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Novel anti-apoptotic mechanism of A20 through targeting ASK1 to suppress TNF-induced JNK activation

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Abstract

The zinc-finger protein A20 has crucial physiological functions as a dual inhibitor of nuclear factor-κB (NF-κB) activation and apoptosis in tumor necrosis factor (TNF) receptor 1 signaling pathway. Although the molecular basis for the anti-NF-κB function of A20 has been well elucidated, the anti-apoptotic function of A20 is largely unknown. Here, we report a novel mechanism underlying the anti-apoptotic function of A20: A20 blocks TNF-induced apoptosis through suppression of c-jun N-terminal kinase (JNK) by targeting apoptosis signal-regulating kinase1 (ASK1). First, the ectopic expression of A20 drastically inhibits TNF-induced JNK activation and apoptosis in multiple cell types including those deficient of NF-κB activation. Unexpectedly, the blunting effect of A20 on TNF-induced JNK activation is not mediated by affecting the TNFR1 signaling complex formation. Instead, A20 interacts with ASK1, an important MAPKK kinase in the JNK signaling cascade. More importantly, overexpression of wild-type A20, but not of mutant A20 (ZnF4; C624A, C627A), promotes degradation of the ASK1 through the ubiquitin-proteasome system. Taken together, the results from this study reveal a novel

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Conflict of interest

The authors declare no conflict of interest.

anti-apoptotic mechanism of A20 in TNF signaling pathway: A20 binds to ASK1 and mediates ASK1 degradation, leading to suppression of JNK activation and eventually blockage of apoptosis.

Keywords

TNF receptor; JNK; NF-κB; apoptosis; cellular signaling

In most cell types, it has been well established that activation of nuclear factor-κB (NF-κB) antagonizes apoptotic cell death triggered by tumor necrosis factor receptor1 (TNFR1) and several other stimuli.[1,2] Although the mechanisms by which anti-apoptotic genes that are regulated by NF-κB inhibit TNF-induced apoptosis remain to be fully explored, it has been well established that the anti-apoptotic function of NF-κB downstream of TNFR1 involves suppression of the c-jun N-terminal kinase (JNK) cascade.[3–5] Indeed, in mouse embryonic fibroblast cells (MEFs) lacking p65, inhibitory κB kinase (IKKβ), or stably expressing superrepressor IκBα, there is persistent JNK activation in response to TNF because of the lack of expression of NF-κB-mediated JNK inhibitors.[6,7] This persistent JNK activation contributes to TNF-induced apoptosis, making those MEFs highly sensitive to TNF-induced apoptosis. Earlier, it has been proposed that a subset of NF-κB target genes including growth arrest and DNA damage-inducible 45β (GADD45β), X-linked inhibitor of apoptosis protein (XIAP), manganese superoxide dismutase (MnSOD), and ferritin heavy chain (FHC) are capable of antagonizing JNK signaling.[3,6,8,9] However, the existing literature on the function of these proteins in TNFR1-mediated JNK signaling cascade is still confusing and controversial. For instance, genetic analysis of the GADD45β- or XIAP-deficient mice showed no defect in TNF-induced JNK activation and apoptosis.[10,11] Moreover, in several NF-κB-deficient cells, MnSOD expression did not substantially inhibit TNF-induced apoptosis,[7,9] suggesting that other proteins might be required cooperatively or independently to suppress the JNK signaling pathway. Thus, it seems that the primary mediators of NF-κB-mediated control of JNK signaling downstream of TNFR1 remain elusive.

As one of the NF-κB target genes, A20 has been well established as a negative feedback loop to block NF-κB activation in response to TNF.[12–15] Recently, substantial progress has been made in understanding the biochemical and molecular mechanisms by which A20 constitutes a negative feedback loop to terminate NF-κB activation. A20 possesses dual ubiquitin editing functions to receptor interacting protein1 (RIP1), a critical signaling molecule in TNF-mediated NF-κB activation. A20 is capable of deubiquitinating K63-linked polyubiquitin chains of RIP1 by its N-terminal ovarian tumor (OTU) domain and adding K48-linked ployubiquitin chains by the C-terminal zinc finger (ZnF) containing domain.[13–15] In addition to RIP1, other ubiquitinated proteins such as TRAF6 and CIAP1 have been identified as A20 targets in the NF-κB signaling pathway.[16–18] Given the well established anti-apoptotic function of NF-κB and the ability of A20 to terminate NF-κB activity, A20 is expected to possess a potent pro-apoptotic function. However, the accumulating evidence suggests the opposite: A20 acts mainly as an anti-apoptotic protein, as it protects most cells from TNF-induced cell death. Overexpression of A20 protects breast cancer MCF-7 cells, fibrosarcoma WEHI164 cells, embryonic fibroblast NIH3T3 cells, and L929 cells from TNF-mediated apoptosis or necrosis.[19–21] A20 also protects the cells from a

number of apoptotic stimuli other than TNF. For instance, A20 protects endothelial cells from Fas/CD95 receptor ligation, lymphoblastic B cells from serum depletion, and macrophages from oxidized low-density lipoprotein.[22–24]

