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Heteromeric complex formation of ASK2 and ASK1 regulates stress-induced signaling

Elisabeth Ortner, Karin Moelling *

Institute of Medical Virology, University of Zurich, Gloriastrasse 30, CH-8006 Zurich, Switzerland

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Abstract

Apoptosis signal-regulating kinase 2 (ASK2) is an interaction partner of the highly related ASK1. Here, we describe a regulatory function of ASK2 in stress signaling-induced cleavage of caspase-3 and poly(ADP-ribose) polymerase (PARP). Increased cleavage of caspase-3 and PARP was demonstrated by overexpression as well as knockdown of ASK2 after stress-induction by serum-starvation. We show that ectopically expressed ASK2 homo-oligomerized while endogenous ASK2 and ASK1 formed hetero-oligomers, which decreased upon serum-starvation. Co-expression of ASK2 and ASK1 stabilized these two proteins and reduced starvation-induced caspase-3 activation and degradation of PARP. Analysis of the intracellular localization of ASK2 exhibited a similar localization compared with ASK1 in the nucleus, cytoplasm, and in mitochondria. We propose that ASK2 regulates stress-induced caspase-3 and PARP cleavage in a dose-dependent manner by heteromeric complex formation with ASK1.

Keywords: Apoptosis signal-regulating kinase 2; ASK2; Serum-starvation; Stress signaling; Caspase-3; Poly(ADP-ribose) polymerase; PARP; Complex formation

Apoptosis signal-regulating kinase 2 (ASK2) has been identified as interaction partner of ASK1 [1]. Both belong to the family of MAPKKK (MAP3K) that is involved in multifunctional signaling networks, regulating cell survival, proliferation, differentiation, and apoptosis [2–5]. Differential hetero-oligomerization between members of the MAP3Ks family has already been described to regulate their kinase activities. Heterodimerization of B-Raf and c-Raf results in elevated kinase activity compared with the monomeric Raf isoforms or their homodimeric complexes [6]. Binding of c-Raf negatively regulates ASK1 in a c-Raf kinase-independent manner [7].

ASK2 is a serine/threonine kinase that activates the stress signaling MAP kinases JNK (c-Jun NH2-terminal protein kinase) and p38 MAPK. ASK2 is closely related to the apoptosis-inducing kinase ASK1 sharing 45% amino acid identity and a similar structure with a central kinase domain and a C-terminal coiled-coil sequence (Fig. 1A). They mainly differ at their N- and C-termini, whereby ASK2 is shorter at either end [1,8]. While this work was in progress, ASK2 was shown to form a heteromeric complex with ASK1 and thereby to function as a MAPKKK by activating JNK and p38 MAPK. This hetero-oligomer was found to positively regulate kinase activation of ASK2 and ASK1 [8].

Stress signaling often leading to apoptosis can be mediated either by the death receptor-mediated extrinsic pathway or the mitochondrial intrinsic pathway (reviewed in [9] and [10]). The extrinsic pathway is triggered by binding of Fas and TNF-α while the intrinsic pathway is induced by cellular stress stimuli, including withdrawal of survival factors, UV-light, γ-irradiation, and cytotoxic drugs. Serum-starvation can activate the mitochondrial intrinsic pathway by inducing the production of cellular reactive oxygen species (ROS) [11–13]. Both apoptotic pathways result in the activation of the executioner caspases-3, -6 and -7, which cleave numerous substrates including poly(ADP-ribose) polymerase (PARP), resulting in self-destruction of the cell [9,14–16].

* Corresponding author. Fax: +41 44 634 4967.
E-mail address: moelling@immv.uzh.ch (K. Moelling).

