain; Protein; Nuclear factor-κB; Apoptosis; p53; DRAL

1. Introduction

Programmed cell death, or apoptosis, is a cell deletion mechanism that is critical for normal development and homeostatic control in multicellular organisms [1]. Activation of the cell death cascade is regulated by homophilic association of proteins containing conserved domains, including the death domain, the death effector domain and the caspase recruitment domain (CARD) [2]. The CARD was originally identified as a conserved sequence present in several proteins involved in regulation of apoptosis, such as RAIDD, several caspases and the *Caenorhabditis elegans* genes CED-3 and CED-4 [3]. However, recent studies indicate that CARD-containing proteins are implicated not only in apoptotic signaling, but also participate in signal transduction pathways leading to activation of NF-κB transcription factor, which, in turn, promotes transcription of genes exerting positive effects on cell survival and proliferation. For example, although sharing structural similarity with the cell death protein Apaf-1, the CARD-containing proteins Nod1/CARD-4 [4,5] and Nod2 [6] are strong activators of NF-κB, rather than inducers of programmed cell death. Induction of NF-κB activation by Nod1 requires an intact CARD domain, proving the involvement of this domain in the activation of non-apoptotic signaling pathways [4]. Also, the CARD-containing proteins RICK [7] and the adapter protein bcl10 [8–11] are potent activators of NF-κB. Very recently, the CARD-containing protein TUCAN/CARDINAL has been identified as a CARD-containing molecule that regulates NF-κB activation and apoptosis in mammalian cells [12,13].

2. Materials and methods

2.1. cDNA cloning and plasmids

The full-length cDNA encoding for TUCAN/CARDINAL was obtained by polymerase chain reaction (PCR) using a proof-reading Pwo polymerase (Roche Biochemicals) and the Marathon Ready cDNAs (Clontech) as template. Nucleotidic sequences of several cDNA clones were determined on both strands. Point-mutants of TUCAN/CARDINAL were generated using PCR-based techniques. Expression vectors used for this study were made using standard cloning techniques or by proof-reading PCRs performed with primers containing appropriate restriction sites or epitope tags as needed. Plasmids constructs were confirmed by partial sequencing and immunoblot analysis. Recombinant proteins were made using the pET system (Novagen), according to the manufacturer's indication.

2.2. Cell culture and antibodies

HEK293 and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. HEK293 and HeLa cells were transfected by calcium phosphate method or with lipofectAMINE (Life Technologies). Anti-FLAG mAb was purchased from Sigma, anti-HA from Roche Biochemicals.

2.3. Co-precipitation and Western blot

For co-immunoprecipitation experiments, transfected cells were lysed in lysis buffer (25 mM HEPES, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% Triton-X 100, 10% glycerol and a protease inhibitor mixture). Lysates were immunoprecipitated with the indicated Abs; immunocomplexes were bound to protein A/G-agarose beads and resolved by SDS–PAGE. Proteins were then transferred to nitrocellulose membrane and incubated with primary antibody followed by a secondary antibody horseradish peroxidase-conjugated (Promega). Blots were developed using SuperSignal (Pierce) and visualized by exposure to autoradiography film.

2.4. In vitro binding assay

HEK293 cells were transfected in Petri dishes with 8 μg of plasmidic DNA. 16–24 h after transfection, cells were lysed in lysis buffer (25 mM HEPES, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% Triton-X

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100, 10% glycerol and a protease inhibitor mixture) and 300 μl of lysates were mixed with 50 μl of the indicated histidine-tagged recombinant protein. Samples were incubated at 4°C, washed several times by pulse centrifugation in the same buffer and resuspend in 50 μl of sample buffer. 10 μl of the reaction was loaded for SDS–PAGE and Western blot analysis.