However, up to date it is still largely unknown how A20 paradoxically protects cells from TNF-induced apoptosis even it is capable of inhibiting NF-κB. Therefore, we aimed to investigate the molecular mechanisms underlying the anti-apoptotic function of A20 by focusing its regulatory function in JNK signaling downstream of TNFR1. The results from this study reveal a novel anti-apoptotic mechanism of A20 in TNF signaling pathway: A20 binds to apoptosis signal-regulating kinase1 (ASK1) and mediates ASK1 degradation, leading to suppression of JNK activation and eventually blockage of apoptosis.

Results

A20 has an essential function in the NF-κB-mediated suppression of JNK activation and apoptosis on TNFR1 ligation

To test whether A20 is involved in TNF-induced JNK activation, we first examined the time course of JNK activation after TNF treatment in the cells overexpressing A20 by ectopic infection of recombinant adenoviral vector carrying A20 (rAd-A20). TNF treatment resulted in a rapid and progressive induction of A20 expression, and overexpression of A20 efficiently blocked TNF-induced IκBα degradation (Figure 1a and b), which is consistent with an earlier report that A20 functions as a negative regulator of NF-κB.[16] Pretreatment with a protein synthesis inhibitor, cycloheximide (CHX), which blocks NF-κB-dependent transcriptional events, not only abolished A20 expression, but also resulted in persistent JNK activation in response to TNF, suggesting that TNF-inducible *de novo* protein synthesis, including A20, is required to suppress the persistent JNK activation. More importantly, overexpression of A20 led to abolishment of JNK activation, as well as IκBα degradation after treatment with TNF and CHX (TNF/CHX) (Figure 1a and b). Consistently, knockdown of A20 by specific small hairpin RNA (shRNA) elicited the persistent IκBα degradation and JNK activation after TNF treatment (Supplementary Figure S1), indicating that A20 has a dual capacity to have in down-regulation of TNF-induced NF-κB and JNK activation. Moreover, cells with overexpression of A20 were found to be more resistant to apoptotic cell death induced by TNF/CHX, as evidenced by the cell viability assay (Figure 1c and d) and cleavage of both caspase-3 and poly(ADP-ribose)polymerase (PARP) (Figure 1e). Pretreatment of JNK-specific inhibitor SP600125 dramatically suppressed TNF/CHX-induced cell death and the cleavage of caspase-3 (Figure 1f and g), confirming that the JNK activation contributes in TNF-induced apoptosis, as reported earlier.[5] On the other hand, pretreatment of an irreversible caspase-8 inhibitor z-IETD-fmk also partially suppressed TNF/CHX-induced apoptosis and caspase-3 cleavage, but with a lesser extent than that of SP600125 (Figure 1f and g). These results suggest that under the condition of NF-κB blockage, persistent JNK activation is a major cell death mechanism in TNF-induced apoptosis.

To investigate directly whether A20 is responsible for the NF-κB-mediated suppression of JNK signaling, rAd-A20 was infected ectopically into MEFs that are null of functional components of NF-κB signaling. Similar to HeLa and HEK293 cells as shown earlier, TNF

treatment induced progressive expression of A20 and transient JNK activation in wild-type (WT) MEFs (Figure 2a). In contrast, in p65−/−, IKKβ−/−, and TNFR-associated factor 2/5 (TRAF2/5) double knockout (DKO) MEFs, there was no induction of A20, but with more persistent JNK activation after TNF treatment (Figure 2b–d, left panels). Remarkably, overexpression of A20 almost completely blocked the persistent JNK activation in these cells deficient of NF-κB (Figure 2b–d, right panels). Earlier, it has been reported that NF-κB-defective cells are highly susceptible to TNF-induced apoptosis, because of persistent JNK activation.[7,25] Consistently, overexpression of A20 in NF-κB-deficient cells offered significant protection against TNF-induced cell death (Figure 2e–g). Taken together, these findings show that the capacity of A20 to block the persistent JNK activation is responsible for its anti-apoptotic function against TNF-induced killing in multiple cell types studied.

