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Apoptosis-linked gene-2 connects the Raf-1 and ASK1 signalings

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Abstract

Raf-1 plays important roles in cell proliferation, differentiation, and survival. However, the unique and essential function of Raf-1 is anti-apoptotic. The molecules that mediate Raf-1's anti-apoptotic function are not known. In the course of identifying new substrates of Raf-1, we found that the Raf-1 kinase domain interacted with apoptosis-linked gene-2 (ALG-2) in yeast two-hybrid system. Our further studies showed that Raf-1 phosphorylated ALG-2 in an in vitro kinase assay. We also found that apoptosis signal-regulating kinase 1 (ASK1) strongly phosphorylated ALG-2. Importantly, Raf-1 blocks the ASK1-dependent ALG-2 phosphorylation. Since ALG-2 associates with ASK1, and both ASK1 and ALG-2 are involved in apoptosis, our observations indicate that Raf-1 may mediate its anti-apoptotic function by interrupting ASK1-dependent phosphorylation of ALG-2.

Keywords: Raf-1; ALG-2; ASK1; Apoptosis; Signal transduction

Raf-1 has been intensively studied and found to interact with many proteins and to be involved in several cellular functions [1–5]. In response to activating signals, the N-terminal domain mediates the interaction of Raf-1 with Ras. The interaction with GTP-bound Ras localizes Raf-1 to the plasma membrane [6,7]. The translocation of Raf-1 to the plasma membrane facilitates further interaction with kinases and other proteins [8]. These interactions lead to the phosphorylation of Raf-1 at multiple tyrosine, serine, and threonine residues, and Raf-1 activation [9]. The activation of Raf-1 can also occur without interacting with Ras [10–12]. Activated Raf-1 then phosphorylates its targets. So far, the well-characterized substrates of Raf-1 are the Meks [13]. Less-characterized substrates include retinoblastoma tumor suppressor protein [14] and the pro-apoptotic Bcl-2 family protein Bad [15].

Recent studies with Raf-1 knockout mice showed that the essential and unique function of Raf-1 is anti-apoptotic, but this function is not mediated by Mek/Erk [1,2]. Furthermore, bFGF and VEGF differentially activate Raf-1 and protect cells from distinct apoptotic pathways. bFGF induced phosphorylation of Raf-1 at serines 338, 339 and protected cells from intrinsic-mediated apoptosis [16], whereas, VEGF induced phosphorylation of tyrosines 340, 341 and protected cells from extrinsic-mediated apoptosis [16]. However, both bFGF and VEGF induce Erk activation [16]. These results indicated that other effectors rather than the Mek/Erk cascade must be the downstream targets of Raf-1 in terms of anti-apoptosis.

Raf-1 translocates to mitochondria and is engaged in anti-apoptosis [16,17]. Transfected active Raf-1 fused with targeting sequences from an outer mitochondrial membrane protein protected cells from apoptosis and resulted in phosphorylation and inactivation of the pro-apoptotic Bcl-2 family protein Bad [15]. Another study showed that mitochondrial Raf-1 protected cells from apoptosis through a Bcl-2-independent pathway [18]. So far, our understanding of Raf-1-mediated anti-apoptosis remains incomplete. Identifying additional

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direct targets of Raf-1 should aid our understanding of the mechanism of Raf-1-mediated anti-apoptotic signaling. Towards this end, we carried out a yeast two-hybrid experiment in which the Raf-1 kinase domain was used as bait to screen a bone marrow cDNA library. We report that the Raf-1 kinase domain interacts with the pro-apoptotic protein ALG-2. Furthermore, an in vitro kinase assay showed that ALG-2 is a substrate of Raf-1.

ALG-2 was discovered originally as a pro-apoptotic protein [19,20], and ALG-2 associates with apoptosis signal-regulating kinase 1 (ASK1) [21]. ASK1 acts upstream of the JNK and p38 Map kinase pathways [22–24]. ASK1/JNK apoptotic signaling is increased in Raf-1 knockout mice, and co-transfection of Raf-1 and ASK1 inhibits the ASK1-induced apoptosis signaling, showing that Raf-1 negatively regulates ASK1 [25,26] but the mechanism is not clear. Here, we show that ASK1 phosphorylates ALG-2 but the ASK1-dependent phosphorylation of ALG-2 is blocked in the presence of Raf-1, indicating that Raf-1 may exert its anti-apoptotic function by interrupting ASK1-dependent ALG-2 phosphorylation.