2.5. NF-κB activation assays

HeLa and HEK293 cells were grown in 6-well plates and co-transfected with 0.2 μg of NF-κB luc (Clontech), 0.1 μg of CMV-βGAL (Clontech) plus each expression plasmid in duplicate. The total amount of transfected plasmidic DNA was kept constant by adding empty vector. 24 h after transfection, cell extracts were prepared and luciferase activity was measured by luminescence spectrophotometry. Relative luciferase activity was normalized on β-gal activity.

2.6. Cell death assay

HeLa cells were transfected in 6-well plates with 2 μg of pcDNA3 expressing the indicated cDNA together with 0.2 μg of CMV β-gal (Clontech). 24 h after transfection, plates were washed and β-gal activity was visualized by fixing the cells in 0.2% glutaraldehyde for 10 min followed by staining in phosphate-buffered saline containing 20 mM each $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$–H_2O and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside for 1–3 h at 37°C. The number of live blue cells and blue cells with apoptotic morphology were counted in at least four fields.

2.7. Two-hybrid screening and β-gal assays

The two-hybrid screenings were conducted using the Matchmaker system III from Clontech according to the manufacturer instructions. Yeast strain AH109 was transformed using the lithium acetate/PEG 4000 procedure and colonies selected on SD plates were then analyzed for expression of GAL4–bait fusion protein by immunoblot analysis. For library screening, yeast AH109 expressing the appropriate GAL4–bait fusion protein was transformed with cDNA libraries obtained from Clontech. Transformed clones were selected on SD/agar plates lacking adenine, leucine, tryptophane and histidine for 5 days at 30°C. Colonies positive for growth on selective media were blotted on filter paper, permeabilized in nitrogen liquid and placed on another filter soaked in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 37.5 mM β-mercaptoethanol) containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-galactoside. Colonies that developed color were re-streaked on selective plates to allow plasmid segregation and tested again for β-galactosidase activity. Yeast colonies were then scored as strong interactors when a bright color developed within 2–5 h and as weak interactors when color developed within 12–24 h. A negative was scored when color failed to develop within 24 h. Assays were done for 5–10 independent transformants.

3. Results

In order to identify novel proteins involved in regulation of apoptosis, we performed a panel of two-hybrid screenings, using as a bait the CARD domains of several CARD-containing proteins fused to the DNA-binding domain of GAL-4. One of the clones isolated encoded for a carboxy-terminus portion of a recently identified CARD-containing protein, termed TUCAN/CARDINAL [12,13]. This protein consists of 431 amino acids, contains a carboxy-terminal CARD and shares homology with NAC/DEFCAP/Nalp1, a CARD-containing protein that interacts selectively with the CARD domain of Apaf1 and regulates caspase activation and apoptosis [14,15].

TUCAN/CARDINAL can form homodimers through homophilic associations involving the CARD region of the protein [12,13]. To investigate the role of specific amino acidic residues of the CARD region in homodimerization of TUCAN/CARDINAL, we generated the mutants TUCAN/CARDINAL (L366R), TUCAN/CARDINAL (L377R) and TU-

Table 1
Specificity of the interaction between TUCAN/CARDINAL and DRAL

Protein fused to GAL-4 domain		β-Galactosidase filter assay
DNA-binding	Activating	
–	DRAL	–
Vector	DRAL	–
pLAM5	DRAL	–
TUCAN 179–341	DRAL	+++
TUCAN 179–431	DRAL	+++
TUCAN 341–431	DRAL	–
TUCAN 179–431	–	–

AH 109 yeast strain was co-transformed with vectors encoding for the indicated polypeptides fused to GAL4 DNA-binding domain and GAL4 transcriptional activation domain. Results from a filter assays for β-galactosidase activity are shown. Yeast colonies were scored as positive when a bright color developed within 2–5 h, a negative colony was scored when color failed to develop within 24 h. Assays were done for 5–10 independent transformants.