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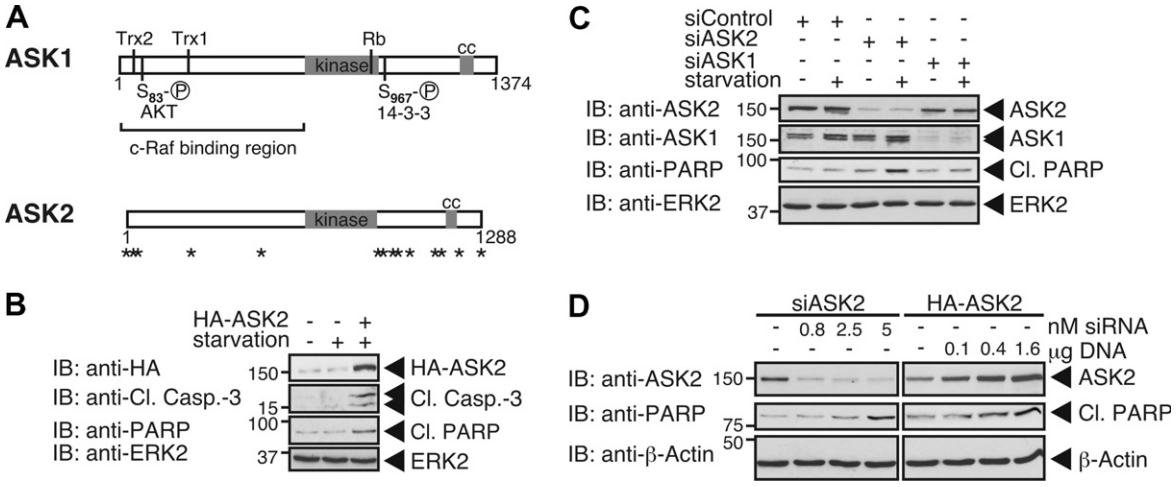

Fig. 1. Modulation of ASK2 protein expression increases stress signaling after serum-starvation. (A) Representation of domain structure (cc: coiled-coil sequence) of ASK2 and ASK1 as well as binding sites on ASK1 for thioredoxin 1 (Trx1) [18], Trx2 [19], retinoblastoma (Rb) protein [17], c-Raf [7] and for 14-3-3 at phosphorylated Ser967 (S_{967}) [25]. ASK1 is phosphorylated by AKT at Ser83 (S_{83}) [26]. Asteriks below ASK2 indicate deletions of 32 and 54 amino acids within the N- and C-terminal region, respectively, compared with ASK1. (B) ASK2 overexpression induces cleavage of caspase-3 and PARP. HeLa cells were transfected with HA-tagged ASK2 or empty vector with equalized amounts of total DNA. Cells were stressed by 24 h serum-starvation. Lysates were prepared and subjected to SDS–PAGE to analyze stress signaling in immunoblots (IB) with antibodies detecting cleaved caspase-3 (Cl. Casp.-3) and PARP, for which only the large cleavage fragment is shown (Cl. PARP). ERK2 expression was used as loading control. Molecular weights in kiloDalton are depicted to the left. (C) Knockdown of ASK2 leads to degradation of PARP. ASK2 or ASK1 were transiently downregulated with the specifically targeted siRNAs siASK2 and siASK1, respectively. An unspecific siRNA (siControl) was used as control. Cells were left untreated or serum-starved for 24 h prior to lysis. Lysates were resolved by SDS–PAGE and immunoblotted with antibodies against ASK2, ASK1, PARP, and ERK2, which was examined for loading control. (D) Cleavage of PARP is dose-dependent on ASK2 expression. Cells were transfected with increasing amounts of siASK2 or HA-ASK2. Amounts of transfected siRNA and DNA were equalized with siControl and empty vector, respectively. Lysates were prepared after 24 h serum-starvation and subjected to SDS–PAGE. Expression of ASK2 and degradation of PARP (Cl. PARP) were examined in immunoblots with the specific antibodies. β-Actin expression was used as loading control.