Materials and methods

The human bone marrow Matchmaker cDNA library, Matchmaker Gal4 two-hybrid system 3, and all reagents for the two-hybrid system, as well as human placental mRNA were purchased from Clontech. Active Raf-1, ASK1, and reaction buffer were purchased from Upstate. Prokaryotic expression vector pET-28c(+) and mammalian expression vector pCMV-Tag2A were purchased from Novagen and Stratagene, respectively. The oligonucleotides for the amplification of Raf-1 and ALG-2 were synthesized by Sigma-Genosys. Plasmid mini-prep purification kits were purchased from Promega. His-bind purification kit and the protein refolding kit were purchased from Novagen.

Two-hybrid system. Raf-1 cDNA from nucleotide 889 to the stop codon (encoding the C-terminal domain of 351 amino acids) was cloned into the pGBKT7 vector (termed pGBKT7-Raf), which expresses a fusion protein of Raf-1 and Gal4 DNA-binding domain as bait protein. Yeast strain AH109 was first transformed with pGBKT7-Raf and grown in SD/−Leu medium according to the manufacturer's instructions. Then, the pGBKT7-Raf-transformed yeasts were sequentially transformed with pGAD-human bone marrow Matchmaker cDNA library expressing fusion proteins of library proteins and Gal4 activation domain. The transformants were plated on 30 of 150-mm SD/−Ade/−His/−Leu/−Trp/X-α-Gal high-stringency plates. After selection by 3 reporters-Ade2, His3, and α-galactosidase, the blue positive clones were grown in 3 ml YPDA medium for DNA extraction. The extracted DNAs were transformed into bacteria DH5α . The pGAD-library plasmids in DH5α were purified with mini-prep kits and were sequenced to identify the genes. To verify the positive interactions, purified positive pGAD-library plasmids and pGADT7 vector expressing GAL4 activation domain (AD) as negative control were transformed into yeasts containing pGBKT7-Raf and pGBKT7 empty vector expressing GAL4 DNA-binding domain and pGBKT7-Lam plasmid expressing a fusion of the GAL4 NDA-BD with human lamin C as negative controls, respectively. The transformed yeasts were grown on both SD/−Ade/−His/−Leu/−Trp/X-α-Gal and SD/−Leu/−Trp low-stringency plates.

Expression and purification of His- and Flag-tagged ALG-2. The full-length human ALG-2 cDNA was amplified from human placental mRNA by RT-PCR and was inserted into an N-terminal His-tag prokaryotic expression vector pET-28c(+) and a Flag-tagged mammalian expression vector pCMV-Tag2C, respectively. After confirmation by sequencing, the plasmid pET-28c(+)-ALG-2 was transformed into bacteria strain DE32. His-tag ALG-2 was purified using His.bind purification kit and protein refolding kit according to manufacturer's instruction. For mammalian expression, 15 μg of pCMV-Tag-ALG-2 was transfected into 1.0×10^7 K562 cells by electroporation. The cells were returned immediately to serum-free RPMI-1640 to be cultured overnight and were lysed with RIPA buffer. After incubation at 4 °C with rocking for 15 min, the cell lysates were centrifuged, and the supernatant fractions were transferred to new tubes containing 80 μl protein A–Sepharose beads. After incubation at 4 °C with rocking for 30 min, the samples were centrifuged for 1 min. Five hundred microliters of supernatant was mixed with 40 μl of agarose-conjugated anti-Flag antibody (Sigma). After incubation at 4 °C with rocking for 2 h, the ALG-2 and antibody complexes were pelleted and washed five times with cold RIPA buffer. ALG-2 was separated from antibody by adding Flag peptide.

Kinase assay and Western blot. The Raf-1 kinase assay was carried out according to the manufacturer's instructions (Upstate). Briefly, 6 μl of 5× ADBI buffer, 10 μl magnesium/ATP cocktail, 1 μl [γ-^{32}P]ATP, 1 μg ALG-2 or 1 μg of inactive Mek, 0.05–0.2 μg Raf-1 or 0.3 μg ASK1 or water as control were mixed. The reaction was incubated at 30 °C for 30 min in a shaking incubator. The mixture was subjected to 12% SDS–PAGE and then transferred electrophoretically to a PVDF membrane (Millipore). After autoradiography analysis, the membrane was washed and then incubated in blocking buffer for 1 h. The membrane was incubated with an anti-His-tag antibody (Qiagen) or anti-Flag antibody (Sigma) in blocking buffer with gentle shaking overnight at 4 °C. After three 5 min washes, the membrane was incubated with secondary antibody (New England Biolabs) in blocking buffer with gentle shaking for 1 h at room temperature. Proteins were detected using CDP-Star Western Blotting Kit (New England Biolabs).