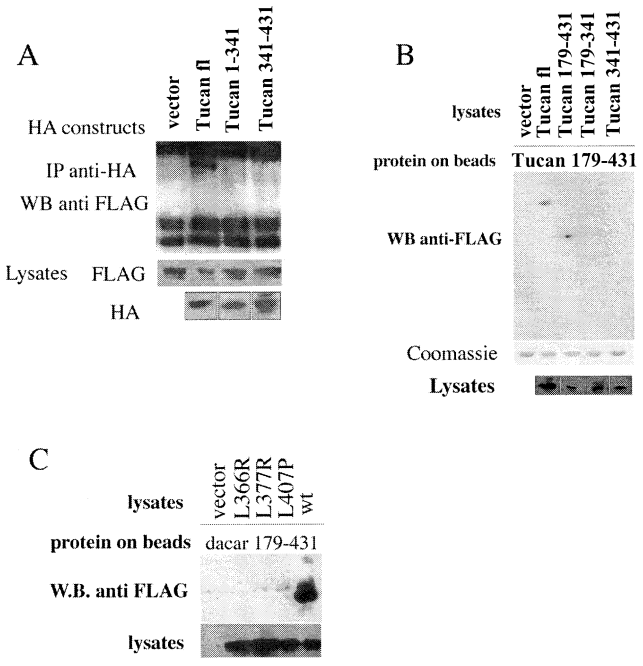

Fig. 1. Homodimerization of TUCAN/CARDINAL. A: HEK293 cells were transfected with FLAG-tagged full-length TUCAN/CARDINAL together with the indicated HA-tagged cDNAs. 16 h after transfection, cell lysates were prepared, immunoprecipitated with anti-HA monoclonal antibody and analyzed for co-precipitating proteins by immunoblot probed with anti-FLAG antibody. Before immunoprecipitation, a fraction of cell lysate was analyzed by Western blot and aligned to visualize protein expression. B: Recombinant histidine-tagged deleted versions of TUCAN/CARDINAL were purified with nickel–nitrilotriacetic acid–agarose beads and mixed with equal amount of proteic lysate extracted from HEK293 cells transfected with FLAG-tagged full-length and deleted forms of TUCAN/CARDINAL. After washing, agarose beads were boiled in SDS-sample buffer, separated by SDS–PAGE and transferred onto nitrocellulose membranes probed with anti-FLAG antibody. A fraction of the reaction mixture was stained with Coomassie to visualize histidine-tagged recombinant proteins. Cell lysates from transfected HEK293 cells were analyzed by Western blot and aligned to visualize relative expression level of TUCAN/CARDINAL constructs. C: Recombinant histidine-tagged TUCAN/CARDINAL was mixed with equal amount of proteic lysate extracted from HEK293 cells transfected with FLAG-tagged wt and mutated versions of TUCAN/CARDINAL, as indicated. After washing, agarose beads were boiled in SDS-sample buffer, separated by SDS–PAGE and transferred onto nitrocellulose membranes probed with anti-FLAG antibody.