Although ASK2 and ASK1 are highly related, the role of ASK2 in stress signaling is unclear. Therefore, we studied the role of ASK2 as part of the ASK1 signaling complex involved in the stress signal transduction process. To this end, we modulated the expression level of ASK2 in HeLa cells and analyzed the contribution of stress by serum-starvation. Both, overexpression and knockdown of ASK2 caused an activation of caspase-3 and cleavage of its downstream substrate PARP upon serum-starvation. In non-stressed cells, overexpressed ASK2 formed homo-oligomers while endogenous ASK2 and ASK1 formed heteromeric complexes. Stress-induction by serum-starvation decreased hetero-oligomerization of ASK2 and ASK1. Concomitant overexpression of ASK2 and ASK1 reduced starvation-induced activation of caspase-3 and degradation of PARP back to the level observed after single expression of ASK2 or ASK1. In addition, both proteins were stabilized when co-expressed. Furthermore, we elucidated the intracellular localization of ASK2 in the nucleus, cytoplasm, and in mitochondria, which is comparable to ASK1 [17–19]. These findings suggest that ASK2 regulates serum-starvation-induced cleavage of caspase-3 and PARP in a dose-dependent manner most likely by homo- or hetero-oligomerization with ASK1.

Materials and methods

Plasmid construction. Human ASK2 was cloned into the pcDNA3.1/Hygro(+)–HA vector (Invitrogen) as follows: The ASK2 sequence from position 834 to 3864 was amplified by two PCR steps using the SuperScript human heart cDNA library (Invitrogen) as template and the following primers: (1) forward 5′-TTGGCGCGCCTGGTATGAACTTGC TGCTCTCCTACCGC-3′ and reverse 5′-CTTGGAGGTGCCGAAGTC AGAAATC-3′ and (2) forward 5′-CACCCGCCAGATCCTGCAGGG A-3′ and reverse 5′-GCGCTCTAGACAGCTCTCAGGGTCCAGAGG TGA-3′. The two amplicons were ligated at the internal SbfI site and cloned into the AscI/XbaI sites of the pcDNA3.1/Hygro(+)–HA vector. The N-terminal fragment of ASK2 from position 1 to 833 was obtained from two EST-clones (IMAGp998B166127, IMAGp998N015354; RZPD) After PCR amplification with the primers forward 5′-TTGGCGCGCCC AGAATGGCGG GGCCGTGTCCC-3′ and reverse 5′-GAATGACACC TACTCAGACAATGCGATGC-3′, this N-terminal fragment was inserted into the AscI/RcaI of the previously cloned pcDNA3.1/Hygro(+)–HA–ASK2 plasmid lacking the N-terminal ASK2 region. ASK2 was subcloned into the pcDNA3.1/Hygro(+)–Myc vector. pcDNA3–ASK1 was provided by H. Gram (Novartis). ASK1 was subcloned by PCR into the AscI and XbaI sites of the pcDNA3.1/Hygro(+)–HA vector using the primers forward 5′-TTGGCGCGCCCAAAATGAGCACGGAGGCGG ACGAGG-3′ and reverse 5′-CACAATAGAATCATAGTCCTGGAT ATCTCTGTAGG-3′.

Cell culture and transfection. HeLa and Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (FCS). Transient transfection was performed using jetPEI™ (Polyplus Transfection, Biopac, France) according to the manufacturer's protocol. When indicated, cells were starved for 24 h by a switch to serum free medium 24 h after transfection.

RNA interference. Validated human siRNAs for ASK2 (Hs_MAP3K6_6 HP Validated siRNA), ASK1 (Hs_MAP3K5_6 HP Validated siRNA), and negative control siRNA (AllStars Negative Control siRNA) were purchased from Qiagen and transfected into cells with HiPerFect according to the manufacturer's protocol.

Antibodies. The following antibodies were used: anti-HA (12CA5) and anti-HA-HRP (3F10) (Roche); anti-Flag (M2), anti-cytokeratin 18

(SIGMA); anti-Myc (9E10), anti-β-actin (C4), anti-ERK2 (C-14), anti-ASK1 (H-300), anti-Lamin A/C (N-18), and anti-c-Raf (C-20) (Santa Cruz Biotechnology); anti-PARP and anti-Cleaved-Caspase-3 (Asp-175) (Cell Signaling Technology Inc.). A rabbit polyclonal anti-ASK2 antibody was generated using the amino acids 926–1288 of human ASK2 fused to glutathione-*S*-transferase (GST) as antigen (G. Fischer, Institute for Labortierkunde, University of Zurich).