Results

In a yeast two-hybrid experiment, Raf-1 kinase domain was used as bait (pGBKT-Raf-1) to screen a bone marrow library (pGADT7-library cDNA). After screening on SD/−Ade/−His/−Leu/−Trp/X-α-gal high-stringency plate, we obtained 169 positive colonies. After sequencing, we found that four of the positive colonies were ALG-2, and we verified the interaction of Raf-1 and ALG-2. The constructs used in verification were:

pGBKT7-Raf-1 plasmid expresses a fusion protein of Raf-1 kinase domain and GAL4 DNA-binding domain (BD) as bait protein;
pGBKT7 vector expresses GAL4 DNA-BD as negative control;
pGBKT7-Lam plasmid expresses a fusion of the GAL4 NDA-BD with human lamin C as negative control;
pGADT7 vector expresses GAL4 activation domain (AD) as negative control.
pGADT7-ALG-2 plasmid expresses a fusion protein of ALG-2 and GAL4 AD.

As shown in Table 1, the yeasts transformed with both pGADT7-ALG-2 and pGBKT7-Raf-1 grew on

Table 1
Verification of Raf-1 and ALG-2 interaction

DNA-binding domain vector	Activation domain vector	High-stringency plate	Low-stringency plate
pGBKT7-Raf-1	pGADT7-ALG-2	Growth	Growth
pGBKT7	pGADT7-ALG-2	No	Growth
pGBKT7-Lam	pGADT7-ALG-2	No	Growth
pGBKT7	pGADT7	No	Growth
pGBKT7-Raf-1	pGADT7	No	Growth

both high-stringency plate and low-stringency plate (SD/−Leu/−Trp). The yeasts transformed with pGADT7-ALG-2 and negative control plasmids pGBKT7, or pGBKT7-Lam, and the yeasts transformed with pGADT7 and pGBKT7, or pGBKT7-Raf-1 did not grow on high-stringency plate but grew on low-stringency plate. This yeast two-hybrid system features the yeast strain AH109, which virtually eliminates false positive by using three reporters-Ade2, His3, and Mel1. Yeast strain AH109 cannot grow on low-stringency plate unless it is transformed with both pGADT7 and pGBKT7 plasmids since pGBKT7 carries the Trp nutritional marker and pGAD7 carries the Leu2 nutritional marker. Therefore, all five transformants grew on low-stringency plate. Only when pGADT7-ALG-2 interacts with Raf-1 to bring GAL4 activation domain and GAL4 DNA-binding domain together, which activates above three reporters, yeast could grow on high-stringency plate.

Then, we carried out co-immunoprecipitation experiments to determine whether there is Raf-1 and ALG-2 interaction in vivo. Since a recent report showed that currently available commercial ALG-2 antibodies from both Becton Dickinson Transduction Laboratories and Santa Cruz Biotechnologies failed to detect bona fide ALG-2 [27], instead of immunoprecipitating endogenous ALG-2, we expressed ALG-2 as a fusion protein with Flag tag at N-terminus for easy immunoprecipitation by anti-Flag antibody. Transiently expressed ALG-2 and endogenous Raf-1 were immunoprecipitated by anti-Flag and anti-Raf-1 antibodies, respectively. Anti-His antibody was used as control. As shown in Fig. 1A, cell lysate was immunoprecipitated by anti-Raf-1 antibody and blotted by anti-Flag antibody, ALG-2 was detected (lane 1), whereas no ALG-2 was detected from the sample immunoprecipitated by anti-His antibody (lane 2). Furthermore, cell lysate was immunoprecipitated by anti-Flag antibody, endogenous Raf-1 was detected (lane 1, Fig. 1B). No Raf-1 was detected in the sample immunoprecipitated by anti-His antibody (lane 2, Fig. 1B). We tested third round wash buffers and did not detect any ALG-2 or Raf-1 (data not shown), indicating that the bands shown are derived from specific immunoprecipitation.

