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Interaction of apoptosis signal-regulating kinase 1 with isoforms of 14-3-3 proteins

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Abstract

Apoptosis signal-regulating kinase 1 (ASK1) is a critical mediator of apoptotic signaling pathways initiated by a variety of death stimuli. Its activity is tightly controlled by various mechanisms such as covalent modification and protein–protein interaction. One of the proteins that control ASK1 function is 14-3-3ζ, a member of the 14-3-3 protein family. Here, we report that ASK1 is capable of binding to other isoforms of 14-3-3, suggesting that binding ASK1 is a general property of the 14-3-3 family. In support of this notion, mutational analysis revealed that the ASK1/14-3-3 interaction was mediated by the conserved amphipathic groove of 14-3-3 with some residue selectivity. Functionally, expression of various isoforms of 14-3-3 suppressed ASK1-induced apoptosis. To understand how 14-3-3 controls the ASK1 activity, we examined intracellular localization of ASK1 upon 14-3-3 co-expression. We found that 14-3-3 co-expression is correlated with the translocation of ASK1 from the cytoplasm to a perinuclear localization, likely the ER compartment. Consistent with this notion, $ASK1^{S967A}$, a 14-3-3 binding defective mutant of ASK, showed no change in intracellular distribution upon 14-3-3 co-expression. These data support a model that 14-3-3 proteins regulate the proapoptotic function of ASK1 in part by controlling its subcellular distribution.

Keywords: ASK1; 14-3-3 isoforms; ER; Subcellular localization

Introduction

Apoptosis signal-regulating kinase 1 (ASK1) is a multifunctional serine/threonine kinase involved in controlling many cellular processes such as apoptosis signaling and cell differentiation [1]. ASK1 was initially discovered as a mitogen-activated protein (MAP) kinase kinase kinase with proapoptotic function [2,3]. Its catalytic activity can be activated by many stress-stimuli such as tumor necrosis factor α (TNFα), oxidative stress, endoplasmic reticulum (ER) stress, and chemotherapeutic agents like cisplatin and taxol. Activation of ASK1 leads to the activation of the c-Jun N-terminal kinase (JNK) and p38 MAP kinase signaling cascades [3]. In support of its role in mediating stress signal induced apoptosis, overexpression of ASK1 can induce apoptotic cell death through a mitochondria-dependent caspase pathway [4], while catalytically inactive mutants of ASK1 can inhibit apoptotic cell death initiated by TNFα, H_2O_2, and cisplatin [3,5–7]. Recent work from ASK1 knock-out mice lends strong support to this notion. In ASK1−/− mouse embryonic fibroblasts, apoptosis induced by TNFα and H_2O_2 is significantly impaired, which is correlated with the drastic decrease of the sustained activation of JNK and p38 MAP kinases [8]. Thus, it is clear that ASK1 plays a critical role in stress signal-induced apoptosis and therefore it is subject to tight controls. Indeed, the ASK1 activity is regulated by both pro- and anti-apoptotic stimuli through various mechanisms, which include reversible phosphorylation and protein–protein interactions. Phosphorylation of T-845 is required for ASK1 activation while phosphorylation of Ser-83 and Ser-967 appears to

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inhibit its proapoptotic function [9–11]. A number of proteins have been found to control ASK1 function by directly binding to ASK1. Binding of TRAF2 promotes ASK1's proapoptotic function while thioredoxin, glutaredoxin, heat shock protein 72, and 14-3-3ζ inhibit ASK1 death-inducing activity [6,11–14]. These intricate regulatory mechanisms allow ASK1 to transmit stress signals to the death machinery in a highly controlled fashion. In particular, the phosphorylation-dependent regulation of ASK1 function, such as phospho-Ser967-mediated 14-3-3ζ interaction, intimately links the ASK1 function to the intracellular kinase/phosphatase-signaling network.

14-3-3 proteins are a family of widely expressed phosphoserine/phosphothreonine-binding eukaryotic proteins [15,16]. There are seven mammalian isoforms (β, ε, γ, η, σ, τ, ζ), which bind to many cellular proteins. Most of these protein–protein interactions are mediated by 14-3-3 binding to a consensus phosphoserine/threonine motif, RSXpS/TXP or its derivatives, where pS/T is the phosphorylated serine or threonine [17,18]. The 14-3-3 binding proteins are composed of a diverse group of signal-transducing molecules, which include protein kinases and phosphatases, such as Raf-1 kinase, phosphatidylinositol 3-kinase, Cdc25, and ASK1 [15]. Depending on the target proteins, 14-3-3 binding may inhibit or activate the catalytic activity of its associated enzymes, alter the interaction of its protein targets with other proteins, or induce relocalization of its binding partners [15,19]. 14-3-3ζ has been shown to associate with ASK1 at Ser^{967}, which lies in a consensus 14-3-3 binding motif, $RSIS^{967}LP$. Interaction of ASK1 with 14-3-3 ζ suppresses the ASK1 pro-apoptotic function [11,20]. How the 14-3-3ζ isoform antagonizes ASK1-mediated apoptosis and whether the other 14-3-3 isoforms can also regulate ASK1 remain elusive. Here, we report that different isoforms of 14-3-3 are capable of interacting with ASK1 and inhibiting ASK1-induced cell death. Furthermore, we demonstrated that co-expression of different 14-3-3 isoforms altered the intracellular localization of ASK1 from the cytoplasm to a perinuclear region, which may in part explain the anti-apoptotic effects of 14-3-3 proteins.

Materials and methods

Plasmids, cell culture, and DNA transfection

14-3-3 isoforms were generated by PCR and subcloned into pDEST26 (Invitrogen), generating His-tagged 14-3-3 fusion vectors for expression in mammalian cells [21]. HA-tagged $ASK1^{wt}$ and $ASK1^{S967A}$ plasmids have been previously described [11]. COS7 and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Mediatech) containing 10% fetal calf serum (Atlanta Biologicals) at 37°C with 5% CO_2. Cells were transfected with plasmids using the FuGENE6 reagent (Roche).

Protein expression, purification, and in vitro binding assays

Recombinant hexahistidine-14-3-3 ζ and mutant proteins were expressed from pET-based plasmids in *E. coli* and purified using Ni^{2+}-charged iminodiacetic acid Sepharose 6B beads as described [22,23]. Radiolabeled ASK1 proteins were generated using the TNT in vitro transcription/translation system according to manufacturer's specifications (Promega). For in vitro binding assays, 5 μg each of immobilized 14-3-3 proteins on Ni^{2+}-charged Sepharose beads were mixed with ^{35}S-labeled ASK1 in NP-40 buffer (1% Nonidet P-40, 137 mM NaCl, 1 mM $MgCl_2$, and 40 mM Tris–HCl, pH 8.0) for 1 h at 4°C with rotation. The 14-3-3/ASK1 complexes were washed once with NP-40 buffer, followed by two radioimmune precipitation assay (RIPA) buffer washes (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 137 mM NaCl, and 20 mM Tris-HCl pH 8.0). The complexes were boiled for 3 min in SDS sample buffer and resolved by SDS-PAGE (12.5%). Presence of radiolabeled products in the 14-3-3 isoform complexes was visualized using a PhosphorImager (Molecular Dynamics, Inc).

Yeast two-hybrid interaction trap assay

A yeast two-hybrid interaction trap [24] was used for studying the in vivo interaction between 14-3-3 isoforms and ASK1. *S. cerevisiae* EGY48 (MATa trp1 ura3 his3 LEU2::pLexAop6-LEU2) containing the *LacZ* reporter plasmid pSH18-34 was used as a host for all interaction experiments. For expression in yeast, we used the 14-3-3 isoforms subcloned into pJG4-5 [21], which generate in-frame HA-fusion constructs. pEG202-ASK1 has been previously described [6]. For protein/protein interaction analysis, EGY48/pSH18-34 was cotransformed with pEG202-ASK1 and pJG4-5 derivatives by the lithium acetate method. Transformants were selected by complementation of auxotrophies using TRP1, URA3, and HIS3 markers. 14-3-3 isoform/ASK1 interactions were determined by either the plate assay with X-gal as an indicator or by the liquid assay with chlorophenyl-red-β-D-galactopyranoside (Roche) as a chromogenic substrate [22].

Apoptosis assays

Morphology-based cell viability assay

HeLa cells were grown in 35-mm dishes at 5×10^4 cells/dish and transfected with a *lacZ* marker and plasmids containing ASK1 and 14-3-3 isoforms using FuGENE 6 Reagent according to the manufacturer's specifications (Roche). Twenty-four hours after transfection, cells were serum starved and cultured for an additional 24 h at 37°C. The cells were washed with PBS and fixed for 5 min at room temperature in 0.5% glutaraldehyde and 2% formaldehyde in PBS. Subsequently, cells were washed twice with PBS and stained with X-gal (0.1% X-gal, 5 mM each

$K_3Fe(CN)_6$ and $K_4Fe(CN)_6$, 2 mM $MgCl_2$ in PBS) overnight at 37°C. The following day, cells were washed twice with PBS and each dish was scored for total blue cells and rounded blue cells under a microscope at 200× magnification in a blinded manner. At least 500 cells/dish were manually counted and the % apoptotic cells were determined by dividing the number of rounded blue cells by the total number of blue cells [25].

Annexin V-based apoptosis assay

COS7 cells (2×10^5/well) were transfected with desired expression vectors. After 24 h, apoptotic cells were detected using AnnexinV-EGFP labeling (K2019-1; BD Biosciences Clontech). Briefly, cells were trypsinized and washed with DMEM media with 10% FBS. Cells (1×10^6) were resuspended in 200 µl binding buffer. AnnexinV-EGFP (5 µl; 40 µg/ml PBS) and propidium iodide (10 µl; 50 µg/ml binding buffer) were added to cells and incubated for 15 min at room temperature in the dark. AnnexinV-EGFP was detected using flow cytometry at 488 nm.

Immunofluorescence and laser confocal microscopy

COS7 cells were grown on one-well Lab-Tek II chamber slides (Nalge Nunc International) at 2×10^5 cells/slide and transfected with test plasmids using FuGene6 (Roche). Forty-eight hours after transfection, cells were fixed with 3% formaldehyde for 5 min. Cells were permeabilized with cold acetone at −20°C for 7 min, and washed with PBS. Slides were blocked with 1% BSA.PBS for 15 min, followed by incubation with primary antibody (1:200) for 1 h at room temperature. We used mouse anti-HA (12CA5, Sigma), M2 mouse anti-Flag (Roche), rabbit anti-His (sc-803, Santa Cruz), rabbit anti-Grp78 (SPA-826, StressGen Biotechnologies), or rabbit anti-Calnexin antibodies (SPA-860, StressGen Biotechnologies). Next, slides were washed with PBS and incubated with Fluorescein-anti rabbit IgG or Texas Red-anti mouse IgG (Jackson Immunoresearch) for 1 h at room temperature. The slides were mounted with Vectashield (Vector Labs) containing 4′, 6-diamidine-2-phenylindole (DAPI) and coverslipped. Cells were analyzed by laser confocal microscopy (Zeiss LSM-410).

Results

ASK1 interacts with different isoforms of 14-3-3

It has been previously established that ASK1 interacts with the ζ isoform of the 14-3-3 protein family in vitro and in vivo [11]. To determine if ASK1 has any isoform-selectivity in its interaction with 14-3-3, we tested ASK1 against different isoforms of 14-3-3 using the yeast two-hybrid system as previously described [22]. As shown in Fig. 1A, all 14-3-3 isoforms tested interacted with ASK1, resulting in activation of the *lacZ* reporter and blue colony formation on induction medium containing X-gal. The control vector without 14-3-3 failed to induce expression of the reporter gene. The well-established Raf-1/14-3-3ζ association was used as a positive control, showing blue

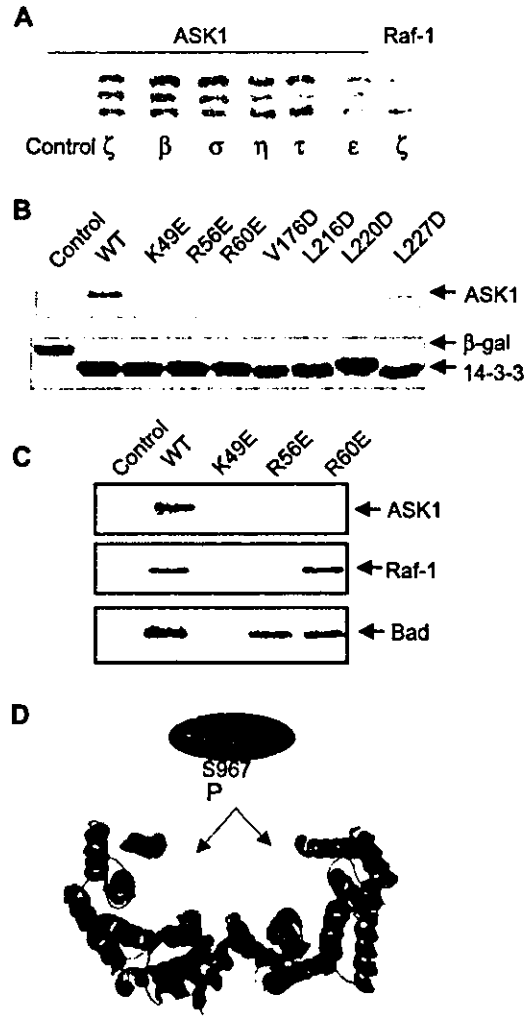

Fig. 1. Interaction of ASK1 with 14-3-3 isoforms. (A) Yeast two-hybrid assay. *S. cerevisiae* strain EGY48/pSH18-34 was transformed with pEG202-ASK1 along with pJG4-5 vectors containing 14-3-3 isoforms. Cells were grown on synthetic medium plates containing galactose (2%), raffinose (1%), and X-gal. Interaction of ASK1 with 14-3-3 isoforms results in blue patches. The last lane is a positive control containing pEG202-Raf-1 and pJG4-5-14-3-3ζ. Control strain contains pEG202-ASK1 and pJG4-5. (B) In vitro solid-phase based assay. ASK1 was radiolabeled in vitro with S^{35}-methionine using the TNT kit from Promega and incubated with equal amount of immobilized recombinant 14-3-3ζ or its mutant derivatives for 2 h at 4°C. The 14-3-3 complexes were extensively washed. The bound ASK1 was resolved by SDS-PAGE and detected by PhosphorImager (upper panel). The bottom panel shows that a similar amount of 14-3-3 proteins was used in the assay. Immobilized β-galactosidase serves as a negative control. (C) Interaction of 14-3-3 mutants with different partners. Similar in vitro solid-phase-based binding assays as in (B) were carried out using radiolabeled Raf-1 and Bad, in addition to ASK1. Selected immobilized 14-3-3 mutant proteins were used. (D) Schematic diagram showing the interaction of phosphorylated ASK1 with 14-3-3 through its amphipathic groove.

colony formation on induction plates [22]. Relative β-galactosidase activity was further quantified with a liquid assay, confirming that ASK1 can bind to different isoforms of 14-3-3 (data not shown).