As NF-κB-mediated suppression of JNK activation is mainly executed through induction of its target genes, we next examined whether A20 affects the transcriptional induction of NF-κB-mediated target genes, which are known to inhibit JNK signaling. Here, we found that in cells infected with the control vector (rAd-GFP), both A20 and GADD45β were transiently induced with TNF treatment, whereas the induction of A20 was more evident and persistent than that of GADD45β (Figure 3a, left panel). Notably, the transcription of other NF-κB target genes (Mn-SOD, XIAP, and FHC) were marginally induced or occurred at late time after TNF treatment. Importantly, TNF-induced up-regulations of these genes, including GADD45β, were impaired by A20 overexpression (Figure 3a, right panel), which is likely because of the inhibitory effect of A20 on NF-κB signaling, as evidenced by the absence of p65 nuclear translocation in those cells with overexpression of A20 (Figure 3b). Taken together, data from this part of the study thus suggest that A20 is an essential NF-κB-mediated target gene to control JNK signaling after the TNF receptor ligation.

A20 does not affect the TRAF2 recruitment into TNFR1 signaling complex on TNF stimulation

It is worth noting that A20 is recruited into TNFR1 signaling complex by interacting with TRAF2, a molecule required for activation of TNF-induced JNK signaling.[26,27] Therefore, it is hypothesized that the inhibitory effects of A20 on TNF-induced JNK activation might be achieved through disruption of the TNFR1 signaling complex involving TRAF2. To assess whether A20 directly affects the formation of the plasma membrane bound TNFR1 signaling complex (complex I), the recruitment of RIP1 and TRAF2 into this complex was examined. Treatment of cells with TNF led to immediate recruitment of RIP1 and TRAF2, as well as A20 into complex I (Figure 4a, left panel). Whereas the recruitment of RIP1 into complex I in rAd-GFP-infected cells was rather transient after TNF treatment, such RIP1 recruitment remained to be hyperubiquitinated in rAd-A20-infected cells (Figure 4a, first row). However, unexpectedly, the extent and duration of TNF-induced TRAF2 recruitment into complex I was not affected by A20 overexpression (Figure 4a, second row), indicating that suppression of TNF-induced JNK activation by A20 is independent of TRAF2 recruitment into complex I. This conjecture also seems to be consistent with the earlier observation that A20 is capable of protecting TRAF2/5 DKO MEFs against TNF-induced cell death (Figure 2g). To further assess whether A20 affects TNF-induced JNK activation by acting on the site of complex I, the binding of RIP1 and TRAF2 with TNFR1 in those NF-κB activation-deficient cells were

monitored. As expected, TNF treatment of p65−/−, IKKβ−/−, and TRAF2/5 DKO MEFs failed to express A20 (Figure 4b, fifth row) and resulted in no detectable recruitment of A20 into TNFR1 (Figure 4b, third row). Interestingly, TNF-induced RIP1 and TRAF2 recruitments were found to be normal in p65−/− and IKKβ−/− MEFs, although TNF-induced RIP1 ubiquitination was more quickly disappeared in these NF-κB activation-deficient MEFs (Figure 4b, top two rows). From the results of above protein-binding experiments, we conclude that the inhibitory effect of A20 on TNF-mediated JNK activation is not achieved through disruption of proximal TNFR1 complex (complex I) formation.

We further examined whether A20 affects the TNFR1-associated cytoplasmic complex (complex II) formation, as reported earlier.[28] Immunoprecipitation assay with a anti-FADD antibody showed that A20 was recruited into complex II in a time-dependent manner after TNF, but not TNF/CHX treatment (Figure 5a, second row). On the other hand, TNF-induced interaction between processed caspase-8 p43/41 and FADD seemed mainly in the presence of CHX (Figure 5a, first row), suggesting that the recruited A20 into complex II may affect the FADD-mediated caspase-8 activity. However, the overexpression of A20 partly suppressed the interaction between caspase-8 and FADD, as well as caspase-8 cleavage after TNF/CHX treatment (Figure 5b, first and fourth rows), whereas it markedly suppressed caspase-3 cleavage (Figure 5b, bottom row). Together with the observations that the potency of the specific caspase-8 inhibitor to suppress TNF-induced apoptosis (Figure 1f and g), these data thus suggest that the recruitment of A20 into complex II is less likely to be of importance for its anti-apoptotic function under the condition of TNF/CHX treatment.