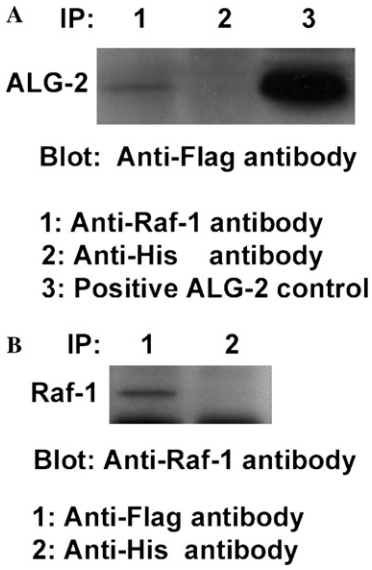

Fig. 1. ALG-2 and Raf-1 form complex in cells. (A) Lysate from K562 cells expressing Flag-tagged ALG-2 was immunoprecipitated with anti-Raf-1 antibody (lane 1) followed by blotting with anti-Flag antibody. Anti-His antibody was used in the immunoprecipitation as a negative control (lane 2) and ALG-2 protein was used as positive control (lane 3). (B) Lysate from K562 cells expressing Flag-tagged ALG-2 was immunoprecipitated with anti-Flag antibody (lane 1) followed by blotting with anti-Raf-1 antibody. Anti-His antibody was used in the immunoprecipitation as a negative control (lane 2).

After confirmation of the interaction between Raf-1 and ALG-2, we carried out a kinase assay to determine whether Raf-1 phosphorylated ALG-2. ALG-2 with a His-tag at the N-terminus was expressed in *E. coli*. After purification by Ni chromatography from inclusion body (single band on SDS–PAGE, Fig. 2A), ALG-2, and inclusion body proteins containing ALG-2 and elution buffer as controls were subjected to kinase assay with active Raf-1 as kinase (since immunoprecipitated endogenous Raf-1 associates with other proteins or kinases, we used purified commercial recombinant active Raf-1). Kinase assay reaction mixtures were subjected to SDS–PAGE gel and then transferred to PVDF membrane and analyzed by autoradiography. As shown in panel a (short exposure of panel b) and panel b of Fig. 2B, Raf-1 phosphorylated recombinant Mek in lane 1, and phosphorylated purified soluble ALG-2 in lane 2, and purified but aggregated ALG-2 in lane 4 (purified soluble ALG-2 aggregated after being stored at −20 °C). Convincingly, Raf-1 only phosphorylated ALG-2, not other inclusion body proteins in lane 6. There was no ALG-2 phosphorylation in elution buffer control in lane 8. Raf-1 is self-phosphorylated in this assay, showing a predominant full-length band and three minor bands, likely degraded fragments during denaturing since only one band appeared on the non-denaturing gel (data not shown). After exposure to X-ray film, the membrane was probed with anti-Mek antibody (Fig. 2B, panel c) and anti-His antibody (Fig. 2B, panel d). These results show that Raf-1