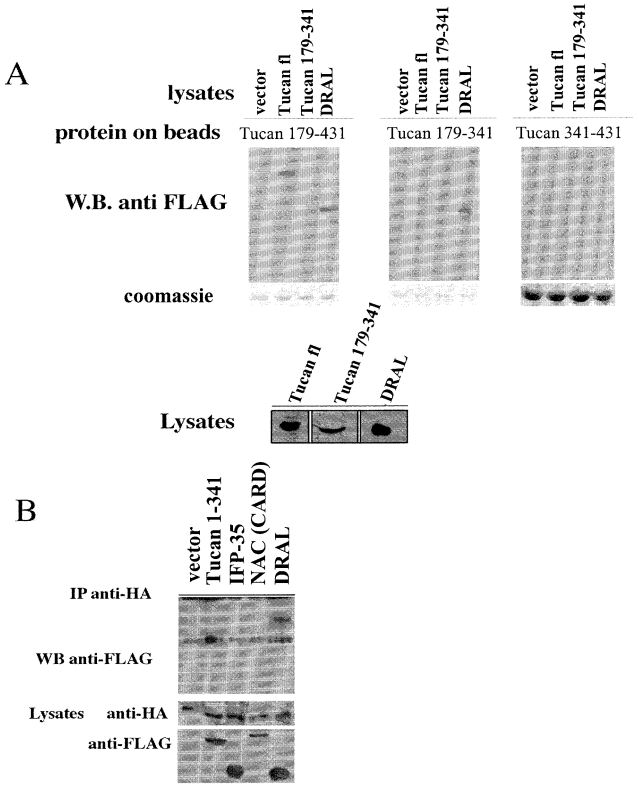

Fig. 2. Association of TUCAN/CARDINAL with DRAL. A: Recombinant histidine-tagged deleted versions of TUCAN/CARDINAL were purified on nickel–nitrilotriacetic acid–agarose beads and mixed with proteic lysates extracted from HEK293 cells transfected with the FLAG-tagged cDNAs. After washing, agarose beads were boiled in SDS-sample buffer, separated by SDS–PAGE and transferred onto nitrocellulose membranes probed with anti-FLAG antibody. A fraction of the reaction mixture was stained with Coomassie to visualize histidine-tagged recombinant proteins. Cell lysates from transfected HEK293 cells were analyzed by Western blot and aligned to visualize relative expression level of TUCAN/CARDINAL constructs. B: HEK293 cells were transfected with HA-tagged full-length TUCAN/CARDINAL together with FLAG-tagged TUCAN/CARDINAL 1–341, a polypeptide containing the CARD of NAC (NAC 1040–1429), FLAG-tagged DRAL and the unrelated protein IFP-35. 16 h after transfection, cell lysates were prepared, immunoprecipitated with anti-HA monoclonal antibody and analyzed for co-precipitating proteins by immunoblot probed with anti-FLAG antibody. Before immunoprecipitation, cell lysates were analyzed by Western blot to visualize protein expression.

CAN/CARDINAL (L407P), which bear mutations in conserved residues of the CARD. Although expressed at levels comparable to the wild-type (wt) protein, the mutants TUCAN/CARDINAL (L366R), TUCAN/CARDINAL (L377R) and TUCAN/CARDINAL (L407P) failed to associate with wt TUCAN/CARDINAL in pull-down experiments (Fig. 1A,B). Thus, an intact CARD domain is essential for dimerization of TUCAN/CARDINAL.

It has been reported that TUCAN/CARDINAL regulates activation of NF-κB transcription factor [13]. To gain insight into the signal transduction pathways in which TUCAN/CARDINAL modulates the activity of this transcription factor, we performed a two-hybrid screen fusing TUCAN/CARDINAL to the DNA-binding domain of GAL-4 and searched a plasmid library of fusion between GAL-4 transcription activation domain and cDNAs from HeLa cells. Sixty six independent clones were identified that were able to grow on selective media and activated the β-galactosidase reporter gene when co-expressed with TUCAN/CARDINAL in the yeast strain AH109. Restriction mapping and partial sequencing of these 66 clones revealed that 47 clones contained inserts of different length encoding for DRAL, also known as SLIM3 or FHL-2, a p53-responsive protein previously identified as being down-regulated in a human embryonal rhabdomyosarcoma cell line [16] (Table 1). To confirm the association between TUCAN/CARDINAL and DRAL in a different experimental system, deleted versions of TUCAN/CARDINAL were expressed as His-tagged fusion proteins in bacteria and tested for binding to FLAG-tagged DRAL expressed in HEK-293 cells. As shown in Fig. 2A, DRAL binds to TUCAN/CARDINAL 179–341 and TUCAN/CARDINAL 179–431, suggesting that the CARD is not involved in this association. In the same experiment, DRAL did not bind to TUCAN/CARDINAL 341–431, proving the specificity of interaction between DRAL and TUCAN/CARDINAL 179–341 and TUCAN/CARDINAL 179–431. We next tested whether TUCAN/CARDINAL can associate to DRAL in mammalian cells. To this end, an expression construct producing HA-tagged TUCAN/CARDINAL was transiently co-transfected in HEK293 together with FLAG-tagged DRAL or control FLAG-tagged constructs. Cell lysates were immunoprecipitated with anti-HA antibody and co-immunoprecipitated proteins were visualized by immunoblotting with anti-FLAG. The results shown in Fig. 2B revealed that TUCAN/CARDINAL specifically co-precipitates with DRAL when co-expressed in these cells.