A20 physically interacts with ASK1 in the JNK signaling cascade

As it seems that the inhibitory effect of A20 on TNF-induced JNK activation is neither associated with TNFR1 signaling complex formation nor the downstream NF-κB-mediated transcription of target genes, we next examined whether A20 physically interacts with the components of JNK signaling cascades. To address this issue, HEK293 cells were transiently transfected with Xp-tagged A20 and flag-tagged kinases [mitogen-activated protein kinase kinase kinase (MAP3K) (GCK, MEKK3, ASK1), mitogen-activated protein kinase kinase (MAP2K) (MKK4/7) or mitogen-activated protein kinase (MAPK) (JNK1/2)], and the protein–protein interactions were assessed. Notably, A20 bound strongly to ASK1, but not to other MAP3K, MAP2K, or MAPK tested (Figure 6a–c). The endogenous association of A20 with ASK1 was also confirmed in HEK 293 cells (Figure 6d and e). More importantly, TNF treatment led to an enhanced interaction between A20 and ASK1, and such interaction was dependent on A20 expression (Figure 6f). To determine the interaction regions, the deletion mutants of ASK1 and A20 were constructed and analyzed for their ability to interact, respectively (Figure 6g and i). ASK1 mutant containing a non-catalytic amino-terminal region (ASK1-N), but not the ASK1-K (containing the kinase domain) or the ASK1-C (containing the carboxy-terminal region), was able to interact with A20 (Figure 6h). In parallel, A20-N, but not A20-C, bound to ASK1 (Figure 6j). The specific association between A20 and ASK1 through the amino-terminal region was further confirmed in the cells co-transfected with each of amino-terminal fragments (Figure 6k), clearing indicating that ASK1 and A20 use their amino-terminal regions for the protein–protein interaction.

A20 reduces the stability and promotes the degradation of ASK1 through the ubiquitination process

A20 restricts TNF-induced NF-κB signaling through its ubiquitin editing activity on RIP1,[14,15] it is thus of interest to test whether A20 would be affecting the stability of ASK1 through a similar mechanism. To address this hypothesis, we first examined whether the protein level of ASK1 was changed by overexpression of A20. TNF/CHX or high concentration of hydrogen peroxide (H2O2) treatment resulted in substantial increase of ASK1 expression (Figure 7a, top row). Such observations are indeed consistent with an earlier report,[29] and indicate that such extracellular stimuli may enhance ASK1 stability. Notably, the level of ASK1 was dramatically decreased in cells with overexpression of A20 (Figure 7a, top row), without alteration of RIP1 and TRAF2 protein levels. In the same blot, TNF- or H2O2-induced JNK activation was suppressed by A20 overexpression (Figure 7a, second row). Moreover, H2O2 induced the cleavage of caspase-3 and PARP was significantly blocked by A20 overexpression (Supplementary Figure S2).

To further evaluate the effect of A20 on ASK1 protein level under the physiological condition, endogenous ASK1 and A20 levels were determined at different time points after TNF treatment. Indeed, there was enhanced ASK1 level at earlier time points (10 to 30 min), but started to decrease by 1 h when A20 was coincidently induced (Figure 7b, left panel). However, the enhanced protein level and kinase activity of ASK1 by TNF continued to maintain up to 2 h in cells treated with TNF/CHX (Figure 7b, right panel), suggesting that induced expression of A20 by TNF may affect the protein stability of ASK1. To confirm this possibility, we examined whether the kinetics of ectopically expressed ASK1 levels are altered by A20 overexpression in the presence of CHX. Consistently, progressive reduction of ASK1 level was observed in cells with overexpression of A20, whereas the ASK1 level remained stable up to 6 h after CHX treatment in cells without A20 expression (Supplementary Figure S3). Furthermore, the proteasome inhibitor MG132 significantly reversed the effect of A20 overexpression on the ASK1 protein level (Supplementary Figure S4), indicating that A20 targets ASK1 degradation through the proteasome-dependent pathway. We next examined the susceptibility of WT and ASK1−/− MEFs in response to TNF/CHX, aiming to understand the function of ASK1-mediated JNK activation in TNF-induced killing. As consistent with an earlier report,[30] ASK1−/− MEFs was defective in persistent JNK activation, and highly resistant to TNF/CHX-induced cell death as compared with WT MEFs (Supplementary Figure S5). These results thus suggest that A20 may target ASK1 degradation, thereby suppress ASK1-mediated JNK activation, and eventually inhibit TNF-induced apoptosis.