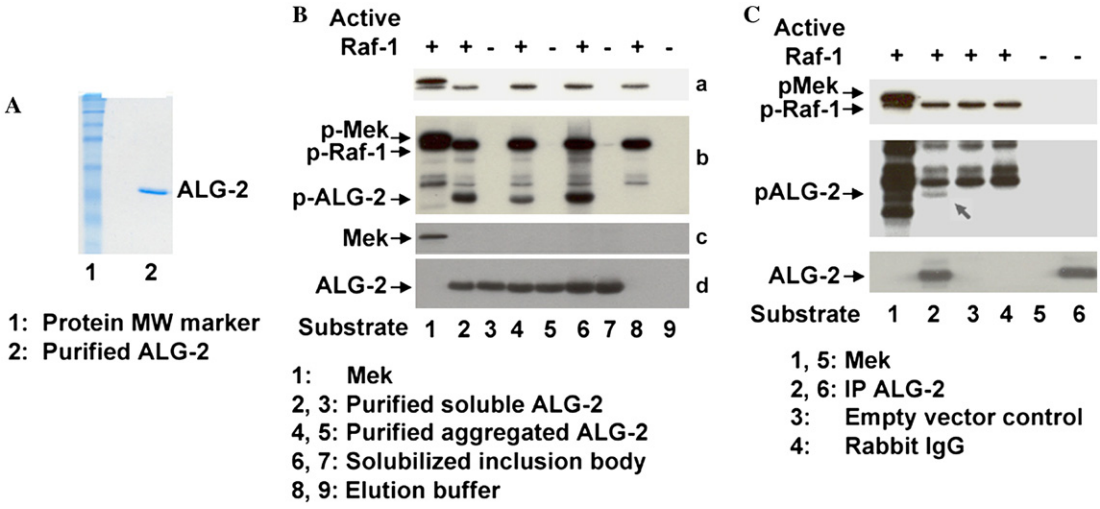

Fig. 2. Raf-1 phosphorylates ALG-2: (A) ALG-2 was expressed in *E. coli*, and purified by Ni chromatography, and then subjected to SDS–PAGE. (B) Raf-1 kinase assay: inactive Mek, purified soluble ALG-2, aggregated ALG-2, solubilized inclusion bodies, and elution buffer were used as substrates respectively, and active Raf-1 was used as kinase. Kinase assay reactions were subjected to SDS–PAGE and then transferred to PVDF membrane and analyzed by autoradiography (upper panel). After autoradiography, membrane was probed with anti-Mek and anti-His antibodies (panels c and d). (C) Raf-1 kinase assay: inactive Mek, anti-Flag antibody IP-ALG-2, IP-sample from empty vector transfected control and rabbit IgG were used as substrates in lanes 1 and 5, 2 and 6, 3 and 4, respectively. The upper and middle panels are autoradiographs and the bottom panel is a Western blot.

specifically phosphorylates ALG-2 and reactions lacking either active Raf-1 (lanes 3, 5, 7, and 9) or ALG-2 (lane 8) show no phosphorylation of ALG-2. We also obtained the same result with ALG-2 expressed in K562 cells (Fig. 2C). Transiently expressed ALG-2 was immunoprecipitated by anti-Flag antibody. Kinase assay was performed with 1 μg of inactive Mek, 10 μl of immunoprecipitated ALG-2, 10 μl of empty vector control sample, and 1 μg of rabbit IgG as substrate, respectively. In Fig. 2C, upper and middle panels, Raf-1 phosphorylated both Mek1 (lane 1) and ALG-2 (lane 2), and did not phosphorylate the sample from empty vector control (lane 3) and IgG (lane 4). Western-blot showed that anti-Flag antibody detected Flag-ALG-2 on lanes 2 and 6 (Fig. 2C, bottom panel).

Since studies have shown that ALG-2 interacts with ASK1 [21], we carried out a kinase assay to investigate whether ASK1 phosphorylates ALG-2. ALG-2, solubilized inclusion bodies containing ALG-2 and mouse IgG as controls were subjected to kinase assay with active ASK1 as kinase. As shown in the upper panel of Fig. 3A, ASK1 phosphorylated ALG-2 in lanes 2 and 3. Other inclusion body proteins were not phosphorylated even when we overloaded inclusion body proteins (the minor band above ALG-2 is self-phosphorylated degraded ASK1). ASK1 did not phosphorylate IgG in lane 1, but Western blot showed the existence of IgG light chain (Fig. 3, middle panel, lane 1) and ASK1 was equally loaded (Fig. 3, bottom panel), indicating that ALG-2 is also the substrate of ASK1. Furthermore, studies have also shown that ASK1 co-immunoprecipitates with Raf-1 [25], and that Raf-1 negatively regulates

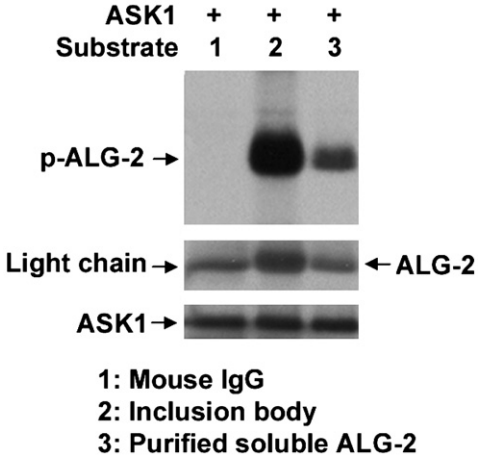

Fig. 3. ASK1 phosphorylates ALG-2. Kinase assay were performed using ASK1 as kinase, mouse IgG as negative substrate control (lane1), solubilized inclusion bodies (lane 2), and purified soluble ALG-2 (lane 3) as substrates. Kinase assay, SDS–PAGE, and Western blot were performed as in Fig. 2. After autoradiography, the membrane was then probed with anti-His antibody, followed by anti-mouse IgG secondary antibody (middle and bottom panels).