The interaction of TUCAN/CARDINAL with DRAL prompted us to investigate whether DRAL may influence the effect of TUCAN/CARDINAL on NF-κB activation. Fig. 3A shows that expression of TUCAN/CARDINAL reduces in a dose-dependent manner activation of NF-κB induced by expression of upstream activators of the IKK complex. Next, HEK293 cells were transiently transfected with TRAF-2, TUCAN/CARDINAL and DRAL in different combinations, along with a luciferase reporter vector for NF-κB activation. As shown in Fig. 3B, although expression of DRAL had no effect on NF-κB activation, this protein enhanced in a dose-dependent manner TRAF-2-mediated activation of NF-κB. However, when co-expressed with TRAF-2 and DRAL, TUCAN/CARDINAL still had an antagonistic effect on NF-κB activation, indicating that the inhibitory effect of TUCAN/CARDINAL occurs downstream of DRAL. The effect of TUCAN/CARDINAL and DRAL on NF-κB activation were also observed when cells expressing TUCAN/CARDINAL and DRAL were exposed to TNF (Fig. 3C).

Conflicting data have been reported with regard to the capability of TUCAN/CARDINAL to regulate apoptosis. For instance, Pathan et al. [12] reported that this protein binds to caspase-9 and can function as an inhibitor of programmed cell death, whereas others failed to reproduce these data in other experimental systems [13]. To test for a pro-apoptotic activity of this protein, HeLa cells were transiently transfected with TUCAN/CARDINAL along with a β-gal reporter vector and the ratio of β-gal positive cells with apoptotic morphology was determined 24 h after transfection. No induction of apoptosis was found following expression of TUCAN/CARDINAL in HeLa cells, and similar results were obtained when TUCAN/CARDINAL was expressed in HEK293 cells