ASK1 binds to the conserved amphipathic groove of 14-3-3

14-3-3 isoforms possess a common structural feature with a conserved amphipathic groove that mediates their interaction with many binding partners [15]. Interaction of ASK1 with different isoforms raised the possibility that ASK1 may target the conserved amphipathic groove of 14-3-3 for its association. To test this model, we utilized recombinant 14-3-3ζ proteins with point mutations in the amphipathic groove. These 14-3-3 proteins were epitope-tagged with hexahistidine and examined for binding to radiolabeled ASK1 in an in vitro solid-phase binding assay [11]. 14-3-3ζ WT or its mutant proteins were mixed with radiolabeled ASK1. After extensive washing, the hexahistidine–14-3-3ζ protein complexes were precipitated with nickel-chelating beads. The radiolabeled ASK1 that was associated with 14-3-3ζ proteins was visualized by SDS-PAGE resolution and autoradiography (Fig. 1B). The lower panel of Fig. 1B shows that similar protein levels of 14-3-3ζ mutants were used. Our data shows that mutations on the hydrophilic face of the groove, K49E, R56E, and R60E, and on the hydrophobic face of the groove, V176D, and L220D, abolished interaction between 14-3-3 and ASK1. L216D and L227D, mutants of hydrophobic amino acids bordering the groove, slightly decreased the 14-3-3/ASK1 interaction. Complementary yeast two-hybrid analysis showed similar results, confirming the importance of the conserved residues in the amphipathic groove of 14-3-3 for ASK1 binding (data not shown).

ASK1 contacts selective residues of 14-3-3 in the amphipathic groove

Like other 14-3-3 ligands, such as Raf-1 and Bad, ASK1 appears to utilize the conserved amphipathic groove of 14-3-3 for binding. However, comparison of the 14-3-3/ASK1 binding with Raf-1 and Bad revealed interesting

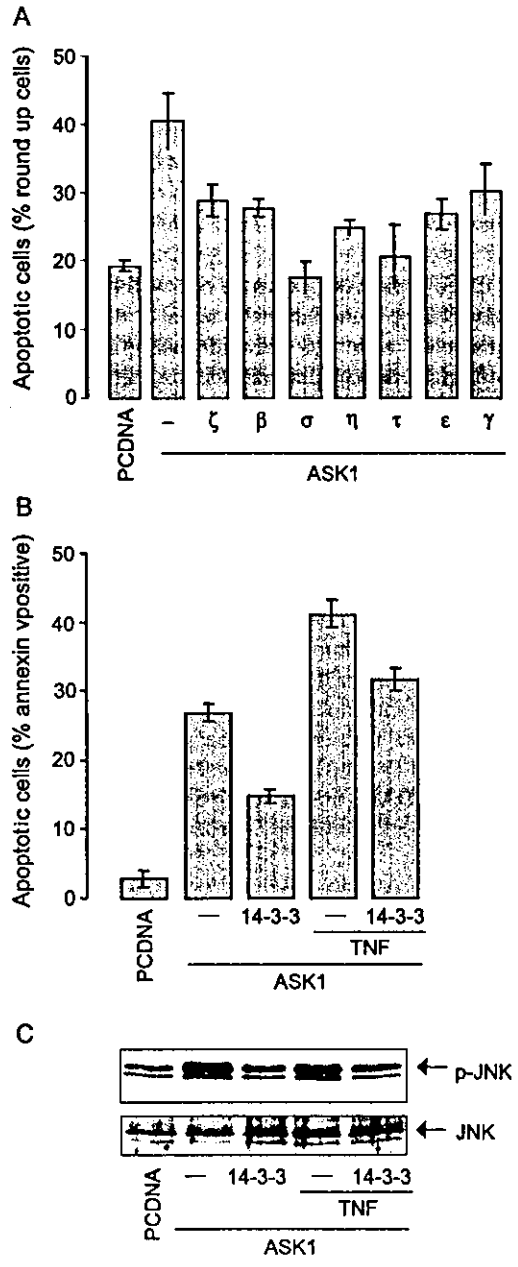

Fig. 2. 14-3-3 isoforms suppress ASK1 induced JNK activation and apoptosis. (A) Inhibition of ASK1-induced apoptosis by 14-3-3 isoforms as revealed by a cell morphology-based assay. HeLa cells (5×10^4 cells/35 mm dish) were transfected in duplicate with a *lacZ* marker plasmid (20% of total DNA) and combinations of pcDNA3-HA-ASK1 (40%) and His-tagged mammalian 14-3-3 isoforms in pDEST vectors (40%). Cells were grown for 24 h in serum and then serum-starved for 24 h. Cells were fixed in glutaraldehyde, and stained with X-gal. Each dish was manually counted (>500 cells/dish) for total blue cells and round-up blue cells in a blind fashion. The percentage of apoptotic cells is calculated as the ratio of round-up blue cells to total transfected cells. (B) Inhibition of ASK1-induced apoptosis by 14-3-3 as revealed by annexin V-based flow cytometry assay. COS7 cells were transfected in triplicate with pcDNA or combinations of pcDNA-HA-ASK1 and pDEST6xHis-14-3-3ζ for 24 h. Cells (1×10^6) were trypsinized, washed with media, and resuspended in 200 μl of binding buffer. Then, annexinV-EGFP (5 μl) and propidium iodide (10 μl) were added to cells and incubated for 15 min at room temperature in the dark. AnnexinV-EGFP was detected using flow cytometry at 488 nm. Cells with TNFα (10 ng/ml) were treated for 12 h before harvesting. Percentage of annexin V positive cells was plotted. (C) Inhibition of ASK1-induced JNK activity by 14-3-3. COS7 cells (2×10^5/well) was transfected in duplicate with pcDNA or a combination of pcDNA-HA-ASK1 and pDEST6xHis-14-3-3ζ. Sixty hours post transfection, cells were treated with TNFα (10 ng/ml) or left untreated for 12 h. Cells were lysed in 1% NP-40 lysis buffer. Twenty-five micrograms of each protein lysate was used for Western blotting with antibodies that recognize phosphorylated JNK (#9251; Cell Signaling) or total JNK (sc-572, Santa Cruz Biotechnology). Cross-reacting materials were detected by ECL (Western Lightning, Perkin-Elmer).

differences (Fig. 1C). Mutating K49 to E abolished the interaction of 14-3-3 to all of these three protein partners tested, while R56E only disrupted the 14-3-3 binding to ASK1 and Raf-1. R56 does not seem to be essential for the 14-3-3/Bad interaction. R60E only diminished the interaction of 14-3-3 with ASK1 and had little effect on the 14-3-3 complex with either Raf-1 or Bad. Although ASK1 appears to dock in the conserved amphipathic groove of 14-3-3 as Raf-1 and Bad, the structural requirement for ASK1 interaction is different from other ligands. These results demonstrate that different 14-3-3 protein partners may selectively interact with residues in the conserved amphipathic groove, although they share some common residues for binding (Fig. 1D).

Inhibition of ASK1-induced apoptosis by different isoforms of 14-3-3

Previous results revealed that 14-3-3ζ is capable of preventing ASK1-mediated cell death [11]. Here, we have demonstrated that different 14-3-3 isoforms can interact with ASK1 via a common amphipathic groove in 14-3-3. However, it is unclear whether all human 14-3-3 isoforms share the function of blocking ASK1-mediated apoptosis.

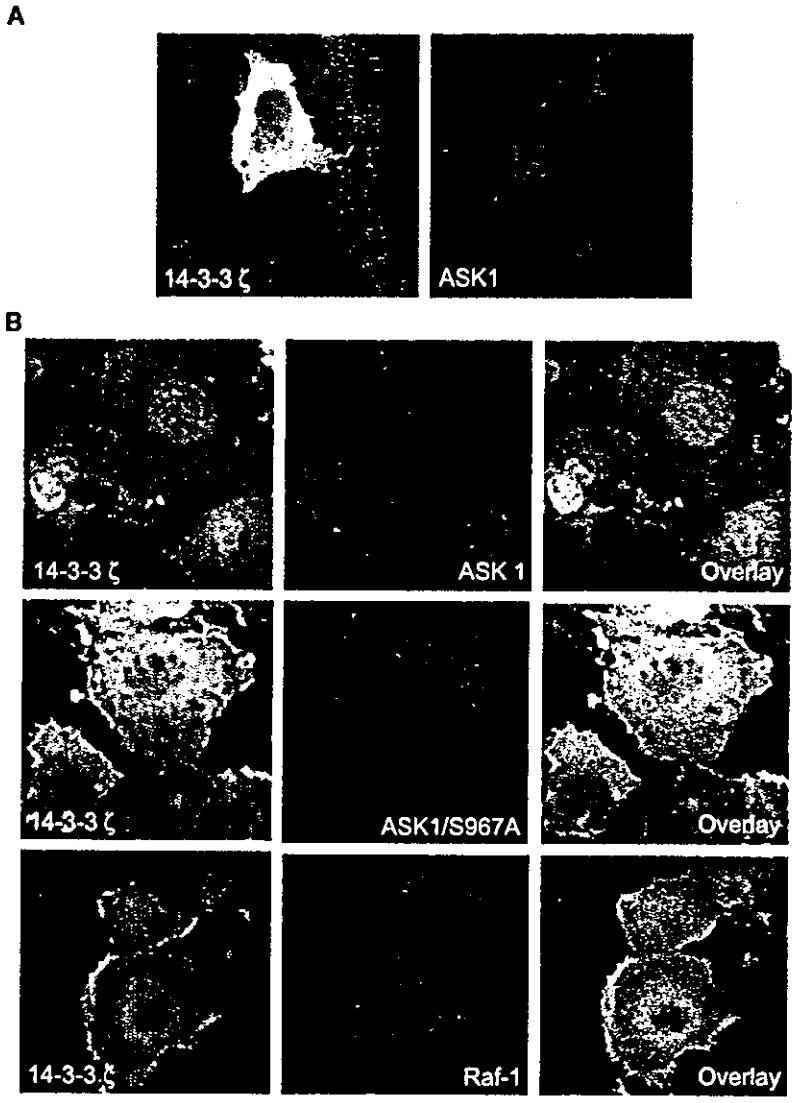

Fig. 3. Intracellular localization of ASK1. (A) Single transfection. COS7 cells (2×10^5) were grown on chamber slides and transfected with pcDNA-HA-ASK1 or pDEST6xHis-14-3-3ζ using FuGene6. Forty-eight hours after transfection, cells were fixed, permeabilized, and proteins were detected by immunofluorescence with anti-HA or anti-histidine antibodies. Localization of HA-ASK1 or 6xHis-14-3-3ζ in cells was by laser confocal microscopy. (B) Co-transfection. COS7 cells were transfected with pDEST6xHis-14-3-3ζ along with pcDNA-HA-ASK1 (upper row), pcDNA-HA-ASK1/S967A (middle row), or pcDNA-Flag-Raf-1 (bottom row), as described above. The expression of Flag-Raf-1 was detected by using an anti-Flag antibody, M2. The right panels show overlayed images.

To address this question, we used a morphology-based apoptosis assay [25]. Apoptotic stimuli often predispose adherent cells to detach from the dish surface and to exhibit rounded-up shape, which provides a morphological readout for apoptosis. To test the effect of 14-3-3 isoforms on ASK1 function, HeLa cells were transfected with a β-gal reporter together with expression vectors for ASK1 and/or 14-3-3 isoforms. Cell morphology of transfected cells was examined. Expression of ASK1 doubled the amount of apoptotic cells compared to control cells that expressed empty vector alone (Fig. 2A). Upon co-expression of 14-3-3 with ASK1, the amount of apoptosis caused by ASK1 was significantly decreased. It appears that all 14-3-3 isoforms are capable of inhibiting ASK1-induced apoptosis (Fig. 2A). Apoptotic cells usually exhibit fragmented DNA, which can be detected by propidium iodide staining [21]. Another feature of apoptotic cells is the translocation of phosphatidylserine from the inner leaflet of the phospholipid bilayer to the cell surface, which can be recognized by annexin V. To confirm the results obtained by the cell morphology-based assay, DNA content-based and annexin V-based flow cytometry analysis was performed [21]. Consistent with the above observation, expression of 14-3-3 isoforms decreased the number of apoptotic cells (sub-G0) induced by ASK1 (data not shown) and the number of annexin V-positive cells (Fig. 2B).