To address whether the conserved ZnF domain of A20 might be responsible for ASK1 degradation, the full-length A20 with point mutations of conserved cystein residue to alanine within OTU (C103A), ZnF3 (C521A, C524A), or ZnF4 (C624A, C627A) domain were generated, as reported earlier.[16] Overexpression of WT- or OTU MT-A20 dramatically decreased ASK1 level, whereas ZnF4 MT- or ZnF3/ZnF4 MT-A20 almost completely eliminated the effect of A20 on ASK1 level (Figure 7c, top row). Such results thus indicate that the conserved ZnF4 domain of A20 is critical for ASK1 degradation. To determine whether A20 ligates ubiquitin chains to ASK1, the immunoprecipitants of endogenous

ASK1 were assayed by immunoblotting with anti-ubiquitin. Strikingly, overexpression of A20 reduced protein level of ASK1 with a concomitant increase of polyubiquitinated ASK1 (Figure 7d, top two rows). Moreover, in cells treated with TNF, there was increased level of polyubiquitinated ASK1 from 30 min onward (Figure 7e, left panel), whereas overexpression of A20 markedly enhanced ASK1 polyubiquitination (Figure 7e, right panel), confirming the ability of A20 to mediate ASK1 ubiquitination and degradation. To rule out the possibility of non-specific interaction of anti-ASK1 polyclonal antibody used in the immunoprecipitation assay, cells were co-transfected with flag-ASK1 and HA-ubiquitin, and then tested the ability of A20 to ubiquitinate the ASK1. Consistently, TNF treatment triggered ASK1 ubiquitination and this ASK1 ubiquitination was dramatically exaggerated by overexpression of A20 (Figure 7f). Taken together, we present clear evidence that A20 blunts TNF-induced JNK activation and apoptosis through interaction with ASK1 to promote its ubiquitination and subsequent proteasomal-dependent degradation.

Enhanced expression of A20 in breast cancer

The specific pathologic context where A20 participates as a predominant player in the cell death and survival process might allow a selective link between A20 and the ASK1–JNK pathway in certain types of human diseases, such as cancer. Thus, we examined the expression levels of A20 in human breast cancer tissues. As consistent with earlier reports,[31,32] the expression levels of A20 were markedly enhanced in breast cancer tissues, as evidenced by immunohistochemistry and immunoblot analysis (Figure 8a and b). Cancer tissues had much higher immunoreactivity than the surrounding normal tissues, and the punctuated granular expression of A20 associated with certain vesicles was observed mainly in the cytoplasm (Figure 8a). On the contrary to the expression pattern of A20, breast cancer tissues expressed lower levels of ASK1 in almost all the samples tested (Figure 8b, top two rows). Taken together, these results suggest that enhanced expression of A20 may contribute to cellular survival through selective inhibition of the ASK1–JNK signaling during the tumorigenic processes.

Discussion

Herein, we have identified a novel mechanism responsible for the anti-apoptotic function of A20 in TNF signaling. A20 acts as a principle mediator in the termination of TNFR1-mediated JNK activation. More importantly, the engagement of A20 in this process is independent of TNFR1 signaling complex formation. Finally, we provide evidence that A20 interacts with ASK1, leading to ASK1 polyubiquitination and proteasomal degradation. Therefore, it is believed that A20 is an important element in the cross-talk between NF-κB and JNK that largely determines the fate of the cell in response to TNF.

It has been well established that suppression of the JNK signaling pathway represents a crucial protective mechanism mediated by NF-κB from TNF-induced apoptosis.[3] Earlier reports suggest that NF-κB-inducible proteins such as GADD45β, XIAP, MnSOD, and FHC have important functions in suppression of JNK activation.[3,8,9] In this study, MnSOD was induced by TNF at late times after the onset of the persistent JNK activation and apoptosis (Figure 3a). Furthermore, XIAP and FHC were marginally or poorly induced by TNF

treatment. Thus, it seems that these factors are unlikely to be important mediators in JNK inhibition by NF-κB. One critical finding from this study is that A20 not only completely blocked the transcription of other NF-κB target genes known to antagonize JNK signaling, but also effectively suppressed TNF-induced JNK activation and apoptotic cell death in NF-κB activation-deficient MEFs (Figure 2). Thus, these findings clearly indicate that A20, rather than other described earlier JNK inhibitors, has a more predominant function in the suppression of TNF-induced JNK signaling under physiological TNFR1 ligation condition.