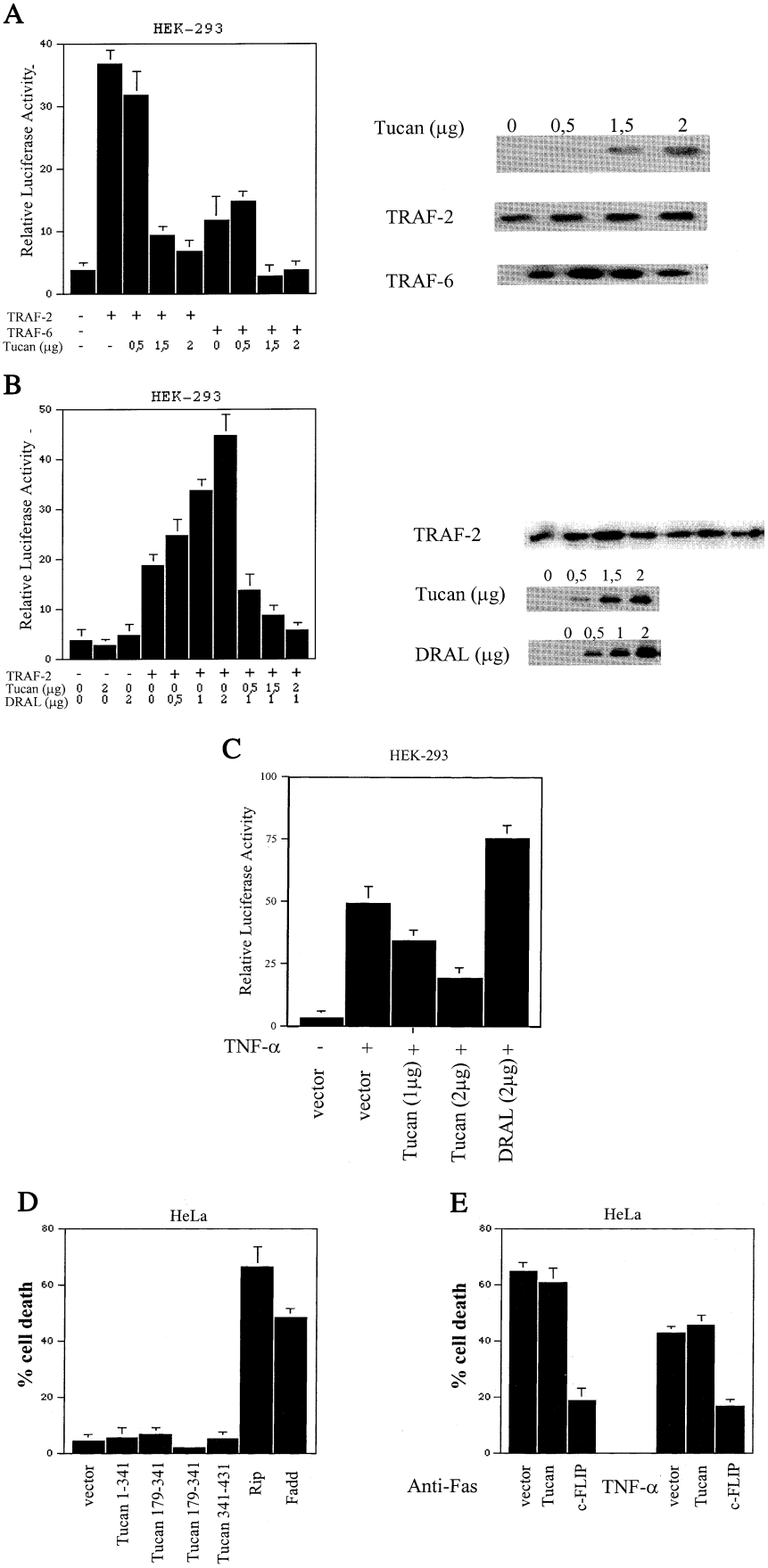
A
HEK-293
Relative Luciferase Activity
TRAF-2
TRAF-6
Tucan (μg)
Tucan (μg) 0 0,5 1,5 2
TRAF-2
TRAF-6
B
HEK-293
Relative Luciferase Activity
TRAF-2
Tucan (μg)
DRAL (μg)
TRAF-2
Tucan (μg) 0 0,5 1,5 2
DRAL (μg) 0 0,5 1 2
C
HEK-293
Relative Luciferase Activity
TNF-α
vector
vector
Tucan (1μg)
Tucan (2μg)
DRAL (2μg)
D
HeLa
% cell death
vector
Tucan 1-341
Tucan 179-341
Tucan 179-341
Tucan 341-431
Rip
Fadd
E
HeLa
% cell death
Anti-Fas
vector
Tucan
c-FLIP
TNF-α
vector
Tucan
c-FLIP