Modulation of intracellular localization of ASK1 by 14-3-3

One mechanism whereby 14-3-3 controls its binding partners is through altered subcellular localization [15,19]. To test the hypothesis that 14-3-3 suppresses ASK1-induced cell death by regulating the intracellular localization of ASK1, we examined ASK1 distribution in response to 14-3-3 co-expression by immunofluorescence staining followed by laser confocal microscopy. When ASK1 or 14-3-3ζ was expressed individually, they were predominantly localized in the cytoplasm with some nuclear 14-3-3 localization (Fig. 3A). When ASK1 was co-expressed with 14-3-3ζ, ASK1 was redistributed in cells and cytoplasmic ASK1 was primarily restricted to a perinuclear region (upper row, Fig. 3B). To examine the specificity of 14-3-3 mediated translocation of ASK1, a 14-3-3-binding defective mutant of ASK1, ASK1S967A, was used. Like ASK1/WT, ASK1/S967A was detected only in the cytoplasm when expressed

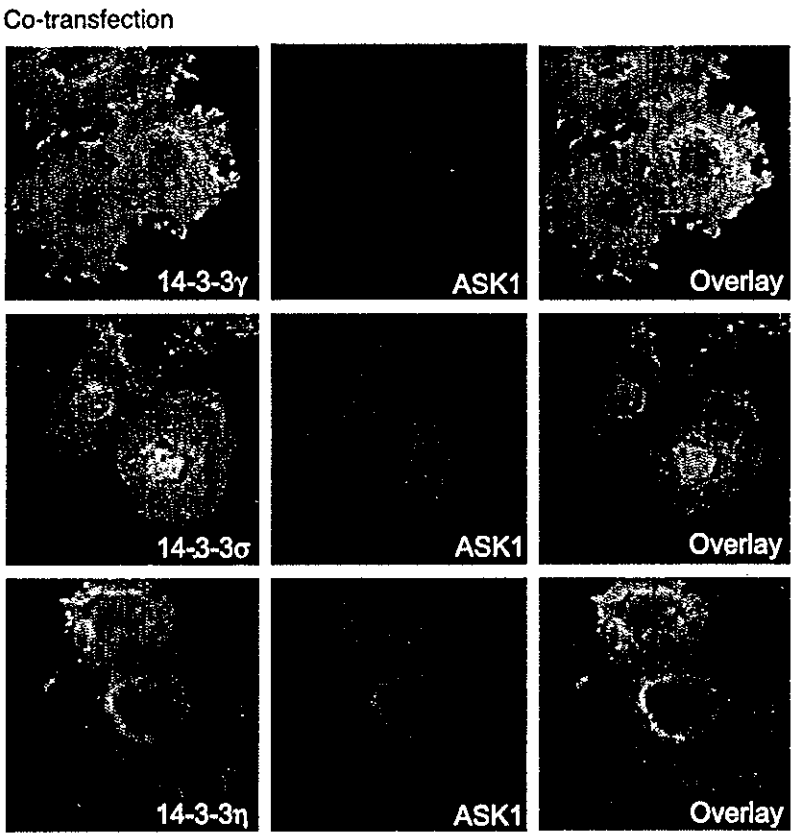

Fig. 4. 14-3-3 isoforms share a common function in relocating ASK1. COS7 cells were transfected with pcDNA-HA-ASK1 along with pDEST6xHis-14-3-3σ (upper row), pDEST6xHis-14-3-3γ (middle row), or with pDEST6xHis-14-3-3η (bottom row). Expression of HA-ASK1 and 6xHis-14-3-3 isoforms was detected with anti-HA or anti-His antibodies as described in legend to Fig. 3.

alone. Interestingly, co-expression with 14-3-3ζ had no effect on the intracellular distribution of ASK1/S967A, which remained cytoplasmic (middle row, Fig. 3B). Reciprocal experiments were carried out to determine whether ASK1-binding defective 14-3-3 mutants, 14-3-3ζ K49E and V176D, had any effect on ASK1 translocation. Consistent with the above data, these ASK1-binding defective 14-3-3 mutants were unable to alter the cytoplasmic localization of ASK1 (data not shown). On the other hand, co-expression of 14-3-3ζ with Raf-1 did not lead to perinuclear localization of Raf-1, suggesting that translocation of ASK1 by 14-3-3ζ may be a specifically regulated event (bottom row, Fig. 3B). Taken together, these data indicate that interaction between 14-3-3ζ and ASK1 is necessary for translocation of ASK1 from the cytoplasm to the perinuclear location.

Consistent with the ability of different 14-3-3 isoforms to interact with ASK1, other 14-3-3 isoforms, such as 14-3-3γ, σ, and η, were all capable of inducing a similar change in the intracellular localization of ASK1 (Fig. 4). The redistribution of ASK1 by 14-3-3 isoforms is not limited to Cos7 cells, as similar results were obtained in HeLa cells (Data not shown).

Translocation of ASK1 to the endoplasmic reticulum by 14-3-3

To establish the intracellular organelle to which ASK1 was relocated, we carried out co-staining experiments using fluorescent markers targeted to various organelles. When co-expressed with 14-3-3, ASK1 was not colocalized with expressed EGFP-Golgi or EGFP-EEA1, markers for Golgi and endosomes, respectively (data not shown). Golgi was seen primarily localized to one side of the nucleus and endosome staining revealed a widespread cytoplasmic distribution, while ASK1 was predominantly perinuclear when co-expressed with 14-3-3 (Fig. 3). It is unlikely that ASK1 is translocated to Golgi and endosomes by 14-3-3. Then, the potential localization of ASK1 in ER was probed by immunostaining with an ER-specific protein marker, Glucose-regulated protein 78 (Grp78). Again, ASK1 was normally localized in the cytoplasm (top row, Fig. 5). However, co-expression of 14-3-3ζ with ASK1 relocated ASK1 to the ER region, showing colocalization of ASK1 and Grp78 (middle row, Fig. 5). A z-scan overlay captured by laser confocal microscopy showed that ASK1 staining overlaps

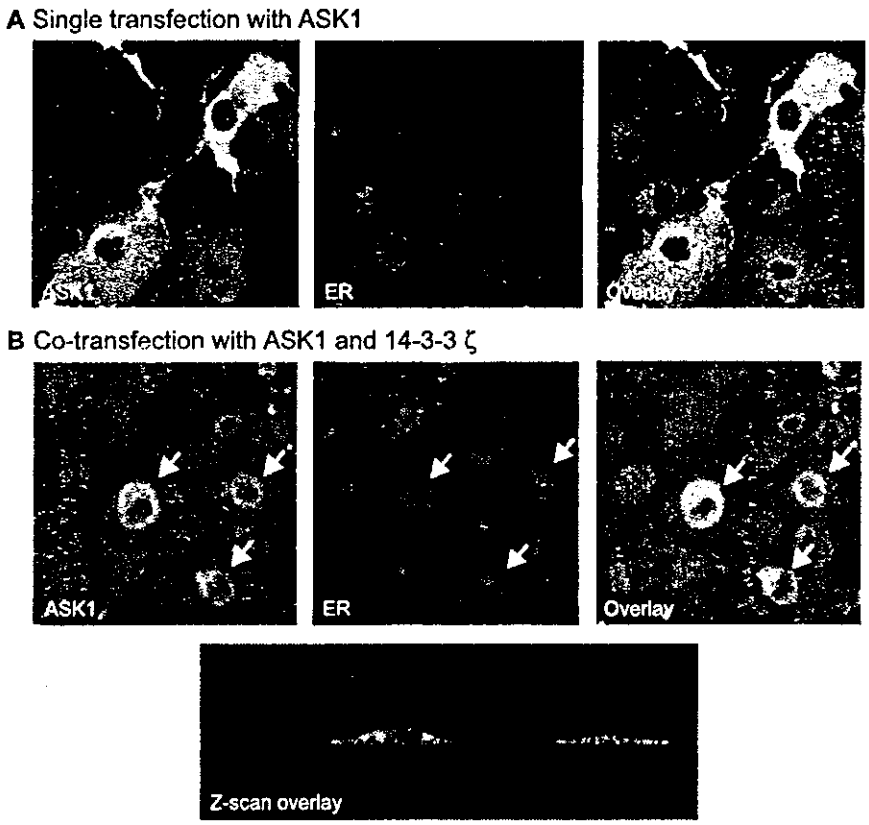

Fig. 5. ASK1 is relocated to the endoplasmic reticulum upon co-expression with 14-3-3. COS7 cells were transfected with pcDNA-HA-ASK1 alone (top row) or pcDNA-HA-ASK1 with pDEST6xHis-14-3-3ζ (middle row). The localization of ASK1 was revealed by immunostaining with anti-HA antibody as described in legend to Fig. 3 and the location of endoplasmic reticulum was revealed by immunostaining with anti-Grp78 antibody. The middle row shows that ASK1 is translocated to the perinuclear region in the presence of overexpressed 14-3-3 and overlaps with the ER immunostain as seen in the overlay image. A z-scan overlay image (bottom) of a cell stained for ASK1 indicates that ASK1 is localized primarily around the nucleus where the ER is present.

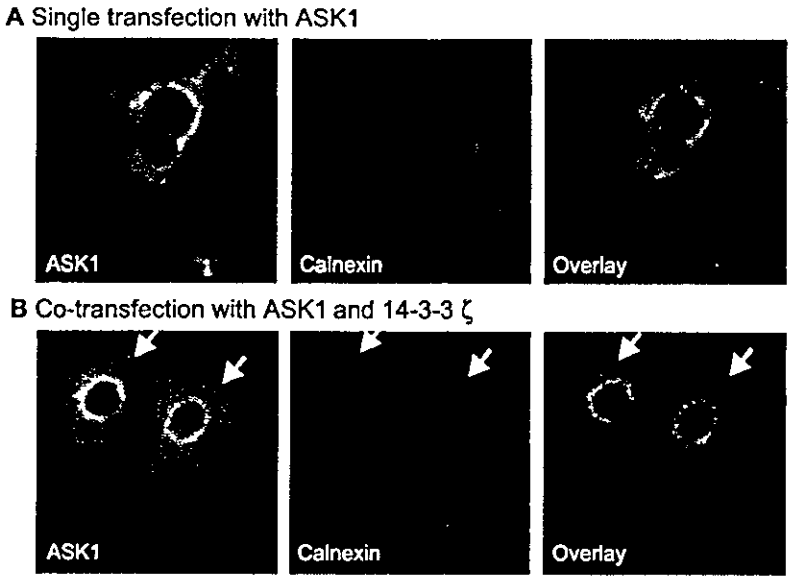

Fig. 6. Co-localization of ASK1 with an ER-resident protein calnexin. COS7 cells were transfected with pcDNA-HA-ASK1 alone (top row) or pcDNA-HA-ASK1 with pDEST6xHis-14-3-3ζ (bottom row). Cells were immunostained for ASK1 and calnexin with anti-HA and anti-calnexin antibodies, respectively. ASK1 is co-localized with calnexin in the ER upon co-expression of 14-3-3 (*arrows*).

with the ER marker staining and is concentrated around the nucleus (bottom panel, Fig. 5). To confirm these results, we used calnexin, a resident ER protein, as an additional ER marker (Fig. 6). ASK1 was distributed cytoplasmically, while calnexin was seen in the perinuclear region indicating presence of the ER (upper row, Fig. 6). Upon co-expression with 14-3-3, ASK1 was translocated to the ER region as evidenced by the overlapping staining of ASK1 and calnexin (bottom row, Fig. 6). Thus, 14-3-3 proteins may function to alter the intracellular distribution of ASK1 and possibly restrict ASK1 to the perinuclear ER region.

Dissociation of the 14-3-3/ASK1 interaction by TNFα

It has been shown that TNFα can activate ASK1 and promotes ASK1-mediated apoptosis [3,12]. It is possible that TNFα could regulate the translocation of ASK1 by 14-3-3, thus neutralizing the 14-3-3 effect. To test this model, we examined the intracellular distribution of ASK1 upon treatment of Cos7 cells with TNFα. Indeed, treatment of cells with TNFα (10 ng/ml) blocked the relocation of ASK1 to the perinuclear region by 14-3-3 (Fig. 7). After 24 h of treatment, some 14-3-3 and ASK1 are co-localized to the perinuclear region, but a greater proportion of both molecules are in the cytoplasm (data not shown). Thus, the 14-3-3/ASK1 interaction may be in part controlled by TNFα signaling.

Discussion

Our results demonstrate that ASK1 is capable of interacting with different isoforms of 14-3-3 proteins. Since isoforms of 14-3-3 are expressed in diverse tissues and cell

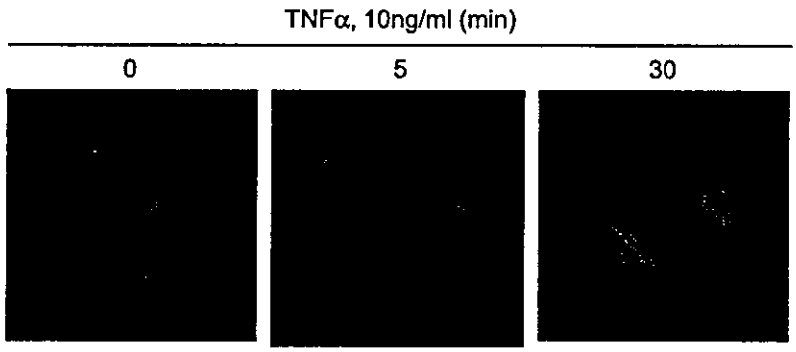

Fig. 7. TNFα maintains cytoplasmic localization of ASK1 when coexpressed with 14-3-3. COS7 cells were transfected with pcDNA-HA-ASK1 and pDEST6xHis-14-3-3ζ for 48 hr. Cells were treated with TNFα (10 ng/ml) for indicated times. Intracellular distribution of ASK1 was revealed by immunofluorescence microscopy as described in legend to Fig. 3.

types, this property would place the widely expressed ASK1 under the strict control of 14-3-3 proteins under a variety of intracellular settings. Upon binding, 14-3-3 proteins can suppress the proapoptotic function of ASK1, which may in part be due to 14-3-3-induced intracellular relocalization of ASK1. Our experiments showed that wild-type ASK1 is relocalized from the diffused cytoplasmic area to a perinuclear region when co-expressed with 14-3-3. This altered intracellular localization of ASK1 appears to be specifically associated with 14-3-3 binding. This notion is supported by the observation that (i) ASK1/S967A, a mutant form of ASK1 with diminished 14-3-3 binding, stays cytoplasmic even in the presence of co-expressed wild-type 14-3-3 and (ii) 14-3-3 mutants defective in ASK1 binding fail to promote wild-type ASK1 translocation to the perinuclear region. Significantly, TNFα, a known ASK1 upstream regulator, interferes with the 14-3-3 induced translocation of ASK1. These results provide a molecular basis whereby 14-3-3 proteins suppress ASK1-induced apoptosis.