Cell lysis and immunoprecipitation. Cells were scrapped and lysed in RIPA buffer (20 mM Tris, pH 7.4; 133 mM NaCl; 2 mM EDTA; 0.1% SDS; 1% Triton X-100; 0.5% DOC; 10% glycerol; 1 mM dithiothreitiol; 50 mM β-glycerophosphate; supplemented with protease inhibitors). Lysates were clarified by centrifugation at 13,000 rpm and boiled in SDS-sample buffer at 95 °C for 5 min. For immunoprecipitation, cells were lysed in NETN buffer (20 mM Tris, pH 7.5; 100 mM NaCl; 1 mM EDTA; 0.5% Nonidet P-40; 1 mM dithiothreitiol; 40 mM β-glycerophosphate; supplemented with protease inhibitors) and lysates were incubated with the appropriate antibody for 2 h at 4 °C. After addition of protein G-Sepharose (Amersham Biosciences), lysates were incubated for another 1 h at 4 °C. Immunoprecipitated proteins were collected by centrifugation at 13,000 rpm, washed in NETN buffer and finally eluted with SDS-sample buffer. Lysates and immunoprecipitates were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and analyzed by immunoblotting using the ECL detection kit (Amersham Biosciences). Quantifications were done using the ImageQuant 5.2 (Molecular Dynamics) software.

Fractionation. Fractions from cytoplasm, membranes and organelles, nucleus, and cytoskeleton were prepared from HeLa cells using the ProteoExtract subcellular proteome extraction kit (Calbiochem) according to the manufacturer's protocol. Fractionation of HeLa cells into cytoplasmic and nuclear extracts was performed as described [20]. Cells were harvested in lysis buffer (10 mM Hepes, pH 7.5, 10 mM NaCl, 1.5 mM $MgCl_2$, 10% glycerol, 1 mM EDTA, 5 mM DTT, 1% NP-40). The cytoplasmic fraction was obtained in the supernatant after centrifugation for 5 min at 4300 rpm. Lysis of the pellet in extraction buffer (25 mM Tris–HCl, pH 8.0; 500 mM NaCl; 1 mM EDTA; 10 mM β-mercaptoethanol; 0.5% Triton X-100) and centrifugation for 30 min at 15,000 rpm yielded the nuclear extract-containing supernatant. Cytoplasmic and mitochondrial fractions of HeLa cells were prepared as described [19]. Cells were collected and resuspended in a buffer consisting of 250 mM sucrose in 70 mM Tris, pH 7.0 supplemented with protease inhibitors. Cells were lysed with 160 μg/ml digitonin and complete lysis was confirmed by Trypan blue staining. The cytosolic fraction was obtained in the supernatant by centrifugation for 2 min. To prepare extracts from mitochondria, cells were collected and resuspended in a hypotonic buffer (10 mM NaCl; 1.5 mM $CaCl_2$; 10 mM Tris, pH 7.5; supplemented with protease inhibitors) followed by stabilization with MS-buffer (525 mM mannitol; 175 mM sucrose; 12.5 mM EDTA, pH 7.5; 12.5 mM Tris, pH 7.5; supplemented with protease inhibitors). Cells were homogenized using a Dounce homogenizer and nuclei were removed by centrifugation for 10 min at 3000 rpm. Mitochondria were pelleted for 10 min at 13,000 rpm and lysed in a buffer containing 50 mM Hepes, pH 7.0; 500 mM NaCl; 1% NP-40; supplemented with protease inhibitors All fractions were separated by SDS–PAGE and analyzed by immunoblotting.