ASK1 signaling [26] but the mechanism is not clear. Since we did not find that Raf-1 and ASK1 phosphorylate each other (data not shown), suggesting that Raf-1 negatively regulates ASK1 signaling by blocking the signal from ASK1 to its downstream, and since both ASK1 and Raf-1 interact with ALG-2 and phosphorylate ALG-2, we carried out kinase assay to determine whether Raf-1 interferes with the ALG-2 phosphorylation by ASK1. As shown in the panel b of Fig. 4A (panel a is short exposure of panel b upper part), in the presence

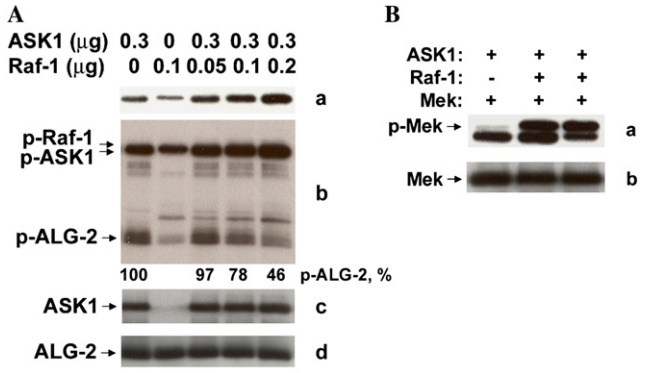

Fig. 4. (A) Raf-1 antagonizes ALG-2 phosphorylation by ASK1. ALG-2 was used as substrate in kinase assay with constant amounts of ASK1 and increasing amount of Raf-1. The density of phosphorylation bands was determined by gel-pro analyzer. After autoradiography (upper panel), the membrane was probed with anti-ASK1 and anti-His antibodies, respectively (panels c and d). (B) ASK1 does not affect Mek phosphorylation by Raf-1. Mek was used as substrate in kinase assay with ASK1, Raf-1, or both ASK1 and Raf-1 as kinase. After autoradiography (upper panel), the membrane was probed with anti-Mek antibody (lower panel).

of Raf-1, the phosphorylation of ALG-2 by ASK1 is decreased, in a concentration-dependent manner. Western blot showed that ASK1 and ALG-2 were added equally in the panels c and d of Fig. 4A. Then, we examined whether ASK1 affected Raf-1's function. As shown in the upper bands of Fig. 4B, we did not find that ASK1 interfered with Mek phosphorylation by Raf-1 (in the Fig. 4B, lower bands of upper panel are auto-phosphorylated ASK1 and Raf-1). Western blot showed that Meks were added equally (Fig. 4B, lower panel). Since single phospho-ALG-2 bands were observed when Raf-1 was used as kinase (Figs. 2B and 4A), but doublet phospho-ALG-2 bands existed when ASK1 was used as kinase (Fig. 4A), we loaded half volume of kinase assay reactions to non-denaturing gel and the other half volume of these to SDS–PAGE (Figs. 5A and B). As shown

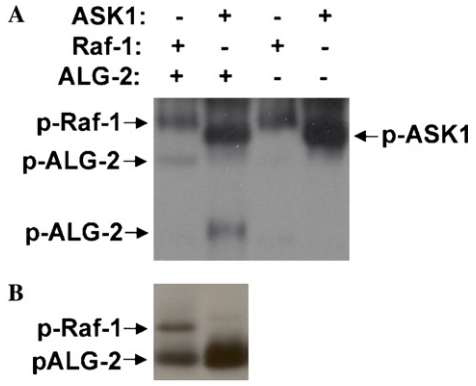

Fig. 5. Raf-1 and ASK1 phosphorylate ALG-2 differently. ASK1 alone, Raf-1 alone, ASK1 plus ALG-2, and Raf-1 plus ALG-2 were subjected to kinase assay, loaded on non-denaturing gel and SDS–PAGE, respectively, and then transferred to membrane and analyzed by autoradiography.

in Fig. 5A, ASK1-phosphorylated ALG-2 migrated faster than Raf-1-phosphorylated ALG-2, indicating that Raf-1 and ASK1 phosphorylate ALG-2 differently, most likely at different sites. Taken together, our results showed that Raf-1 specifically interfered with the ALG-2 phosphorylation by ASK1.