It has been well documented that 14-3-3 proteins can serve as sequestration proteins [15,19]. 14-3-3 proteins can change intracellular localization of their binding partners, leading to altered function of their associated proteins [26–29]. For example, 14-3-3 interacts with Bad upon phosphorylation at Ser136 by specific kinases including Akt/protein kinase B. The 14-3-3/Bad interaction is associated with the cytoplasmic localization of Bad, which keeps Bad away from mitochondrially situated Bcl-2, leading to inhibition of Bad-mediated apoptosis [26]. Cdc25 is sequestered in the cytoplasm during interphase by interaction with 14-3-3 thereby preventing cell cycle entry [27]. Similarly, 14-3-3 binding is correlated with the cytoplasmic localization of cdc2 [30], histone deacetylase 4 and 5 [31], and NFAT [32]. Cytoplasmic sequestration is not the only mechanism. It has been demonstrated that binding to 14-3-3 is associated with the nuclear localization of telomerase [33]. Recently, it has been shown that the exit of membrane-targeted proteins from the ER, such as Iip35 and potassium channel protein KCNK3, requires 14-3-3 binding [34,35]. Our report reveals a novel activity of 14-3-3 in locating ASK1 to a perinuclear compartment. As we show here, ASK1 is normally localized in the cytoplasm, which appears to be required for its proapoptotic function. However, ASK1 is localized to the perinuclear rim/ER region upon co-expression with 14-3-3 isoforms, which correlates with a decrease in ASK1-induced apoptosis (Fig. 2). It is likely that its perinuclear localization may hamper the ability of ASK1 to transmit stress signals.

The exact intracellular organelle to which ASK1 is relocated is unknown. Our results suggest that ASK1 is unlikely relocated to the Golgi or endosomes by 14-3-3. Using the EGFP or EYFP Living Colors organelle-targeted expression vectors, we found that upon 14-3-3 co-expression, ASK1 is colocalized with two ER-resident proteins, Grp78 (Fig. 5) and calnexin (Fig. 6), suggesting that ASK1 is likely relocated to the ER compartment in the perinuclear region. These results are intriguing because recent results suggest that ASK1 plays an essential role in mediating ER stress-induced apoptotic responses in neuronal cells [36]. However, in this case, accumulation of proteins in the ER activates the ER-resident transmembrane kinase, IRE1, which recruits TRAF2 and ASK1 to its cytoplasmic domain, leading to activation of JNK and subsequently stress-induced apoptosis. Our study suggests that the ASK1/14-3-3 interaction localizes ASK1 to the ER, which may remove ASK1 from the cytoplasmic milieu where it is required for interaction with and the subsequent activation of JNK. Indeed, overexpression of 14-3-3ζ inhibited ASK1-induced activation of JNK (Fig. 2C). In addition, it is unclear whether ASK1 retains its kinase activity after relocation to the ER with 14-3-3. It is possible that this ER localization occurs only when 14-3-3 proteins are overexpressed. On the other hand, there appears to be two potential ER localization signals in ASK1, K^{945}KTQ, and C^{1352}LRLRGG [37]. These signals may be regulated by phosphorylation of Ser-967 and the 14-3-3 binding. In support of our model, it is recently reported that upon co-expression of IGF1 receptor, ASK1 is redistributed to the perinuclear region in HEK293 cells [38]. Another possibility exists whereby 14-3-3/ASK1 interaction may enhance binding of reduced thioredoxin to ASK1, which is known to prevent ASK1-induced apoptosis [6]. Thioredoxin has recently been reported to enhance the ubiquitination and degradation of ASK1 [39]. It is possible that 14-3-3 may cooperate with thioredoxin in the regulation of ASK1.

How the ASK1/14-3-3 interaction is controlled remains unclear. However, TNFα is a known activator of ASK1. Our experiments reveal that TNFα interferes with the localization of ASK1 to the ER region in the presence of co-expressed 14-3-3. This finding suggests that the ASK1/14-3-3 interaction or the ASK1 localization may be controlled in part by the TNFα signaling pathways or a TNFα regulated gene product. ASK1 may be present in the ER region under physiological conditions in association with 14-3-3. Cellular stresses, such as TNFα, may activate a pathway to dissociate 14-3-3 from ASK1 and relocate ASK1 to the cytoplasm where it can activate JNK and prime the cell for apoptosis. Detailed studies are needed to test this hypothesis.

It appears that the ASK1 activity can be controlled by several mechanisms, (i) phosphorylation in the activation loop of the catalytic domain [9] as well as in the regulatory domain [10,11], (ii) homo-oligomerization of ASK1 [5,9], (iii) protein-protein interactions, such as TRAF2, thioredoxin, Hsp72, ALG2, and 14-3-3 proteins [6,11,12,14,40]. Interaction of ASK1 with these regulatory proteins can either directly alter the catalytic activity of ASK1 or indirectly impact ASK1 function through altering its stability, oligomerization status, or subcellular localization. For example, thioredoxin binding in endothelial cells induces ASK1 ubiquitination and degradation [39], while the reported ALG2 interaction leads to nuclear localization of ASK1 [40]. As we present here, ASK1 may be localized to a

perinuclear region through binding to 14-3-3 proteins. These complementary mechanisms together ensure the appropriate activation of ASK1 and efficient operation of ASK1-mediated signaling pathways.

It has been well established that 14-3-3 proteins utilize their conserved amphipathic groove for interaction with diverse ligands. Comparison of ASK1 with Raf-1 and Bad for 14-3-3 binding reveals interesting differences. Specifically, ASK1 appears to require Lys-60 of 14-3-3 for interaction while this residue is largely dispensable for either Raf-1 or Bad (Fig. 1). These data suggest that within the conserved amphipathic groove, there are some ligand-specific residues, which may allow a certain degree of ligand specificity for 14-3-3 interaction. Such information can be used to design ligand-specific binding mutants of 14-3-3 for future functional analyses. The R60E mutant may be used to preferentially disrupt 14-3-3/ASK1 interaction without a pleiotropic effect on most other 14-3-3 binding partners.

Taken together, our studies define a novel mechanism wherein 14-3-3 isoforms bind ASK1 and relocate ASK1 from the cytoplasm to the ER region thereby suppressing ASK1-mediated apoptosis. This model is not mutually exclusive with other mechanisms. For example, 14-3-3 binding may impact multiple ASK1 activities, including its catalytic activity (Goldman and Fu, unpublished). Through controlling both subcellular localization and the catalytic activity of ASK1, isoforms of 14-3-3 may provide a sensitive mechanism for the control of ASK1 function and ASK1-mediated apoptosis.

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References

[1] K. Takeda, A. Matsuzawa, H. Nishitoh, H. Ichijo, Roles of MAPKKK ASK1 in stress-induced cell death, Cell Struct. Funct. 28 (2003) 23–29.

[2] X.S. Wang, K. Diener, D. Jannuzzi, D. Trollinger, T.H. Tan, H. Lichenstein, M. Zukowski, Z. Yao, Molecular cloning and characterization of a novel protein kinase with a catalytic domain homologous to mitogen-activated protein kinase kinase kinase, J. Biol. Chem. 271 (1996) 31607–31611.

[3] H. Ichijo, E. Nishida, K. Irie, P. ten Dijke, M. Saitoh, T. Moriguchi, M. Takagi, K. Matsumoto, K. Miyazono, Y. Gotoh, Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways, Science 275 (1997) 90–94.

[4] T. Hatai, A. Matsuzawa, S. Inoshita, Y. Mochida, T. Kuroda, K. Sakamaki, K. Kuida, S. Yonehara, H. Ichijo, K. Takeda, Execution of apoptosis signal-regulating kinase 1 (ASK1)-induced apoptosis by the mitochondria-dependent caspase activation, J. Biol. Chem. 275 (2000) 26576–26581.

[5] Y. Gotoh, J.A. Cooper, Reactive oxygen species- and dimerization-induced activation of apoptosis signal-regulating kinase 1 in tumor necrosis factor-alpha signal transduction, J. Biol. Chem. 273 (1998) 17477–17482.

[6] M. Saitoh, H. Nishitoh, M. Fujii, K. Takeda, K. Tobiume, Y. Sawada, M. Kawabata, K. Miyazono, H. Ichijo, Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1, EMBO J. 17 (1998) 2596–2606.

[7] Z. Chen, H. Seimiya, M. Naito, T. Mashima, A. Kizaki, S. Dan, M. Imaizumi, H. Ichijo, K. Miyazono, T. Tsuruo, ASK1 mediates apoptotic cell death induced by genotoxic stress, Oncogene 18 (1999) 173–180.

[8] K. Tobiume, A. Matsuzawa, T. Takahashi, H. Nishitoh, K. Morita, K. Takeda, O. Minowa, K. Miyazono, T. Noda, H. Ichijo, ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis, EMBO Rep. 2 (2001) 222–228.

[9] K. Tobiume, M. Saitoh, H. Ichijo, Activation of apoptosis signal-regulating kinase 1 by the stress-induced activating phosphorylation of pre-formed oligomer, J. Cell. Physiol. 191 (2002) 95–104.

[10] A.H. Kim, G. Khursigara, X. Sun, T.F. Franke, M.V. Chao, Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1, Mol. Cell. Biol. 21 (2001) 893–901.

[11] L. Zhang, J. Chen, H. Fu, Suppression of apoptosis signal-regulating kinase 1-induced cell death by 14-3-3 proteins, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 8511–8515.

[12] H. Nishitoh, M. Saitoh, Y. Mochida, K. Takeda, H. Nakano, M. Rothe, K. Miyazono, H. Ichijo, ASK1 is essential for JNK/SAPK activation by TRAF2, Mol. Cell 2 (1998) 389–395.

[13] J.J. Song, J.G. Rhee, M. Suntharalingam, S.A. Walsh, D.R. Spitz, Y.J. Lee, Role of glutaredoxin in metabolic oxidative stress. Glutaredoxin as a sensor of oxidative stress mediated by H_2O_2, J. Biol. Chem. 277 (2002) 46566–46575.

[14] H.S. Park, S.G. Cho, C.K. Kim, H.S. Hwang, K.T. Noh, M.S. Kim, S.H. Huh, M.J. Kim, K. Ryoo, E.K. Kim, W.J. Kang, J.S. Lee, J.S. Seo, Y.G. Ko, S. Kim, E.J. Choi, Heat shock protein hsp72 is a negative regulator of apoptosis signal-regulating kinase 1, Mol. Cell. Biol. 22 (2002) 7721–7730.

[15] H. Fu, R.R. Subramanian, S.C. Masters, 14-3-3 proteins: structure, function, and regulation, Annu. Rev. Pharmacol. Toxicol. 40 (2000) 617–647.

[16] A. Aitken, Functional specificity in 14-3-3 isoform interactions through dimer formation and phosphorylation. Chromosome location of mammalian isoforms and variants, Plant Mol. Biol. 50 (2002) 993–1010.

[17] A.J. Muslin, J.W. Tanner, P.M. Allen, A.S. Shaw, Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine, Cell 84 (1996) 889–897.

[18] M.B. Yaffe, K. Rittinger, S. Volinia, P.R. Caron, A. Aitken, H. Leffers, S.J. Gamblin, S.J. Smerdon, L.C. Cantley, The structural basis for 14-3-3:phosphopeptide binding specificity, Cell 91 (1997) 961–971.

[19] A.J. Muslin, H. Xing, 14-3-3 proteins: regulation of subcellular localization by molecular interference, Cell Signal 12 (2000) 703–709.

[20] H. Xing, S. Zhang, C. Weinheimer, A. Kovacs, A.J. Muslin, 14-3-3 proteins block apoptosis and differentially regulate MAPK cascades, EMBO J. 19 (2000) 349–358.

[21] R.R. Subramanian, S.C. Masters, H. Zhang, H. Fu, Functional conservation of 14-3-3 isoforms in inhibiting bad-induced apoptosis, Exp. Cell Res. 271 (2001) 142–151.

[22] L. Zhang, H. Wang, D. Liu, R. Liddington, H. Fu, Raf-1 kinase and exoenzyme S interact with 14-3-3zeta through a common site involving lysine, J. Biol. Chem. 272 (1997) 13717–13724.

[23] H. Wang, L. Zhang, R. Liddington, H. Fu, Mutations in the hydrophobic surface of an amphipathic groove of 14-3-3zeta disrupt its interaction with Raf-1 kinase, J. Biol. Chem. 273 (1998) 16297–16304.