Results and discussion

Modulation of ASK2 expression induces increased cleavage of caspase-3 and PARP after stress-induction

To determine whether ASK2 is involved in a stress-signaling cascade, we examined the effects of modulated ASK2 expression on the cleavage of caspase-3 and PARP after stress-induction by serum-starvation. We approached this by overexpression as well as knockdown studies of ASK2 (Fig. 1). Stress-induction was analyzed by immunoblots (IB) of cell lysates by monitoring the cleavage and thus activation of caspase-3 (Cl. Casp.-3) and the cleavage of PARP (Cl. PARP) [9,14]. Transiently transfected HA-tagged ASK2 (HA–ASK2) increased degradation of PARP compared with transfection of empty vector in 24 h serum-starved HeLa cells (Fig. 1B). Unexpectedly, downregulation of ASK2 in HeLa cells by transient transfection of an ASK2-specific siRNA (siASK2) also led to PARP cleavage under the same conditions (Fig. 1C). This ASK2 knockdown-induced PARP cleavage was specific, since non-targeted control siRNA (siControl) only resulted in a weak serum-starvation-induced PARP degradation that was inhibited by knockdown of ASK1 (siASK1). The antibody generated against the C-terminal amino acid sequence of the human ASK2 did not cross-react with ASK1 confirming its specificity. Interestingly, downregulation of ASK1 weakly reduced the amount of ASK2 protein and serum-starvation led to a weak increase of ASK1. Furthermore, titration of ASK2 expression by downregulation and overexpression in serum-starved HeLa cells revealed a correlation between the expression level of ASK2 and the amount of PARP cleavage (Fig. 1D). Such a dose-dependence has not been shown for ASK1, which is indispensable for extrinsic TNF-α-induced and intrinsic oxidative stress-induced apoptosis [21]. Knockdown of ASK2 has previously been shown [8] to inhibit JNK activation upon oxidative stress, indicating that the cleavage of caspase-3 and PARP induced by decreased ASK2 expression after serum-starvation is mediated in a JNK-independent signaling pathway. Thus, in the absence of ASK2, serum-starvation may induce caspase-3 cleavage via the intrinsic mitochondria-mediated apoptosis pathway through the activation of ASK1.

Homo- and heteromeric complex formation of ASK2 and ASK1

Since homo-oligomerization is a prerequisite for kinase activation of ASK1 [22], we tested ASK2 for homo-oligomerization. Furthermore, we investigated a possible differential complex formation of ASK2 and ASK1 upon cellular stress (Fig. 2). Using a co-immunoprecipitation (IP) approach, we found that Myc-tagged ASK2 co-precipitated with HA-tagged ASK2 from HEK293 cells and vice versa (Fig. 2A). Endogenous co-precipitation analysis showed that ASK2 and ASK1 formed heteromers in non-stressed cells, which was decreased after serum-starvation (Fig. 2B, second panel). Immunoprecipitation of ASK1 was hardly detectable in non-stressed cells according to a previous study [8] and was increased after stress-induction (third panel). By contrast, immunoprecipitation of ASK2 was slightly decreased upon serum-starvation. Consistent with Fig. 1C (second panel, lanes 2 and 4), we observed a weak starvation-induced increase of the expression of ASK1 but not of ASK2 in whole cell lysates.

Since ASK2–ASK1 hetero-oligomerization was reduced after serum-starvation, we next studied an influence of concomitant overexpression of these two proteins on stress