Fig. 3. TUCAN/CARDINAL and DRAL modulate NF-κB activation. A,B: HEK293 cells were transiently co-transfected with the indicated amount of expression vector, together with pNF-κB-luc and a β-galactosidase reporter vector. The total amount of transfected plasmidic DNA was maintained constant by adding empty vector. 16 h after transfection, cell lysates were prepared and luciferase activity was measured. Expression level of transfected cDNAs is shown for a representative experiment. Data represent relative luciferase activity normalized on β-galactosidase activity and are representative of three independent experiments done in triplicate. C: HEK293 cells were transiently co-transfected with the indicated amount of expression vector, together with pNF-κB-luc and a β-galactosidase reporter vector. 16 h after transfection, cells were treated with TNF-α (20 ng/ml) for 4 h and luciferase activity normalized on β-galactosidase activity was determined. Data shown is representative of two independent experiments done in triplicate. D: HeLa cells were transiently co-transfected with the indicated expression vector, together with a β-galactosidase reporter vector. 24 h after transfection, β-gal positive cells were stained and examined for apoptotic morphology. Data represent percentage of dead blue cells over the total blue cells counted (mean ± S.D.) and is representative of four independent experiments done in triplicate. E: HeLa cells were transiently co-transfected with the indicated expression vector, together with a β-galactosidase reporter vector. 24 h after transfection, cells were treated with anti-Fas (50 ng/ml) or TNFα (20 ng/ml) for 16 h in the presence of cycloheximide (0.8 μg/ml). The number of apoptotic cells was determined as described in (A). The experiment shown is representative of four independent experiments done in triplicate
←

(Fig. 3D). We also tested the effect of TUCAN/CARDINAL expression in a cellular model of induced cell death. To this end, HeLa cells transfected with TUCAN/CARDINAL together with a β-gal reporter gene, were subsequently treated with TNF and anti-Fas/*Apo*I to induce apoptosis, and the number of β-gal positive cells with apoptotic morphology was determined. However, expression of TUCAN/CARDINAL had no effect on cell survival in this experimental system (Fig. 3E), and similar results were obtained when apoptosis was evoked by treatment with staurosporine (data not shown).

4. Discussion

CARD-containing proteins have been implicated in signal transduction pathways leading either to apoptotic cell death or to induction of the transcription factor NF-κB. This transcription factor has emerged as a critical regulator of cytoprotective responses, controlling expression of genes promoting cell survival and proliferation. Retained in the cytoplasm by the inhibitory proteins I-κBs, NF-κB translocates to the nucleus upon different stimuli and activates transcription of target genes, including anti-apoptotic proteins such as IAPs. Thus, the implication of TUCAN/CARDINAL in regulation of NF-κB activity may represent an additional mechanism by which CARD-containing proteins modulate signaling pathways involved in cell death and proliferation.

We have shown that TUCAN/CARDINAL can associate with the p53-responsive protein DRAL. The p53 tumor suppressor gene encodes for a nuclear protein that plays an important role in cell cycle regulation and apoptosis and, significantly, p53 is the most frequently mutated gene in human cancer. In responses to cellular stress, p53 exerts its biological activity by inducing or repressing transcription of specific target genes. These downstream effectors have been characterized with respect to p53-mediated growth arrest, but the signaling cascades associated with p53-regulated cell death remain elusive. DRAL was originally identified because of its differential expression in normal myoblast and a corresponding malignant cell line [16]. DRAL belongs to a subfamily of proteins containing four and half LIM domain, a cysteine-rich domain essentially involved in protein–protein interactions. Expression of DRAL was shown to efficiently induce apoptosis in different cell lines, suggesting that this protein may be directly involved in p53-dependent apoptosis [17]. In contrast, our investigations in this direction failed to find a pro-apoptotic role for DRAL in the cellular systems used for this study. Some of the discrepancy may relate to the different cellular and/or expression systems adopted. However, our data have now revealed an additional role for DRAL, indicating that this protein may also participate in regulation of NF-κB activation. Thus, DRAL appears functionally related to CARD-containing proteins, being implicated in signaling pathways that regulate apoptosis and activation of NF-κB.

Many cellular stimuli result in the induction of both the tumor suppressor p53 and NF-κB transcription factors. However, whereas activation of p53 is associated with induction of apoptosis, stimulation of NF-κB promotes resistance to programmed cell death. In this context, our observations suggest that DRAL and TUCAN/CARDINAL may participate in a regulatory mechanism that integrates and coordinates cellular proliferation and apoptosis.

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