[24] J. Gyuris, E. Golemis, H. Chertkov, R. Brent, Cdi1, a human G1 and

S phase protein phosphatase that associates with Cdk2, Cell 75 (1993) 791–803.
[25] J. Chen, K. Fujii, L. Zhang, T. Roberts, H. Fu, Raf-1 promotes cell survival by antagonizing apoptosis signal-regulating kinase 1 tough a MEK-ERK independent mechanism, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 7783–7788.
[26] J. Zha, H. Harada, E. Yang, J. Jockel, S.J. Korsmeyer, Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L), Cell 87 (1996) 619–628.
[27] C.Y. Peng, P.R. Graves, R.S. Thoma, Z. Wu, A.S. Shaw, H. Piwnica-Worms, Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216, Science 277 (1997) 1501–1505.
[28] S. Basu, N.F. Totty, M.S. Irwin, M. Sudol, J. Downward, Akt phosphorylates the Yes-associated protein, YAP, to induce interaction with 14-3-3 and attenuation of p73-mediated apoptosis, Mol. Cell 11 (2003) 11–23.
[29] A.L. Eilers, E. Sundwall, M. Lin, A.A. Sullivan, D.E. Ayer, A novel heterodimerization domain, CRM1, and 14-3-3 control subcellular localization of the MondoA-Mlx heterocomplex, Mol. Cell. Biol. 22 (2002) 8514–8526.
[30] T.A. Chan, H. Hermeking, C. Lengauer, K.W. Kinzler, B. Vogelstein, 14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage, Nature 401 (1999) 616–620.
[31] C.M. Grozinger, S.L. Schreiber, Regulation of histone deacetylase 4 and 5 and transcriptional activity by 14-3-3-dependent cellular localization, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 7835–7840.
[32] C.W. Chow, R.J. Davis, Integration of calcium and cyclic AMP signaling pathways by 14-3-3, Mol. Cell. Biol. 20 (2000) 702–712.
[33] H. Seimiya, H. Sawada, Y. Muramatsu, M. Shimizu, K. Ohko, K. Yamane, T. Tsuruo, Involvement of 14-3-3 proteins in nuclear localization of telomerase, EMBO J. 19 (2000) 2652–2661.
[34] T. Kuwana, P.A. Peterson, L. Karlsson, Exit of major histocompatibility complex class II-invariant chain p35 complexes from the endoplasmic reticulum is modulated by phosphorylation, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 1056–1061.
[35] I. O'Kelly, M.H. Butler, N. Zilberberg, S.A. Goldstein, Forward transport. 14-3-3 binding overcomes retention in endoplasmic reticulum by dibasic signals, Cell 111 (2002) 577–588.
[36] H. Nishitoh, A. Matsuzawa, K. Tobiume, K. Saegusa, K. Takeda, K. Inoue, S. Hori, A. Kakizuka, H. Ichijo, ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats, Genes Dev. 16 (2002) 1345–1355.
[37] N. Zerangue, M.J. Malan, S.R. Fried, P.F. Dazin, Y.N. Jan, L.Y. Jan, B. Schwappach, Analysis of endoplasmic reticulum trafficking signals by combinatorial screening in mammalian cells, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 2431–2436.
[38] V. Galvan, A. Logvinova, S. Sperandio, H. Ichijo, D.E. Bredesen, Type 1 insulin-like growth factor receptor (IGF-IR) signaling inhibits apoptosis signal-regulating kinase 1 (ASK1), J. Biol. Chem. 278 (2003) 13325–13332.
[39] Y. Liu, W. Min, Thioredoxin promotes ASK1 ubiquitination and degradation to inhibit ASK1-mediated apoptosis in a redox activity-independent manner, Circ. Res. 90 (2002) 1259–1266.
[40] I.S. Hwang, Y.S. Jung, E. Kim, Interaction of ALG-2 with ASK1 influences ASK1 localization and subsequent JNK activation, FEBS Lett. 529 (2002) 183–187.

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Identification of novel direct transcriptional targets of glucocorticoid receptor

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Transcription of the genes *Granzyme A* (*GZMA*), *FK506 binding protein 51* (*FKBP5*), and *Down syndrome critical region gene 1* (*DSCR1*) is upregulated in leukemic cells upon treatment with glucocorticoids (GCs). Several lines of evidence suggest that these genes are implicated in GC-induced apoptosis upstream of the Bcl-2 family of proteins. These genes were upregulated by GC even in the presence of an inhibitor of protein synthesis, cycloheximide, indicating that they are direct target genes of glucocorticoid receptors. *DSCR1* is reported to have four isoforms, each of which has a distinct first exon, E1–E4. Among these isoforms, the one with E1 was selectively upregulated by GC. *GZMA* and *FKBP5* have a cluster of putative glucocorticoid response elements (GREs) in introns 1 and 2, respectively, that was identified to be responsible for the response to GC. They were composed of one complete (A/T)G(A/T)(A/T)C(A/T) sequence surrounded by two incomplete (A/T)G(A/T)(A/T)C(A/T) sequences separated by one to four nucleotides. *DSCR1*, however, did not have a functional GRE upstream or downstream of exon 1. These studies may lead to improved therapeutic uses of GCs in leukemia and lymphoma based upon the expression of these GC target genes.
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Introduction

Glucocorticoids (GCs) are known to be potent immunosuppressive, antiallergic, and anti-inflammatory drugs. They exert their effects on target cells by binding to an intracellular glucocorticoid receptor (GR). The ability of GCs to induce apoptosis and cell cycle arrest in lymphoid cells has resulted in their widespread use as chemotherapeutic agents for various leukemias, lymphomas, and multiple myelomas, although the precise mechanism of their actions is yet to be elucidated.[1,2] Glucocorticoid receptor (GR) is a member of the nuclear hormone receptor superfamily localized in the cytoplasm. Inactive GR is bound to a large protein complex that includes heat shock protein 90 (Hsp90). When GC binds to GR, Hsp90 dissociates and the GC/GR complex translocates to the nucleus where it binds to specific palindromic sequences, termed glucocorticoid response elements (GREs), resulting in the transcriptional upregulation of various genes.[3]

We previously investigated transcriptional changes during GC-induced apoptosis in GC-sensitive human pre-B leukemia 697 cells harboring the t(1;19) chromosomal translocation[4] using oligonucleotide microarrays.[5] Among 93 genes induced by a synthetic GC, dexamethasone (DEX), were *Granzyme A* (*GZMA*), and two other genes encoding calcineurin inhibitors, *FK506 binding protein 51* (*FKBP5*) and *Down syndrome critical region gene 1* (*DSCR1*). Granzymes are serine proteases and are packaged in cytotoxic granules of CTL and NK cells, together with the pore-forming protein perforin. The concerted action of these molecules induces apoptosis of target cells, such as infected cells or transformed tumor cells.[6,7] *DSCR1*, a gene located on chromosome 21, is highly expressed in fetal brain and is suggested to have a role in brain development. The product of *DSCR1* interacts with the catalytic A subunit of calcineurin and inhibits its phosphatase activity, and thus is also called modulatory calcineurin-interacting protein 1 (MCIP1).[8] *FKBP5* encodes FK506 binding protein of 51 kDa, which is known as an immunophillin, and also regulates the inhibition of calcineurin.[9,10] These three genes are suggested to mediate GC-induced apoptosis in lymphoid cells by the following findings. First, the granzyme inhibitor 3,4-dichloroisocoumarin has been reported to inhibit DEX-induced apoptosis of 697 cells.[11] Second, the activation of calcineurin protects T cells from GC-induced apoptosis.[12,13] Moreover, using a variety of pre-B leukemic cells, the expression of the latter two genes evoked by GC and the induction of apoptosis were found to be closely correlated.[5] However, the induction of these genes does not necessarily mean that they are direct transcriptional targets, because they may be regulated via a secondary effect induced by the primary targets of GC. In addition, GR regulates transcription by binding to and inhibiting or enhancing the function of other transcription factors such as AP-1, NF-κB, and the STAT family of transcription factors, which can change the transcriptional profile.[14,15] Therefore, we investigated the genomic organization of these genes and whether they are direct target genes of GR.

Materials and methods

Cells and reagents

Pre-B human leukemia 697 cells were routinely grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 50 U/ml of penicillin, and 0.1 mg/ml of streptomycin. HeLa and COS-7 cells were maintained in DMEM with the same supplements. DEX and cycloheximide (CHX) were purchased from Sigma.

Plasmid construction

To generate pGRE-Luc, the oligonucleotides 5′-TCGATCAGAA CACTGTGTTCTGA-3′ and 5′-TCGATCAGAACACAGTGTTCT GA-3′ were annealed and subcloned into the *Xho*I site of pGV-P2 (Wako Chemicals, Osaka, Japan). Genomic sequences corresponding to human *GZMA*, *FKBP5*, and *DSCR1* were amplified by PCR with appropriate primers using the Expand

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High Fidelity PCR system (Roche Diagnostics) and subcloned into a luciferase vector, pGV-B2 or pGV-P2 (Wako Chemicals). Reporter constructs with mutated sequences were generated by the PCR-based method described previously.[16] Detailed information on the primers used for the PCR is available at Leukemia's website. All the constructs were verified by DNA sequencing.

Analysis of gene expression by RT-PCR

To analyze the isoform-specific regulation of *DSCR1*, 500 ng of total RNA extracted from 697 cells was reverse-transcribed and cDNA was amplified by PCR using a forward primer specific to *DSCR1* exon 1 or 4, and a reverse primer for exon 5. Logarithmically amplifying PCR product was subjected to agarose gel electrophoresis. For real-time RT-PCR, one-step RT-PCR was performed using a 7700 ABI PRISM Sequence Detector System (Perkin Elmer-Applied Biosystems). Fluorogenic probes carrying 5′ 6-carboxy-fluorescein as a reporter dye and 3′ 6-carboxy-tetramethyl-rhodamine as a quencher dye were used to detect the PCR product. In every experiment, the *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene was amplified using a series of dilutions of a known amount of the standard RNA supplied by Perkin Elmer to prepare a standard curve. Data analysis was performed as described[17] with slight modifications. Detailed information on the primers used for the PCR is available at Leukemia's website.

Western blot analysis

Western blot analysis was performed as described previously[5] using goat anti-FKBP51 polyclonal antibody (F-13, Santa Cruz) followed by horseradish peroxidase-conjugated donkey anti-goat immunoglobulins (Santa Cruz). The proteins were visualized using an enhanced chemiluminescence method (Amersham).

Transfection and luciferase assay

HeLa cells growing in six-well plates were transfected with 750 ng of the reporter gene plasmid along with 500 ng of pCMVβGal and 750 ng of an expression plasmid for rat GR, p6RGR[18] (a gift from Keith Yamamoto), using Effectene reagent (Qiagen). The medium was replaced with DMEM without phenol red (Invitrogen) with 10% charcoal-treated fetal calf serum just before the lipofection. The cells were harvested at 16 h after transfection and used for a luciferase assay. Luciferase activities were measured as described previously and normalized for transfection efficiency based on β-galactosidase activities.[19]

Electrophoretic mobility shift assay

COS-7 cells were transfected with p6RGR and 10^{-6} M of DEX was added. Nuclear extract was prepared 48 h after transfection by three cycles of freezing and thawing in cell resuspension buffer (40 mM HEPES-KOH, pH 7.9, 0.4 M KCl, 1 mM dithiothreitol, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1% aprotinin) and incubated with an end-labeled double-stranded oligonucleotide probe: FKBP5 GRE2 5′-AGTAACA CAATGTACAGGTTTGTAGCATTG-3′; GZMA GRE3 5′-TGG GAGAATCCAAGAACATCTGGTGCAGGA-3′; GZMA GRE4 5′-TGTGTTTAGTTCTCACTGTTCC-3′. The reaction was performed in 15 μl of binding buffer (4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 0.05 mg/ml poly(dI-dC)) for 20 min at room temperature. The supershift analysis was performed by including anti-GR polyclonal antibody (Affinity Bioreagents) in binding reactions. Samples were fractioned on a nondenaturing 6% polyacrylamide gel and visualized by autoradiography.

Results

Three candidate target genes are upregulated by GC in the absence of de novo *protein synthesis*

We and others have identified *GZMA*, *FKBP5*, and *DSCR1* as GC-responsive genes using microarray technology.[5,20–23] However, it is likely that some of the GC-induced genes identified are regulated because of secondary effects induced by the primary targets of GC. To rule out this possibility, we analyzed the effect of GC in the presence and absence of an inhibitor of protein synthesis, CHX. Evidently, protein synthesis was indeed shut down by CHX as the level of FKBP5 protein declined in cells growing in the presence of CHX even after DEX treatment, in contrast to the experiment without CHX (Figure 1a). Hence, direct target genes of GC will be transcriptionally activated, but because of the inhibition of protein synthesis, the GC-induced proteins will not be synthesized and will not induce secondary

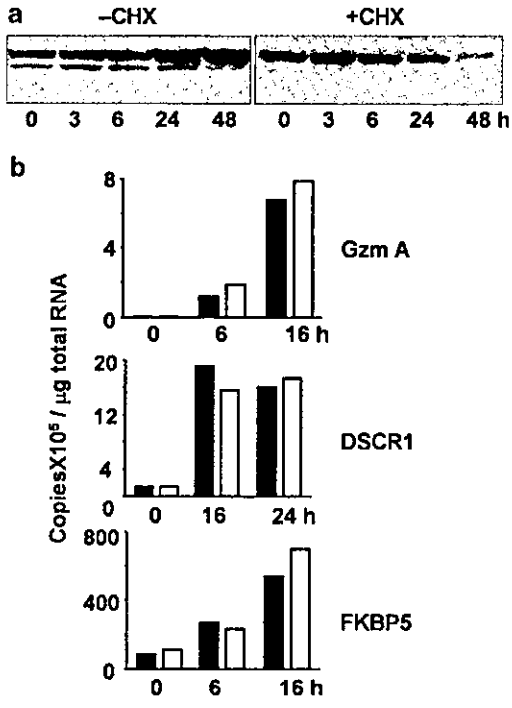

Figure 1 Three genes are directly upregulated by DEX. (a) Cell lysates were obtained from 697 cells treated with 10^{-6} M of DEX for the periods indicated and subjected to Western blotting to detect FKBP5. In some experiments (indicated by +CHX), 10 μg/ml of CHX was added at 16 h prior to DEX treatment. (b) Transactivation of the three genes by DEX is CHX-insensitive. 697 cells growing in the presence (open columns) or absence (closed columns) of CHX for 16 h were treated with DEX for the periods indicated and then total RNA was extracted. cDNA encoding the three genes was amplified by RT-PCR and analyzed with a 7700 ABI PRISM Sequence Detector System. The expression of the genes normalized to that of *GAPDH* is shown. The analysis was performed in triplicate.

or indirect targets in this experimental system. As shown in Figure 1b, these three genes were induced significantly irrespective of the presence of CHX as assessed by real-time RT-PCR, suggesting that their expression was induced directly by GC without the need for protein synthesis.