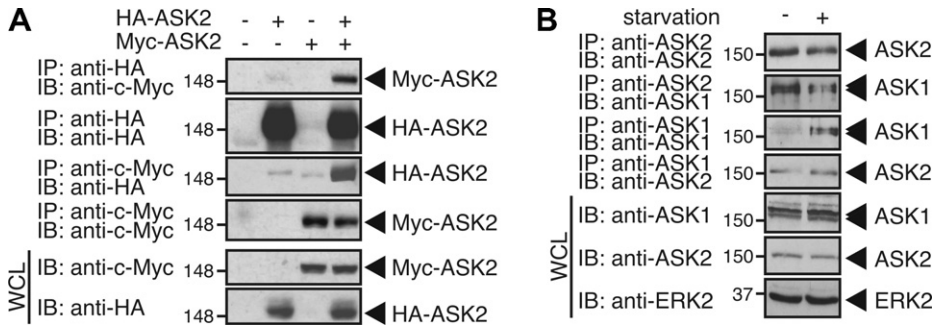

Fig. 2. Heteromeric complex formation of ASK2 and ASK1. (A) ASK2 is capable to homo-oligomerize. HEK293 cells were transfected with HA- and Myc-tagged constructs of ASK2 as indicated with constant amounts of total DNA. The cell lysates were immunoprecipitated (IP) with anti-HA or anti-Myc antibodies. The immunocomplexes were subjected to SDS–PAGE and homomeric complex formation was detected in immunoblots (IB) with the tag-specific antibody not used for immunoprecipitation. Protein expression was controlled in immunoblots of whole cell lysates (WCL). Molecular weights in kiloDalton are depicted to the left. (B) Co-precipitation of endogenous ASK2 and ASK1 is decreased after serum-starvation. HeLa cells were left untreated or serum-starved for 24 h prior to immunoprecipitation of ASK2 or ASK1 with the anti-ASK2 or anti-ASK1 specific antibodies. After resolving of the immunoprecipitates by SDS–PAGE, complex formation of ASK2 and ASK1 was analyzed by immunoblotting with anti-ASK2 and anti-ASK1 antibodies. Protein expression and equal loading were confirmed in immunoblots of whole cell lysates.

signaling. Immunoblot analyses demonstrated that overexpression of HA–ASK1 and Myc–ASK2 in 24 h serum-starved HeLa cells led to increased caspase-3 activation and PARP degradation (Fig. 3A). However, co-transfection of HA–ASK1 and Myc–ASK2 reduced cleavage of caspase-3 and PARP to the levels observed in cells transfected with empty vector only. Notably, the protein amounts of ASK2 and ASK1 were increased when both proteins were co-expressed compared to individual expression (Fig. 3B). This finding is in agreement with a recent report [8] showing that ASK1 stabilizes ASK2 by a kinase-independent mechanism. In addition, we found that ASK2 also stabilizes ASK1.

Taken together, we propose that in non-stressed cells at physiological or equimolar levels ASK2 and ASK1 reside as a heteromeric complex, which negatively regulates activation of caspase-3 and cleavage of PARP. A modulation of ASK2 expression together with serum-starvation disturbs the balance between ASK2 and ASK1, thereby leading to stress signaling (Fig. 3C). Additionally, overexpression but not downregulation of ASK1 leads to stress-induced cell death [21,23]. We suggest that over-