Discussion

Raf-1 has been intensively studied for years, and many kinases and proteins have been found to interact with it [8,11,28,29]. However, the biological role of Raf-1 is not fully understood. A recent study with Raf-1 knockout mice showed that the essential and unique function of Raf-1 is anti-apoptotic [1,2]. The molecule that mediates Raf-1's anti-apoptotic signal is not known. Therefore, we carried out a yeast two-hybrid experiment to identify the Raf-1 substrate that mediates its anti-apoptotic function. We found that the Raf-1 kinase domain interacts with a pro-apoptotic protein ALG-2 in addition to its interaction with Mek1. In further kinase assay studies, we found that Raf-1 specifically phosphorylated ALG-2. We used solubilized inclusion bodies that contain ALG-2 and many other proteins as substrate, yet Raf-1 only phosphorylated ALG-2 (Fig. 2B). We also found that ASK1 strongly phosphorylated ALG-2. Importantly, Raf-1 interferes with the ALG-2 phosphorylation by ASK1.

ALG-2 is a Ca^{2+}-binding protein belonging to the penta-EF hand protein family and was originally discovered as a pro-apoptotic protein required for T cell receptor (TCR)-, Fas-, and glucocorticoid-induced cell death [19,20]. How ALG-2 is involved in apoptosis is not clear [19,30], although early analyses indicated that ALG-2 was located downstream of the ICE/Ced-3 signaling cascade activated by TCR and Fas [31]. A recent study showed that ALG-2 was associated with ASK1 [21]. ASK1 has been found to be involved in apoptosis [23,32]. ASK1 interacts with Fas receptor associated protein Daxx and TNF receptor associated factors (TRAF), and acts upstream of the JNK and p38 Map kinase pathways when stimulated by a variety of stimuli such as Fas, oxidative stress, and DNA damage [22–24,33]. We have now shown that ASK1 phosphorylates ALG-2, indicating that ALG-2 may be a component of the ASK1-related apoptotic pathway. Whereas, it has been shown that Raf-1 co-immunoprecipitated with ASK1 [25] and blocked ASK1-induced apoptosis, and in the Raf-1 knockout mouse cells, ASK1/JNK or/p38 signaling increased significantly [26], suggesting that Raf-1 negatively regulates ASK1-mediated apoptotic signaling. However, the mechanism of Raf-1 negative regulation of ASK1 is not clear. Although Raf-1 and ASK1 can be co-immunoprecipitated, it has not been determined whether Raf-1 and ASK1 directly interact

since they may form a ternary complex with other proteins. The molecules that interact with both Raf-1 and ASK1 include 14-3-3 protein and ALG-2. However, a study has shown that 14-3-3 protein was not involved in the formation of the complex of Raf-1 and ASK1 [25]. Thus, ALG-2 is likely to be the candidate that connects Raf-1 and ASK1 signalings.

Indeed, we did not find that the two kinases, ASK1 and Raf-1, phosphorylate each other, suggesting that Raf-1 inhibits ASK1-induced apoptosis by blocking the signal from ASK1 to its downstream target instead of affecting ASK1 directly. Here, we show that Raf-1 and ASK1 phosphorylate ALG-2 differently and that Raf-1 interferes with ASK1-dependent ALG-2 phosphorylation. Most likely, phosphorylation by Raf-1 causes ALG-2 conformation change, which is not favorable for ASK1 phosphorylation. It is also possible that the binding of Raf-1 to ALG-2 blocks the access of ASK1 to ALG-2. This may explain why overexpression of Raf-1 mutation K375M also can block ASK1-induced apoptosis [25]. Furthermore, although ALG-2 connects Raf-1 and ASK1 signals, the signal seems to be one way from Raf-1 to ASK1 since ASK1 has no effect on the phosphorylation of Mek by Raf-1. These observations indicate that Raf-1 may exert its anti-apoptotic function by interrupting the ASK-dependent-ALG-2 phosphorylation.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2005.05.074.

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