The exon 1 variant of DSCR1 *is responsive to GC*

The human *DSCR1* gene was reported to express four variant mRNAs with each of four alternative exons (exons 1–4) incorporated selectively at the 5′ terminus of the expressed transcript.[8] The majority of these transcripts were identified to represent isoforms that include exon 1 or 4 (Figure 2a) and the other two were not detectable in human fetal and adult tissues by Northern analysis.[8] Exon 1 and 4 isoforms use distinct promoters and their transcription is differentially regulated. For example, expression of the exon 4 isoform, but not the exon 1 isoform, is selectively increased by calcineurin activity, creating a negative feedback circuit.[24] To address the issue of which variant is responsible for the upregulation of *DSCR1* by GC, we constructed RT-PCR primers that specifically recognize these isoforms (Figure 2a). As shown in Figure 2b, transcription of the exon 1 isoform (Exon1-5) is upregulated by DEX showing a similar time course as total *DSCR1* expression (Exon7-7). However, the exon 4 isoform (Exon4-5) was barely detectable in 697 cells and its expression level was not increased after the treatment with DEX. The exon 4 isoform was detected in the heart and kidney in which this variant was reported to be significantly expressed,[8] demonstrating that these primers were capable of efficiently recognizing the exon 4 isoform (Figure 2c). Collectively, GCs were demonstrated to upregulate the exon 1 isoform of *DSCR1*. Since *DSCR1* spliced from exons 1 and 4 would generate DSCR1 proteins with distinct N-terminus, it would be interesting to see if there is a difference in calcineurin inhibition between these two protein variants.

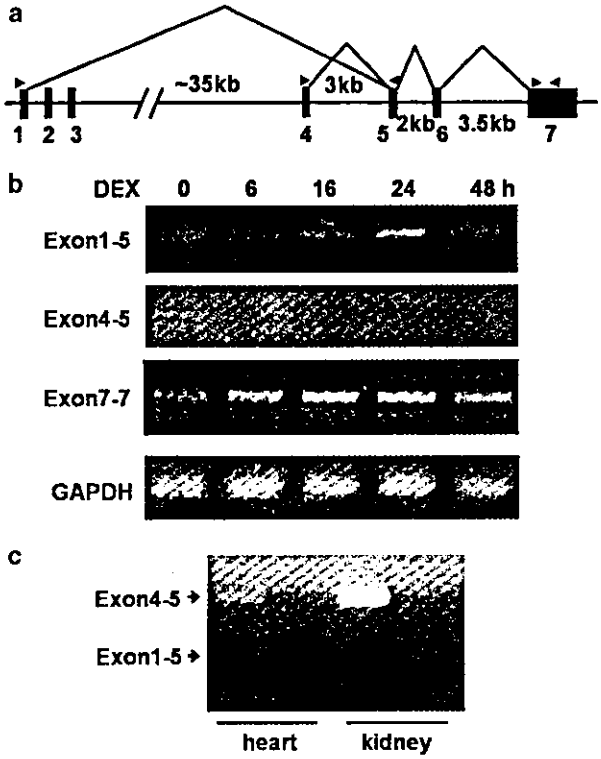

Figure 2 Variant-specific transcriptional regulation of DSCR1. (a) Genomic organization of the human *DSCR1* gene, indicating four alternative initial exons (1–4) and three exons common to all forms of *DSCR1* mRNA (5–7). Locations of two forward primers in initial exons and a reverse primer in exon 5, as well as a pair of primers in exon 7 to detect the total amount of *DSCR1* mRNA are indicated by arrowheads. (b) Semiquantitative RT-PCR analysis to detect *DSCR1*. 697 cells were treated with 10^{-6} M of DEX for the time periods indicated and total RNA was extracted for RT-PCR. Locations of the primers used for RT-PCR are indicated on the left. The *GAPDH* gene was amplified as an internal control. (c) RT-PCR analysis using adult human tissues. RT-PCR was performed similarly except that RNAs from human tissues were used as templates.

Intronic regions of GZMA *and* FKBP5 *are required for a transcriptional response to GC*

Having shown that the expression of the three genes was induced directly by GC, we amplified the promoter and intron fragments from human genomic DNA by PCR and subcloned them into a luciferase vector in order to further substantiate the role of the transcriptional activation of these genes. Despite extensive analysis of the reporter gene, no sequence responsive to GC was identified in the region around exon 1 for *DSCR1* (at least not up to ~2.2 kb and down to ~2.0 kb from exon 1), although a couple of candidate GREs were found in this region (data not shown). These results suggest that the functional GRE is located at a position distant from exon 1 or the transactivation of *DSCR1* is mediated by a nonclassical mechanism.

The *GZMA* promoter was also unresponsive to GC at least up to ~2.3 kb from exon 1 (Figure 3a, pGV-GzmPro). However, when an ~2.7 kb fragment of intron 1 was subcloned into a reporter vector, it was significantly upregulated by DEX (pGV-GzmInt). The degree of induction was more than two-fold that with pGV-GRE in which the consensus 1 × GRE (5′-AGAA CACTGTGTT-3′) was subcloned. To narrow down the region reactive to GC, the 2.7 kb segment was divided into three and various deletion mutants were tested for the response to DEX. As shown in Figure 3b, the response to DEX was completely eliminated when the middle segment of ~1 kb was deleted from the construct (pGV-GzmIntA and pGV-GzmIntC) indicating the presence of a GC-responsive element in this fragment. It was also suggested that the downstream sequence in intron 1 has an inhibitory effect on GC-induced transactivation since the constructs lacking this segment showed significantly higher induction (pGV-GzmIntAB and pGV-GzmIntB). In fact, inspection of the DNA sequence in this middle segment revealed the presence of four candidate GREs that partially match the previously reported consensus sequence (numbered GRE1 to GRE4 as shown in Figure 3c). We therefore introduced a series of nucleotide substitutions at these sites to map the actual GREs. When either GRE3 or GRE4 was mutated, DEX-mediated transactivation was markedly compromised down to less than 10-fold (pGV-GzmIntB3, pGV-GzmIntB4). In addition, their combined mutations virtually eliminated DEX-mediated transactivation (pGV-GzmIntB234, pGV-GzmIntB1234). In contrast, reporter constructs with a mutation of either GRE1 or GRE2 still showed more than 20-fold activation by DEX (pGV-GzmIntB1, pGV-GzmIntB2). These results imply that GRE3 and GRE4 are functional GREs, although GRE1 and GRE2 may have an auxiliary role.

The *FKBP5* gene was similarly examined for the presence of a functional GRE. Again, the promoter sequence of ~2 kb did not respond to the treatment with DEX (Figure 4a, pGV-FKBPPro).

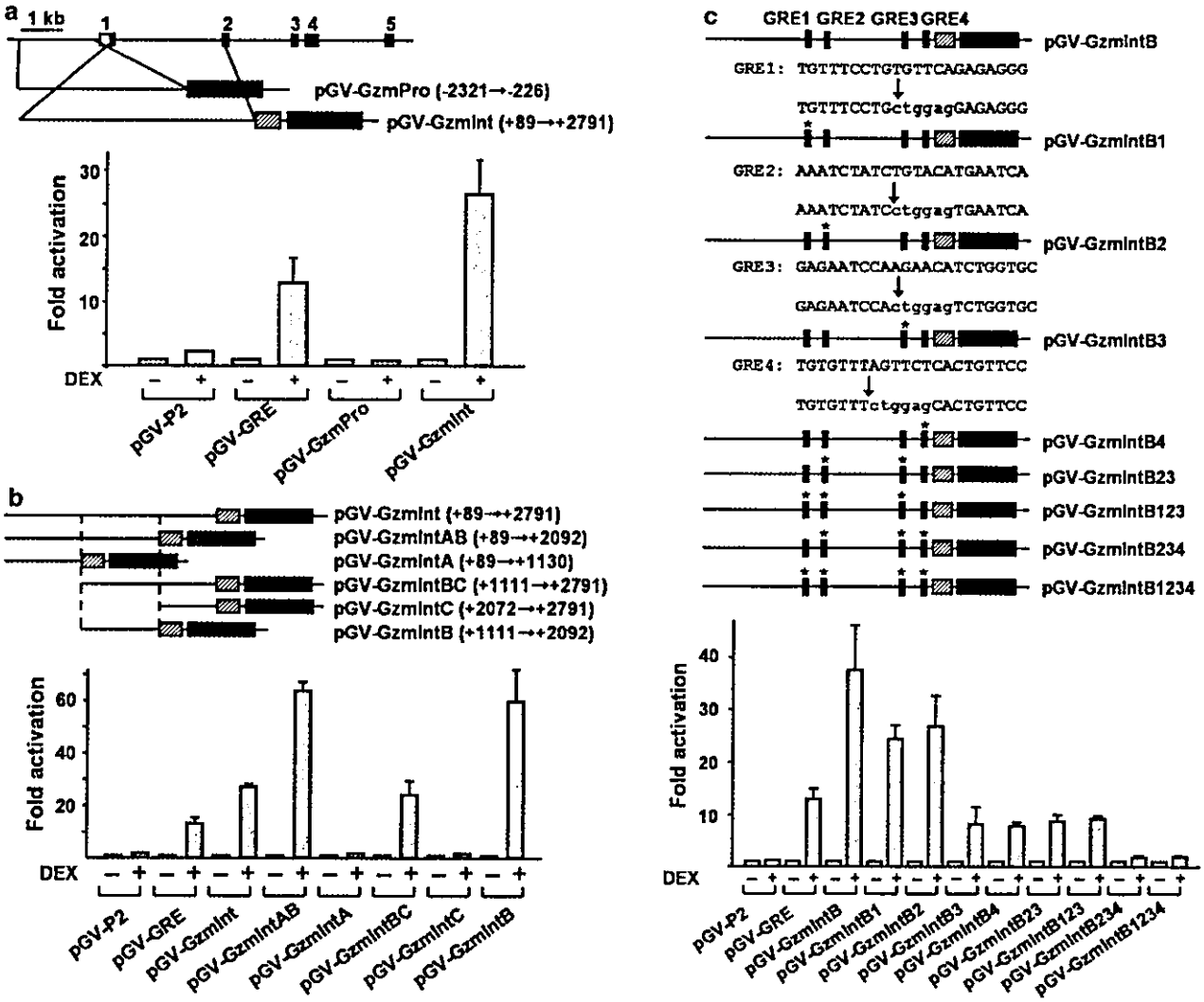

Figure 3 Regulation of the *GZMA* intron by GC. (a) The genomic organization of the human *GZMA* gene and reporter gene constructs used for luciferase assays are schematically depicted at the top. Open and filled boxes indicate noncoding and coding exons, respectively. Striped and gray boxes indicate DNA sequences encoding the SV40 promoter and luciferase, respectively. Numbers in parentheses denote nucleotide positions relative to the translational initiation site based on the genomic sequence, AC091977. Results of transient transfection assays of GZMA-luciferase reporter plasmids are shown in the bottom panel. HeLa cells were transfected with the indicated reporter gene plasmid along with pCMVβGal and p6RGR. The medium was replaced with DMEM containing 10% charcoal-treated fetal calf serum without phenol red (Invitrogen) just before the lipofection. Cells grown in the presence or absence of 10^{-6} M DEX were harvested at 16 h after transfection and used for a luciferase assay. Luciferase activities normalized for β-galactosidase activities were expressed as fold activation. Data are representative of three experiments with similar results. (b) The middle part of intron 1 is GC-responsive. To narrow down the region reactive to DEX, intron 1 was divided into three and subcloned into the reporter vector as depicted in the upper panel. Luciferase assays were performed and expressed as described in (a). (c) Identification of GRE in intron 1. Candidate GRE sequences were mutated as indicated by a PCR-based method. Lowercase letters indicate mutated nucleotides. Thick vertical bars represent the location of candidate GREs. Mutated GREs are marked by asterisks. Luciferase assays were performed and the results were expressed as described in (a).

Since introns 1 and 2 are ~46 and ~5.5 kb long, respectively, we inspected the vicinity of exons 1 and 2 for the presence of possible GREs. Four candidate GREs found in this region were subcloned into a luciferase vector and subjected to reporter gene analyses (Figure 4a). The luciferase constructs containing two candidate GREs in intron 2 (pGV-FKBPIntC) significantly responded to DEX to the same degree as pGV-GRE, whereas the constructs containing the other two candidate GREs in intron 1 were unresponsive to DEX (pGV-FKBPIntA and pGV-FKBPIntB). When the downstream GRE of pGV-FKBPIntC, but not the upstream one, was mutated, the response was virtually eliminated, suggesting that the downstream GRE is functional (Figure 4b, pGV-FKBPIntCmt2).