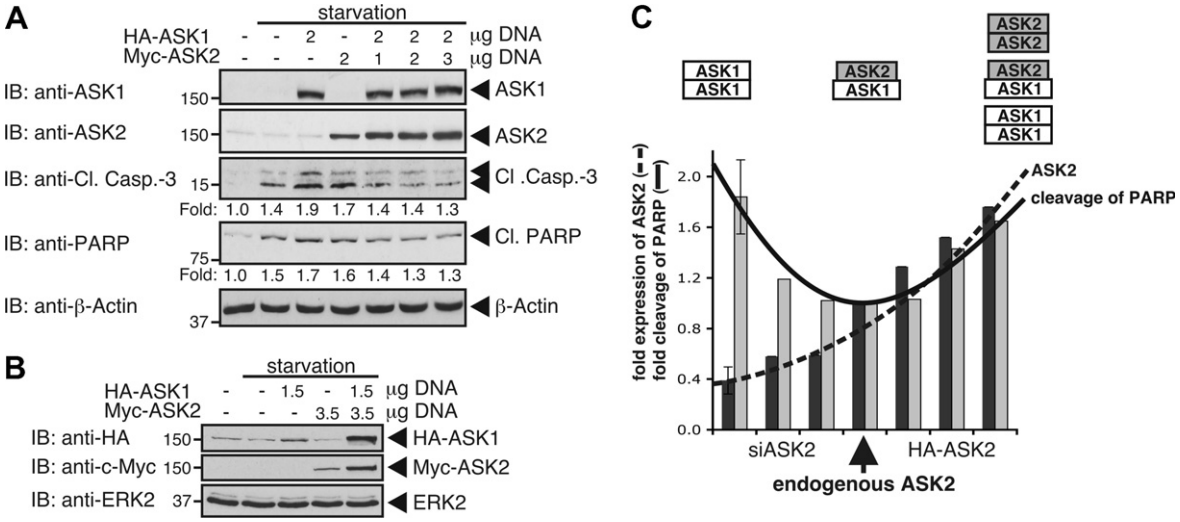

Fig. 3. Co-expression of ASK2 and ASK1 reduces serum-starvation-induced stress signaling. (A) HeLa cells were transfected with HA–ASK1 and/or Myc–ASK2 as indicated. Total amount of transfected DNA was equalized with empty vector. After 24 h serum-starvation, lysates were prepared, resolved by SDS–PAGE, and subjected to immunoblotting (IB) with antibodies against ASK1, ASK2, cleaved caspase-3 (Cl. Casp.-3), and PARP. The larger degradation-derived fragment of PARP (Cl. PARP) is shown. Expression of β-Actin was used as loading control. Pixel density was quantified with the ImageQuant 5.2. software and numbers indicate fold activation of caspase-3 and cleavage of PARP. Molecular weights in kiloDalton are depicted to the left. (B) Protein stabilizing effect of the co-expression of ASK2 and ASK1. HeLa cells were transfected with HA–ASK1 and/or Myc–ASK2 with equalized total amounts of transfected DNA using empty vector. Cells were serum-starved for 24 h prior to lysis. The lysates were subjected to SDS–PAGE and immunoblotted with anti-HA and anti-Myc antibodies to examine protein expression levels. ERK2 expression was used as loading control. (C) A change of ASK2 expression (black bars; the arrow indicates endogenous expression of ASK2) either by downregulation (left) or overexpression (right) together with a stress challenge by serum-starvation leads to elevated cleavage of PARP (grey bars). Quantifications are obtained from Fig. 1D; standard deviation is calculated from Fig. 1B–D.