With regard to TNFR1 signaling, TRAF2 binds to the amino-terminal region of A20, and this interaction is required for the recruitment of A20 into TNFR1 signaling complex.[26,27] Therefore, it is possible that A20 may regulate JNK signaling pathway by affecting this signaling complex formation. Indeed, an early study reported that A20 inhibited the recruitment of death domain proteins TRADD and RIP1 into TNFR1 signaling complex and thus protected Jurkat T-cell from TNF-induced apoptosis.[33] In this study, A20 is also found to be transiently recruited into TNFR1 complex I as well as complex II after TNF treatment (Figures 4 and 5). Notably, TNF-induced TRAF2 recruitment into TNFR1 signaling complex was not affected by A20, indicating that the inhibitory effect of A20 on TNF-induced JNK activation is unlikely to be achieved through TRAF2. This conclusion was further supported by the fact that TNF-induced TRAF2 recruitment was normal in the condition of CHX pretreatment (data not shown) or in NF-κB activation-deficient MEFs (Figure 4b). These discrepancies with an early report[33] might be resulted from cell-type difference and/or differences in experimental systems (transient adenoviral infection *versus* stable transfection of A20). Although TRAF2 is important to activate ASK1 and JNK in response to TNF or H2O2,[4,34] we observed that overexpression of A20 sufficiently blocked the TNF-induced JNK activation and apoptosis in TRAF2/5 DKO MEFs (Figure 2). In this regard, it was of interest to determine whether ASK1 might be activated in TRAF2/5DKOMEFs after TNF treatment. Interestingly, TNF-induced persistent phosphorylation of ASK1 preserved in TRAF2/5 DKO MEFs (Supplementary Figure S6), indicating that TRAF2 is not absolutely required for TNF-induced ASK1 activation. Earlier, it has been proposed that ROS have an important function in TNF-induced persistent JNK activation under NF-κB-deficient cells including TRAF2/5 DKO MEFs.[7] As TRAF6 is also required for ROS-mediated activation and ASK1 and JNK signaling pathway,[35] one possibility is that, in case of TRAF2/5 deficiency, TRAF6 might be involved in TNF-mediated ROS formation and JNK activation.

It is well known that the duration of JNK activation is a critical factor in determining the susceptibility of cells to TNF-induced apoptosis: persistent JNK activation contributes to TNF-induced cell death.[36] Acute JNK induction by TNFR1 likely involves MAP3K of the MAPK/ERK kinase (MEK) and mixed-lineage kinase (MLK) groups.[37] Conversely, the persistent JNK activation downstream of TNFR1 occurs through activation of the ASK1.[30] In this respect, the fact that A20 binds to ASK1, but not to GCK or MEKK3, and inhibits TNF- or H2O2-induced JNK activation is particularly interesting. Furthermore, the results of domain mapping experiments show that A20 binds to the non-catalytic amino-terminal region of ASK1, which has been shown to be responsible for the interaction with E3 ligase-CIAP1, -TRAF2, or thioredoxin,[38,39] suggesting that the association of ASK1 through this amino-terminal region with A20 has an essential function in down-regulation of JNK

signaling through regulating ASK1 stability and degradation. Earlier, it has been shown that the protein stability of ASK1 mediated through interaction with E3 ligases is an important mechanism for the regulation of ASK1–JNK signaling cascade in response to ROS or TNF.[29,38,39] Therefore, it is conceivable that the complex formation with ASK1 and A20 is a critical step for suppression of the ASK1–JNK signaling cascade and subsequent apoptotic cell death. However, as there are multiple signaling molecules including A20 and other ASK1-binding proteins recruited into the ASK1 signalosome,[35] further elucidation of dynamic interactions of these signaling molecules within the ASK1 complex is required to provide a better understanding of physiological functions of such a complex. One important hint from this study is that the expression levels of A20 were increased significantly in breast cancer tissues (Figure 8), suggesting that A20 may have an important function in promoting the carcinogenic processes through its pro-survival function. However, intriguingly, some recent genetic analysis showed that A20 was frequently inactivated by somatic mutation or deletion in certain types of tumors including B-lineage lymphomas.[40,41] Therefore, further studies are required to define the functional characteristics of A20 in tissue-specific malignancies such as breast cancer.

Materials and Methods

Antibodies

All commercial antibodies were purchased from the following: anti-JNK, anti-RIP1, and anti-PARP (BD Biosciences, San Jose, CA, USA); anti-phospho-JNK1/2 (Invitrogen, Carlsbad, CA, USA); anti-actin and anti-flag (Sigma-Aldrich, St. Louis, MO, USA); anti-IKKβ (Upstate Biotechnology, Waltham, MA, USA); anti-caspase-3 (Cell Signaling Technology, Beverly, MA, USA); anti-TNFR1 (R&D Systems, Minneapolis, MN, USA); anti-A20, anti-p65, anti-ubiquitin, anti-IKKα, anti-TRAF2, anti-Xpress, anti-HA, anti-ASK1, anti-IκBα, and anti-GFP (Santa Cruz Biothenology, Santa Cruz, CA, USA). Anti-phospho-ASK1 antibody was a kind gift from Dr. H Ichijo (The University of Tokyo, CREST, Japan).