GR can bind in vitro *to an oligonucleotide probe representing the* GZMA *and* FKBP5 *gene region*

To determine whether the GR protein can bind to the sequences corresponding to the GREs in *GZMA* and *FKBP5* that proved to be functional in reporter gene assays, electrophoretic mobility shift assays were performed. As shown in Figure 5a, when nuclear extracts were obtained from COS-7 cells transiently transfected with expression plasmid for GR and incubated with a radiolabeled DNA probe containing a GRE sequence in the intronic region, a complex with a shift in gel mobility was detected (lanes 1, 4, and 7). When anti-GR antibody was included in binding reactions, the bands detected in the lanes

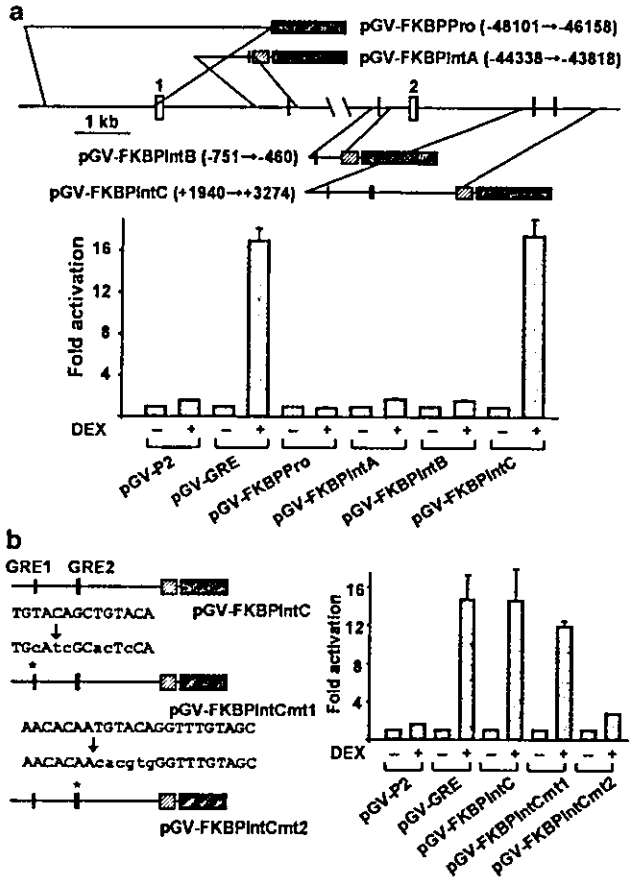

Figure 4 Regulation of the *FKBP5* intron by GC. (a) The genomic organization of the human *FKBP5* gene and reporter gene constructs used for luciferase assays are schematically depicted at the top. Open and filled boxes indicate a noncoding and a coding exon, respectively. Exons 1 and 2 are separated by a sequence ~46 kb long. Numbers in parentheses denote nucleotide positions relative to the translational initiation site in exon 2 based on the genomic sequence, AL033519 and AL590400. Thick vertical bars represent the location of candidate GREs. Luciferase assays were performed and the results were expressed as described in Figure 3. (b) Identification of GRE in intron 2. Candidate GRE sequences were mutated as indicated by a PCR-based method. Lowercase letters indicate mutated nucleotides. Mutated GREs are marked by asterisks. Luciferase assays were performed and the results were expressed as described in Figure 3.

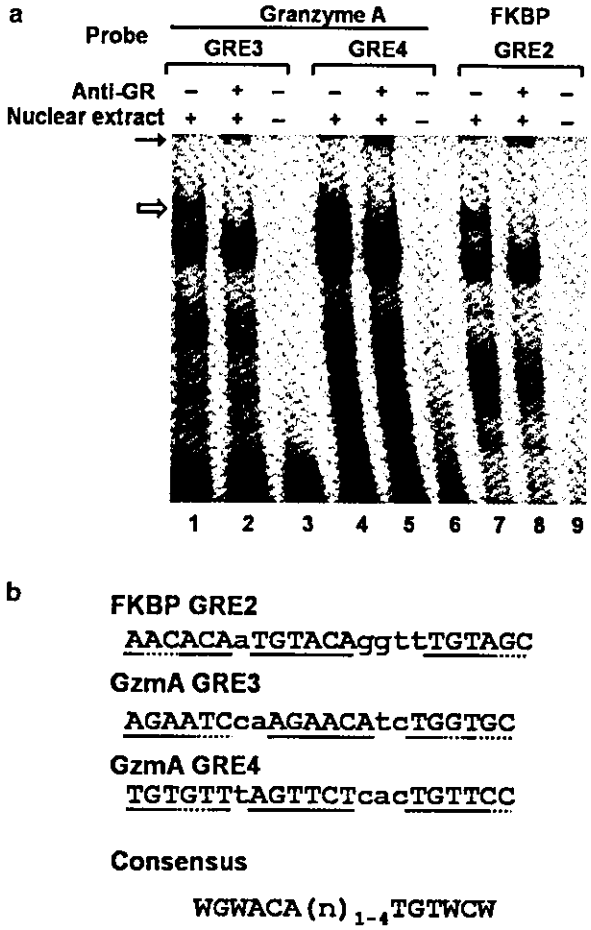

Figure 5 Electrophoretic mobility shift assays and alignment of GC-reactive sequences. (a) GR can bind *in vitro* to an oligonucleotide probe representing the *GZMA* and *FKBP5* gene region. Nuclear extracts were obtained from COS-7 cells transiently transfected with expression plasmid for GR and incubated with ^{32}P-labeled oligonucleotide DNA probes containing putative GRE sequences with or without polyclonal antibody against GR as indicated. DNA–protein complexes were size-fractionated in a nondenaturing polyacrylamide gel and detected by autoradiography. The shifted and supershifted complexes are indicated by open and closed arrows, respectively. (b) DNA sequences demonstrated to be reactive to GC are aligned. The consensus motif deduced from the alignment is indicated at the bottom. Nucleotides that fit and do not fit into the consensus motif are indicated by solid and broken underlines, respectively. Lowercase letters indicate nucleotides that separate 5-bp consensus sequences. W indicates the nucleotide A or T.

mentioned above were replaced by supershifted bands (lanes 2, 5, and 8), demonstrating the specificity of the complex formation. These results indicate that GR binds to the GRE sequences in the *GZMA* and *FKBP5* genes *in vitro*, which were identified by reporter gene assays.

Discussion

Although a number of reports have been published on the genome-wide screening of GC-induced genes,[5,20–23,25] to our knowledge, no reports have dealt with the molecular mechanism of how screened genes are transactivated by GC. Among the genes screened in our previous study, we focused on *GZMA*, *DSCR1*, and *FKBP5*, which have reportedly been relevant to GC-induced apoptosis, and found that all three are direct targets of GC. In the *GZMA* and *FKBP5* genes, GC-responsive intronic sequences to which GR can bind *in vitro* have been determined. None of the promoter sequence we analyzed responded to GC. However, this is not unexpected because a number of transcription factors, for example p53, transactivate target genes by binding to intronic sequences.[26–28] Recently, it was reported that ~400 bp of promoter sequence of human *FKBP5* exhibited upregulation by progestin in human breast cancer cells.[29] In contrast, our reporter construct, which contained this region, was unresponsive to GC, although the receptors for GC and progestin are known to recognize a sequence that is similar, if not the same.[30] Given their findings that no classical progestin-responsive element was identified in this region and the activity of luciferase was stimulated only 2.5-fold by a progesterone analog, much weaker than >10-fold effect observed in our study, it is speculated that progestin mainly regulates the

transcription of *FKBP5* through the intronic sequence we identified rather than a promoter sequence.

The DNA sequences that were responsive to DEX in our study were aligned in Figure 5b. The recognition sequence of GR is defined as a 15-bp motif with partial dyad symmetry such as 5′-GGTACAnnnTGTTCT-3′.[31] However, a mutagenesis study indicated that some sequences that do not perfectly fit into the consensus sequence are still GC-responsive.[30] Based on our results, three repeats of 5′-WGWWCW-3′ separated by 1–4 bp are responsive to GC in which the second motif needs to have a perfect match. However, the presence of this sequence alone does not result in a functional GRE. Indeed, the sequence in *GZMA* that fulfills this consensus sequence, for example GRE2 in Figure 3c, was not functional, because disruption of this candidate GRE had little effect on the response to GC. Therefore, the induction potential of a given site is speculated to be a complex function in the context of the higher gene structure.

The molecular mechanism of GC-induced apoptosis is not fully understood. In the first place, it is necessary for GR to function as a transactivator in order to induce apoptosis, because mutant GR incapable of binding DNA fails to induce thymocyte apoptosis[32] and inhibition of *de novo* protein synthesis by CHX virtually eliminated DEX-induced apoptosis in 697 cells (data not shown). In addition to the genes investigated in this study, the *Bim* gene (*BCL2L11*) has recently been reported to be induced by GC in murine lymphoma cell lines.[25] Bim protein is a BH3-only member of the Bcl-2 family that is capable of directly activating the apoptotic cascade.[33] It is therefore an attractive candidate for a GC target gene that mediates apoptosis. However, at least in our microarray analysis, *Bim* expression was not induced at any time points examined after DEX treatment, although its expression at low levels was observed (data not shown). Thus, we conclude that *Bim* is unlikely a target of GC that generally mediates GC-induced apoptosis. Nevertheless, the levels of *Bim* expression may determine the sensitivity of leukemic cells to GC, since GC-induced apoptosis is partially impaired in hematopoietic cells from $Bim^{-/-}$ mice.[34]

Is one of the genes investigated here solely responsible for GC-induced cell death? We established a lentivirus-mediated small interference RNA delivery system to selectively knock down *FKBP5* or *DSCR1*. The knocking-down of a single gene, however, was not sufficient to significantly inhibit GC-induced apoptosis, suggesting that multiple GR-induced genes activate a network of pathways that contribute to apoptosis (data not shown). This is reminiscent of p53-induced apoptosis. More than 16 target genes of p53 have been proposed to mediate apoptosis, but it is still unclear whether any single target gene is critical. Several lines of evidence suggest that the signaling pathway of p53-induced apoptosis is cell type-dependent and two or more genes cooperate to induce cell death in certain situations.[35,36] In this regard, it should be noted that the *granzyme K* gene, *GZMK*, is also significantly upregulated by GC (data not shown). *GZMK* encodes a serine protease that has features common to granzyme A, and the two genes are only ~70 kb apart on human chromosome 5. Thus, *GZMK* may be another target of GC that mediates apoptosis. Generating mice doubly deficient in GC target genes would help to clarify the signal transduction of GC-induced apoptosis. Expression profiling of the genes described in this study will contribute to predictions of responsiveness or resistance to GC therapy. Further study of GC target genes will provide the rationale for the optimal use of GC to improve treatment outcome in leukemia.

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Supplementary Information

Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu).

References

1 Distelhorst CW. Recent insights into the mechanism of glucocorticosteroid-induced apoptosis. *Cell Death Differ* 2002; **9**: 6–19.
2 Tissing WJ, Meijerink JP, den Boer ML, Pieters R. Molecular determinants of glucocorticoid sensitivity and resistance in acute lymphoblastic leukemia. *Leukemia* 2003; **17**: 17–25.
3 Riccardi C, Cifone MG, Migliorati G. Glucocorticoid hormone-induced modulation of gene expression and regulation of T-cell death: role of GITR and GILZ, two dexamethasone-induced genes. *Cell Death Differ* 1999; **6**: 1182–1189.
4 Barker PE, Carroll AJ, Cooper MD. t(1;19)(q23;p13) in pre-B acute lymphocytic leukemia cell line 697. *Cancer Genet Cytogenet* 1987; **25**: 379–380.
5 Yoshida N-L, Miyashita T, U M, Yamada M, Reed JC, Sugita Y *et al.* Analysis of gene expression patterns during glucocorticoid-induced apoptosis using oligonucleotide arrays. *Biochem Biophys Res Commun* 2002; **293**: 1254–1261.
6 Beresford PJ, Kam CM, Powers JC, Lieberman J. Recombinant human granzyme A binds to two putative HLA-associated proteins and cleaves one of them. *Proc Natl Acad Sci USA* 1997; **94**: 9285–9290.
7 Shresta S, Graubert TA, Thomas DA, Raptis SZ, Ley TJ. Granzyme A initiates an alternative pathway for granule-mediated apoptosis. *Immunity* 1999; **10**: 595–605.
8 Fuentes JJ, Pritchard MA, Estivill X. Genomic organization, alternative splicing, and expression patterns of the *DSCR1* (Down syndrome candidate region 1) gene. *Genomics* 1997; **44**: 358–361.
9 Baughman G, Wiederrecht GJ, Campbell NF, Martin MM, Bourgeois S. FKBP51, a novel T-cell-specific immunophilin capable of calcineurin inhibition. *Mol Cell Biol* 1995; **15**: 4395–4402.
10 Vega RB, Yang J, Rothermel BA, Bassel-Duby R, Williams RS. Multiple domains of MCIP1 contribute to inhibition of calcineurin activity. *J Biol Chem* 2002; **277**: 30401–30407.
11 Yamada M, Hirasawa A, Shiojima S, Tsujimoto G. Granzyme A mediates glucocorticoid-induced apoptosis in leukemia cells. *FASEB J* 2003; **17**: 1712–1714.
12 Zhao Y, Tozawa Y, Iseki R, Mukai M, Iwata M. Calcineurin activation protects T cells from glucocorticoid-induced apoptosis. *J Immunol* 1995; **154**: 6346–6354.
13 Asada A, Zhao Y, Kondo S, Iwata M. Induction of thymocyte apoptosis by Ca^{2+}-independent protein kinase C (nPKC) activation and its regulation by calcineurin activation. *J Biol Chem* 1998; **273**: 28392–28398.
14 McEwan IJ, Wright AP, Gustafsson JA. Mechanism of gene expression by the glucocorticoid receptor: role of protein–protein interactions. *BioEssays* 1997; **19**: 153–160.
15 Stöcklin E, Wissler M, Gouilleux F, Groner B. Functional interactions between Stat5 and the glucocorticoid receptor. *Nature* 1996; **383**: 726–728.
16 Imai Y, Matsushima Y, Sugimura T, Terada M. A simple and rapid method for generating a deletion by PCR. *Nucleic Acids Res* 1991; **19**: 2785.