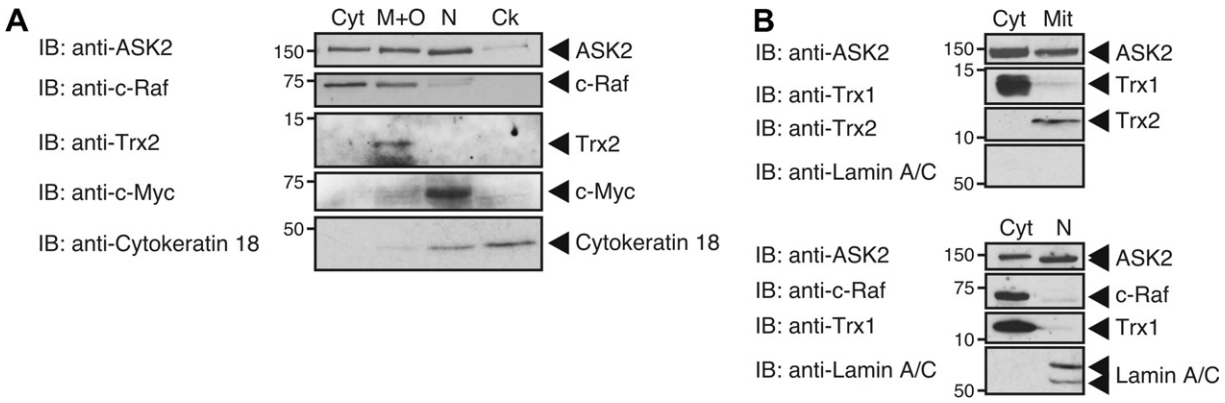

Fig. 4. Intracellular localization of ASK2 in HeLa cells. (A) Subcellular proteome extracts from cytoplasm (Cyt), membrane/organelle (M + O), nucleus (N), and cytoskeleton (Ck) were resolved by SDS–PAGE for immunoblot (IB) analysis. Expression of ASK2 was analyzed with the ASK2-specific antibody and purity of each fraction was controlled with antibodies against c-Raf, Trx2, c-Myc, and cytokeratin 18. Molecular weights in kiloDalton are depicted to the left. (B) Fractions from cytoplasm (Cyt) and mitochondria (Mit) (upper panel) or cytoplasm (Cyt) and nuclei (N) (lower panel) were immunoblotted for endogenous ASK2 with the anti-ASK2 antibody. Purity of each fraction was controlled using antibodies against Trx1, Trx2, c-Raf and Lamin A/C.

expression of ASK2 leads to formation of homomeric complexes and also to hetero-oligomerization of ASK2 and ASK1. In these cells, serum-starvation-induced stress would then be mainly mediated by ASK1 homo-oligomers [22]. It remains to be clarified why overexpression of ASK2 together with kinase-inactive ASK1 increases kinase activation of ASK2 and caspase-3 activation in non-stressed cells [8]. Presumably, disturbing the endogenous balance between ASK2 and ASK1 may be sufficient for stress-induction. Additionally, the coiled-coil sequences of ASK2 and ASK1 differ in amino acid composition and also in their length, which most likely results in different structural configurations. Therefore, overexpressed kinase-inactive ASK1 may bind to endogenous ASK1 with a higher affinity than to ASK2, thereby inducing dissociation of the ASK2–ASK1 heteromeric complex. Consequently, ASK2 could form homo-oligomers leading to phosphorylation within the activation loop in the kinase domain as described for homo-oligomerized ASK1 [24]. The observed protein stabilization of co-expressed ASK2 and ASK1 further supports a negative regulatory function of the ASK2–ASK1 heteromeric complex on stress signaling.

Intracellular localization of ASK2

To investigate whether the intracellular localization of ASK2 coincide with the described localization of ASK1 in the cytoplasm, nucleus and in mitochondria [17,19,23], we fractionated HeLa cells by subcellular proteome extraction. Immunoblot analyses revealed a localization of ASK2 in all fractions except the cytoskeletal extract (Fig. 4A). Expression of ASK2 was further confirmed in the cytoplasm, nucleus, and in mitochondria by two additional fractionations (Fig. 4B). The band shift observed for ASK2 in the nuclear fraction (Fig. 4B, lower panel) is most likely caused by different salt concentrations of the extraction buffers. Summarizing, ASK2 and ASK1 exhibit a similar subcellular localization.

Interestingly, ASK2 is mainly expressed in tissues containing high amounts of mitochondria such as heart and skeletal muscle [1] and only weakly induces activation of JNK and p38 MAPK [1,8]. Hence we speculate that mitochondria-located ASK2 may be able to induce caspase-3 activation in a JNK-independent manner when forming a homomeric complex as already described for mitochondrial ASK1 [19]. On the other hand, we assume that ASK2 negatively regulates stress signaling by hetero-oligomerization with ASK1 in mitochondria. Thus, stress signaling via mitochondria may be partly regulated by differential complex formation of ASK2 and ASK1. Since ASK2 and ASK1 also co-localize in the cytoplasm and nucleus, other interacting proteins might influence their complex formation in these compartments. Cytoplasmic and nuclear thioredoxin 1 (Trx1) [18], c-Raf [7], 14-3-3 [25], and retinoblastoma (Rb) protein [17,19] are described binding partners of ASK1 (Fig. 1A). Furthermore, AKT negatively regulates apoptosis by phosphorylation of ASK1 at Ser83 [26]. It remains to be elucidated whether these proteins may influence complex formation of ASK2 and ASK1.

Acknowledgments

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