Plasmids and adenovirus production

Xpress-tagged full-length A20 were prepared by amplifying the human normal colon tissue and MEFs of cDNA library in-frame with corresponding primers into pcDNA 3.1C (Invitrogen). Flag-tagged constructs (flag-GCK, flag-MEKK3, flag-ASK1, flag-MKK4, flag-MKK7, flag-JNK1, and flag-JNK2) were generated using pENT/CMV vector (Stratagene, La Jolla, CA, USA). HA-Ub was provided by E Junn (University of New Jersey-Robert Wood Johnson Medical School, NJ, USA). Deletion mutants (A20-N, A20-C, ASK1-N, ASK1-K, and ASK1-C) were generated by standard PCR methods and point mutants at cystein 103 (OTU-MT-A20, C103A), 521/524 (ZnF3-MT-A20, C521A, C524A), and 624/627 (ZnF4-MT-A20, C624A, C627A) were created using a Quickchange kit as directed by the manufacturer (Stratagene). Adeonoviruses encoding the full-length A20 and GFP were created using Virapower adenovirus expression system according to the manufacturer's instruction (Invitrogen). Site-specific recombination between entry vector (A20-pENT/CMV-flag) and adenoviral destination vector (pAd/PL-DEST) were established with LR clonase II (Invitrogen).

Cell culture, adenovirus infection, and transfection

HeLa cells, HEK293 cells, and MEFs (WT, p65−/−, IKKβ−/−, TRAF2/5 DKO, and ASK1−/−) were maintained in DMEM supplemented with 10% fetal bovine serum, 2mmol/l of glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin (Life Technology, Carlsbad, CA, USA). HEK293 cells were transfected with Lipofectamine PLUS reagent by following the instruction provided by the manufacturer (GIBCO/BRL). Alternatively, HeLa and MEFs were infected with recombinant virus with the titers of 200 PFU per cells obtained A20-pAd/PL-DEST-transfected 293A Phoenix packaging cells.

Tissue samples and preparation of homogenates

Tissue samples were obtained from sequential patients during surgical operations for malignant breast cancers at Chungnam National University Hospital, Daejeon, Korea. The study was approved by the hospital institutional review board (approval number 07–21) according to the Declaration of Helsinki, and written informed consent was obtained from each patient by research team before surgery. After washing with cold saline, normal and cancerous tissues were homogenized in 100mM Tris–HCl (pH 8.0) and 1% Triton X-100. Tissue homogenates were centrifuged at 1500 × *g* for 15 min to remove debris. After clarification, the homogenates were centrifuged at 100 000 × *g* for 1 h at 4°C. Protein concentration of homogenates was determined for each sample using the Bradford method (Bio-Rad, Hercules, CA, USA).

Immnunoblot analysis and immunoprecipitation

After treating with different conditions as described in the figure legends, the cells and human breast tissues were lysed in M2 buffer (20mM Tris, pH 7.6, 0.5% NP-40, 250mM NaCl, 3mM EDTA, 3mM EGTA, 2mM dithiothreitol, 0.5mM PMSF, 20mM β-glycerol phosphate, 1mM sodium vanadate, and 1 μg/ml leupeptin). For immunoblots, 50 μg of the lysates was fractionated by SDS–polyacrylamide gel and visualized by enhanced chemiluminescence (Amersham). For immunoprecipitation assays, the cells were collected in M2 buffer after treatments as described in the figure legends. The resulting lysates were precipitated with the relevant antibody and protein G-sepharose beads by incubation at 4 °C overnight or for 4 h. Immunoprecipitates were washed three times with M2 buffer, and the bound proteins were resolved in 10% SDS–polyacrylamide gels for immunoblot analysis. Immune complex kinase assay for ASK1 was performed as described in detail earlier.[29]