17 Pawlowski V, Revillion F, Hornez L, Peyrat JP. A real-time one-step reverse transcriptase-polymerase chain reaction method to quantify c-erbB-2 expression in human breast cancer. *Cancer Detect Prev* 2000; **24**: 212–223.
18 Miesfeld R, Rusconi S, Godowski PJ, Maler BA, Okret S, Wikstrom AC *et al*. Genetic complementation of a glucocorticoid receptor deficiency by expression of cloned receptor cDNA. *Cell* 1986; **46**: 389–399.
19 Shikama Y, Yamada M, Miyashita T. Caspase-8 and caspase-10 activate NF-κB through RIP, NIK and IKKα kinases. *Eur J Immunol* 2003; **33**: 1998–2006.
20 Galon J, Franchimont D, Hiroi N, Frey G, Boettner A, Ehrhart-Bornstein M *et al*. Gene profiling reveals unknown enhancing and suppressive actions of glucocorticoids on immune cells. *FASEB J* 2002; **16**: 61–71.
21 Planey SL, Abrams MT, Robertson NM, Litwack G. Role of apical caspases and glucocorticoid-regulated genes in glucocorticoid-induced apoptosis of pre-B leukemic cells. *Cancer Res* 2003; **63**: 172–178.
22 Tonko M, Ausserlechner MJ, Bernhard D, Helmberg A, Kofler R. Gene expression profiles of proliferating *vs* G1/G0 arrested human leukemia cells suggest a mechanism for glucocorticoid-induced apoptosis. *FASEB J* 2001; **15**: 693–699.
23 Chauhan D, Auclair D, Robinson EK, Hideshima T, Li G, Podar K *et al*. Identification of genes regulated by dexamethasone in multiple myeloma cells using oligonucleotide arrays. *Oncogene* 2002; **21**: 1346–1358.
24 Yang J, Rothermel B, Vega RB, Frey N, McKinsey TA, Olson EN *et al*. Independent signals control expression of the calcineurin inhibitory proteins MCIP1 and MCIP2 in striated muscles. *Circ Res* 2000; **87**: E61–E68.
25 Wang Z, Malone MH, He H, McColl KS, Distelhorst CW. Microarray analysis uncovers the induction of the proapoptotic BH3-only protein Bim in multiple models of glucocorticoid-induced apoptosis. *J Biol Chem* 2003; **278**: 23861–23867.
26 Kastan MB, Zhan Q, el Deiry WS, Carrier F, Jacks T, Walsh WV *et al*. A mammalian cell cycle checkpoint pathway utilizing p53 and *GADD45* is defective in ataxia-telangiectasia. *Cell* 1992; **71**: 587–597.
27 Oda K, Arakawa H, Tanaka T, Matsuda K, Tanikawa C, Mori T *et al*. *p53AIP1*, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. *Cell* 2000; **102**: 849–862.
28 de Stanchina E, Querido E, Narita M, Davuluri RV, Pandolfi PP, Ferbeyre G *et al*. PML is a direct p53 target that modulates p53 effector functions. *Mol Cell* 2004; **13**: 523–535.
29 Hubler TR, Denny WB, Valentine DL, Cheung-Flynn J, Smith DF, Scammell JG. The FK506-binding immunophilin FKBP51 is transcriptionally regulated by progestin and attenuates progestin responsiveness. *Endocrinology* 2003; **144**: 2380–2387.
30 Strähle U, Klock G, Schütz G. A DNA sequence of 15 base pairs is sufficient to mediate both glucocorticoid and progesterone induction of gene expression. *Proc Natl Acad Sci USA* 1987; **84**: 7871–7875.
31 Beato M, Chalepakis G, Schauer M, Slater EP. DNA regulatory elements for steroid hormones. *J Steroid Biochem* 1989; **32**: 737–747.
32 Reichardt HM, Kaestner KH, Tuckermann J, Kretz O, Wessely O, Bock R *et al*. DNA binding of the glucocorticoid receptor is not essential for survival. *Cell* 1998; **93**: 531–541.
33 O'Connor L, Strasser A, O'Reilly LA, Hausmann G, Adams JM, Cory S *et al*. Bim: a novel member of the Bcl-2 family that promotes apoptosis. *EMBO J* 1998; **17**: 384–395.
34 Bouillet P, Metcalf D, Huang DC, Tarlinton DM, Kay TW, Kontgen F *et al*. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science* 1999; **286**: 1735–1738.
35 Lindsten T, Ross AJ, King A, Zong WX, Rathmell JC, Shiels HA *et al*. The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. *Mol Cell* 2000; **6**: 1389–1399.
36 Villunger A, Michalak EM, Coultas L, Müllauer F, Böck G, Ausserlechner MJ *et al*. p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. *Science* 2003; **302**: 1036–1038.

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Silencing of imprinted *CDKN1C* gene expression is associated with loss of CpG and histone H3 lysine 9 methylation at *DMR-LIT1* in esophageal cancer

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The putative tumor suppressor *CDKN1C* is an imprinted gene at 11p15.5, a well-known imprinted region often deleted in tumors. The absence of somatic mutations and the frequent diminished expression in tumors would suggest that *CDKN1C* expression is regulated epigenetically. It has been, however, controversial whether the diminution is caused by imprinting disruption of the *CDKN1C/LIT1* domain or by promoter hypermethylation of *CDKN1C* itself. To clarify this, we investigated the CpG methylation index of the *CDKN1C* promoter and the differentially methylated region of the *LIT1* CpG island (differentially methylated region (*DMR*)-*LIT1*), an imprinting control region of the domain, and *CDKN1C* expression in esophageal cancer cell lines. *CDKN1C* expression was diminished in 10 of 17 lines and statistically correlated with the loss of methylation at *DMR-LIT1* in all but three. However, there was no statistical correlation between *CDKN1C* promoter MI and *CDKN1C* expression. Furthermore, loss of CpG methylation was associated with loss of histone H3 lysine 9 (H3K9) methylation at *DMR-LIT1*. Histone modifications at *CDKN1C* promoter were not correlated with *CDKN1C* expression. The data suggested that the diminished *CDKN1C* expression is associated with the loss of methylation of CpG and H3K9 at *DMR-LIT1*, not by its own promoter CpG methylation, and is involved in esophageal cancer, implying that *DMR-LIT1* epigenetically regulates *CDKN1C* expression not through histone modifications at *CDKN1C* promoter, but through that of *DMR-LIT1*.
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Introduction

CDKN1C (*p57^{KIP2}*) is considered to be a tumor suppressor because it maps to 11p15.5, where it has shown frequent loss of heterozygosity in various tumors (Henry *et al.*, 1989; Bepler and Garcia-Blanco, 1994; Shibagaki *et al.*, 1994; Baffa *et al.*, 1996). It encodes a cyclin-dependent kinase inhibitor (Lee *et al.*, 1995; Matsuoka *et al.*, 1995), suppresses myc/RAS-mediated transformation (Watanabe *et al.*, 1998), and causes the cancer-predisposing Beckwith–Wiedemann syndrome (BWS) by gene mutation (Hatada *et al.*, 1996b). While no somatic mutation in tumors has been found (Tokino *et al.*, 1996; O'Keefe *et al.*, 1997), expression was diminished in various tumors (Hatada *et al.*, 1996a; Thompson *et al.*, 1996; Matsumoto *et al.*, 2000; Oya and Schulz, 2000; Shin *et al.*, 2000a; Schwienbacher *et al.*, 2000), suggesting that the diminution might be caused by disruption of epigenetic regulation and may be associated with tumorigenesis.

CDKN1C, an imprinted gene showing maternal preferential expression, is located within an imprinted region at 11p15.5 (Hatada and Mukai, 1995). This well-known region is divided into two domains, *CDKN1C/LIT1* and *IGF2/H19* (Feinberg, 2000). The expression of imprinted genes within these domains is regulated by an imprinting control region (ICR) specific to each domain. In *IGF2/H19*, the differentially methylated region (DMR) upstream of *H19* is known as an ICR. As for *CDKN1C/LIT1*, DMR at the 5′ CpG island of noncoding *LIT1* transcript (*DMR-LIT1*) is a putative ICR (Figure 1a). *DMR-LIT1* is normally methylated on the maternal allele and unmethylated on the paternal (Lee *et al.*, 1999; Mitsuya *et al.*, 1999; Smilinich *et al.*, 1999). *DMR-LIT1* is only a gametic methylated region, methylated in oocyte, within *Cdkn1c/Lit1* (Yatsuki *et al.*, 2002). Targeted deletion of *DMR-Lit1* on the paternally inherited chromosome resulted in the derepression in *cis* of imprinted genes, including *Cdkn1c*

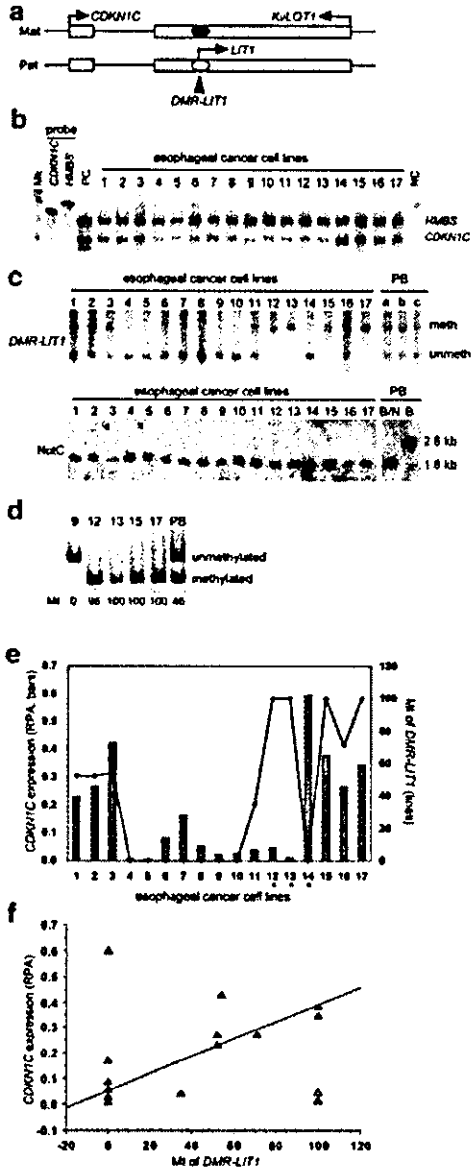

Figure 1 Positive correlation between *CDKN1C* expression and CpG methylation of *DMR-LIT1*. (**a**) *CDKN1C/LIT1* imprinted domain. Representative genes are shown. Arrows indicate transcriptional direction. Black and white ovals indicate methylated and unmethylated *DMR-LIT1*, respectively. Mat: maternal allele. Pat: paternal allele. (**b**) *CDKN1C* expression was quantified by ribonuclease protection assay normalized with a housekeeping *HMBS* gene. Protected bands. 201 bp for *CDKN1C* and 255 bp for *HMBS*. are indicated. Mk: marker; PC: positive control; NC: negative control. (**c**) Methylation-sensitive Southern blotting of *DMR-LIT1*. Methylated (meth, 6.0 kb) and unmethylated (unmeth, 4.2 kb) bands are detected by *DMR-LIT1* probe. The blot probed with NotC control probe indicates complete digestion. PB: normal peripheral blood; B/N: double digestion with *Bam*HI and *Not*I; B: digestion with *Bam*HI. (**d**) Methylation status analysed by hot-Stop COBRA. (**e**) Plots of *CDKN1C* expression by RPA (bars) and *DMR-LIT1* MI (lines). *CDKN1C* expression is apparently inconsistent with *DMR-LIT1* MI in #12, #13, and #14 (asterisked). (**f**) Correlation between *CDKN1C* expression and *DMR-LIT1* MI. Data from all cell lines were plotted in a graph. White triangles were #12–14 (asterisked in (e)). Statistical analysis, excluding #12–14, revealed a close correlation between *CDKN1C* expression and *DMR-LIT1* MI ($\rho = 0.832$, $P = 0.0027$)

(Fitzpatrick *et al*., 2002). Targeted deletion of human *DMR-LIT1* using microcell hybrids also showed reactivation of *CDKN1C* expression (Horike *et al*., 2000). These results suggested that demethylation of maternally methylated *DMR-LIT1* would diminish *CDKN1C* expression in tumors. It had been reported that loss of maternal *DMR-LIT1* methylation was common in various adult tumors (Scelfo *et al*., 2002), and the abnormal imprinting status of *CDKN1C* was correlated with loss of maternal methylation of *DMR-LIT1* in hepatocarcinoma (Schwienbacher *et al*., 2000).

However, it is well known that hypermethylation of the promoter contributes to silencing of tumor suppressor genes in human cancer (Jones and Laird, 1999; Baylin *et al*., 2001). It was recently reported that in several tumors, *CDKN1C* silencing was associated with dense methylation of the region around the transcription start site (Shin *et al*., 2000b; Kikuchi *et al*., 2002; Li *et al*., 2002). Thus, it has been controversial whether reduction of *CDKN1C* expression in cancer is caused by disruption of an imprinting control mechanism or by its own promoter hypermethylation.

In all eukaryotes, the covalent modification of histone N-terminal tails is important in the regulation of transcription, mitosis, and heterochromatin formation (Strahl and Allis, 2000; Jenuwein and Allis, 2001). H3K9 methylation was associated with the formation of stably silenced chromatin in mammals (Lachner *et al*., 2001). Histone acetylations and histone H3 lysine 4 (H3K4) methylation changed the chromatin structure to increase accessibility to transcriptional factors and correlated with transcriptional activity, respectively (Strahl *et al*., 1999; Strahl and Allis, 2000). To date, histone modifications at several imprinted loci, such as *SNRPN*, *IGF2/H19*, *Igf2r*, and *U2af1-rs1*, have been examined. Recently, we clarified histone modification status at *DMR-LIT1* in mouse and human tissues (Higashimoto *et al*., 2003). However, in human cancer, the modification at *DMR-LIT1* and *CDKN1C* promoter has not yet been examined.

Here, we describe the relationships between *CDKN1C* expression and epigenetic factors; CpG methylation at *DMR-LIT1* and *CDKN1C* promoter, and histone modifications at those loci. Our results suggest that diminished expression of *CDKN1C* due to disruption of imprinting regulation caused by loss of CpG and H3K9 methylation at *DMR-LIT1* is involved in esophageal cancer.

Results

CDKN1C expression is diminished in esophageal cancer cell lines

We analysed 17 esophageal cancer cell lines (#1–17, Table 1). First, the expression level of the *CDKN1C* gene was quantified with ribonuclease protection assay (RPA) and real-time RT–PCR normalized with that of a housekeeping hydroxymethylbilane synthase gene