RT–PCR analysis

After total RNA was extracted from HeLa cells using a TRIZOL reagent (Life Technologies), the oligo(dT)-primed cDNA was synthesized using a RT–PCR kit (Stratagene). PCR amplication was carried out using the primer pairs specific to each human genes (A20, 5′-GCACACTGTGTTTCATCG-3′ and 5′-GGCATACATTGCTTGAAC-3′; Gadd45, 5′-ATGACGCTGGAAGAGCT-3′ and 5′-GGGCCTCAGCGTTCCT-3′; XIAP, 5′-AGAGCTGGATTTTATGCT-3′ and 5′-TTCAGCACATTGTTTACA-3′; IκBα, 5′-AAGACGAGGAGTACGAGC-3′ and 5′-TGACATCAGCACCCAAGG-3′; FHC1, 5′-CTGCTTCAACAGTGCTTG-3′ and 5′-GTTTGTGCAGTTCCAGTA-3′; Mn-SOD, 5′-

AACGCGCAGATCATGCA-3′ and 5′-CTCCCAGTTGATTACATTC-3′; GAPDH, 5′-GACCCCTTCATTGACCTC-3′ and 5′-GCCATCCACAGTCTTCTG-3′).

Determination of cell death

After treatment, as described in the figure legends, cells were trypsinized and collected. Each sample was stained with trypan blue (Bio-Whittaker, Walkersville, MO, USA) and counted with a hemacytometer. The stained cells (blue) were counted as dead cells and were expressed as a percentage of total cells. Data are expressed as the mean ± S.E. from at least three independent experiments.

Lentiviral packaging and transduction of A20 shRNA

The lentiviral vector pLKO.1 and an shRNA lentiviral plasmid stock for targeting human A20 to pLKO.1 were purchased from Sigma-Aldrich (catalog no. for shRNA: SHCLNG-NM 006290). The sequence of the clone targeting human A20 was GTACCGGGATGAAGGAGAAGCTCTTAAACTCGAGTTTAAGAGCTTCTCCTTCATCTTTTTTG. Lentivirus was produced by cotransfection of HEK 293 T cells with the lentiviral vector, together with three packaging plasmids (pMDL-RRE, pRSV-REV, pVSV-g) using the lipofectamin, as described earlier.[42] The lentiviral particles infections into Hela cell lines, followed by selection with puromycin (3 μg/ml), were performed according to the manufacturer's instructions (Sigma-Aldrich).

Confocal imaging analysis

HeLa cells were grown on glass coverslips and then transfected with pEGFP-C1-A20. After 24 h, the cells were treated as indicated in the figure legend and fixed in 4% paraformaldehyde at room temperature for 10 min. Cells were washed in cold PBS and permeablized with 0.2% Triton X-100, and incubated with mouse anti-p65 antibody (Santa Cruz Biothenology). The PE-conjugated secondary antibody (BD Pharmingen, San Diego, CA, USA) was used and mounted with Fluromount-G (Vector Laboratories, Burlingame, CA, USA) and visualized using an OLYMPUS confocal microscope.

Immunohistochemistry

Expression of A20 was analyzed by immunohistochemistry (IHC) on paraffin-embedded or frozen tissue sections from 33 malignant breast cancer tissues; 3 μm thick sections from the paraffin blocks were used for IHC with mouse EnVision-HRP detection system (Dako, Carpinteria, CA, USA). After deparaffinization and antigen retrieval by a pressure cooker in 10mM sodium citrate buffer (pH 6.0) at full power for 4 min, tissue sections were treated with 3% H2O2 for 10 min. The anti-A20 antibody was diluted (1:50) with background reducing diluent (Dako) and incubated overnight at 4°C in humid chamber. The slides were then incubated with the EnVision reagent for 30 min, and then sequentially incubated with DAB chromogen for 5 min, counterstained with Meyer's hematoxylin and mounted. Careful rinses with several changes of TBS-0.3% tween buffer were performed at each step. Negative control used was mouse IgG1 isotype control to exclude the primary antibody. Cytoplasmic granular staining was considered as positive cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations

TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRAF2	TNFR-associated factor 2
RIP1	receptor interacting protein 1
TRADD	TNFR1-associated death domain protein
MEF	mouse embryonic fibroblast cells
ASK1	apoptosis signal-regulating kinase1
ZnF	zinc finger
GADD45β	growth arrest and DNA damage-inducible 45β
XIAP	X-linked inhibitor of apoptosis protein
MnSOD	manganese superoxide dismutase
FHC	ferritin heavy chain
MAP3K	mitogen-activated protein kinase kinase kinase
MAP2K	mitogen-activated protein kinase kinase
MAPK	mitogen-activated protein kinase
MLK	mixed-lineage kinase
NF-κB	nuclear factor-κB
JNK	c-jun N-terminal kinase
IKKβ	inhibitory κB kinase
PARP	poly(ADP-ribose)polymerase
CHX	cycloheximide

WT wild type

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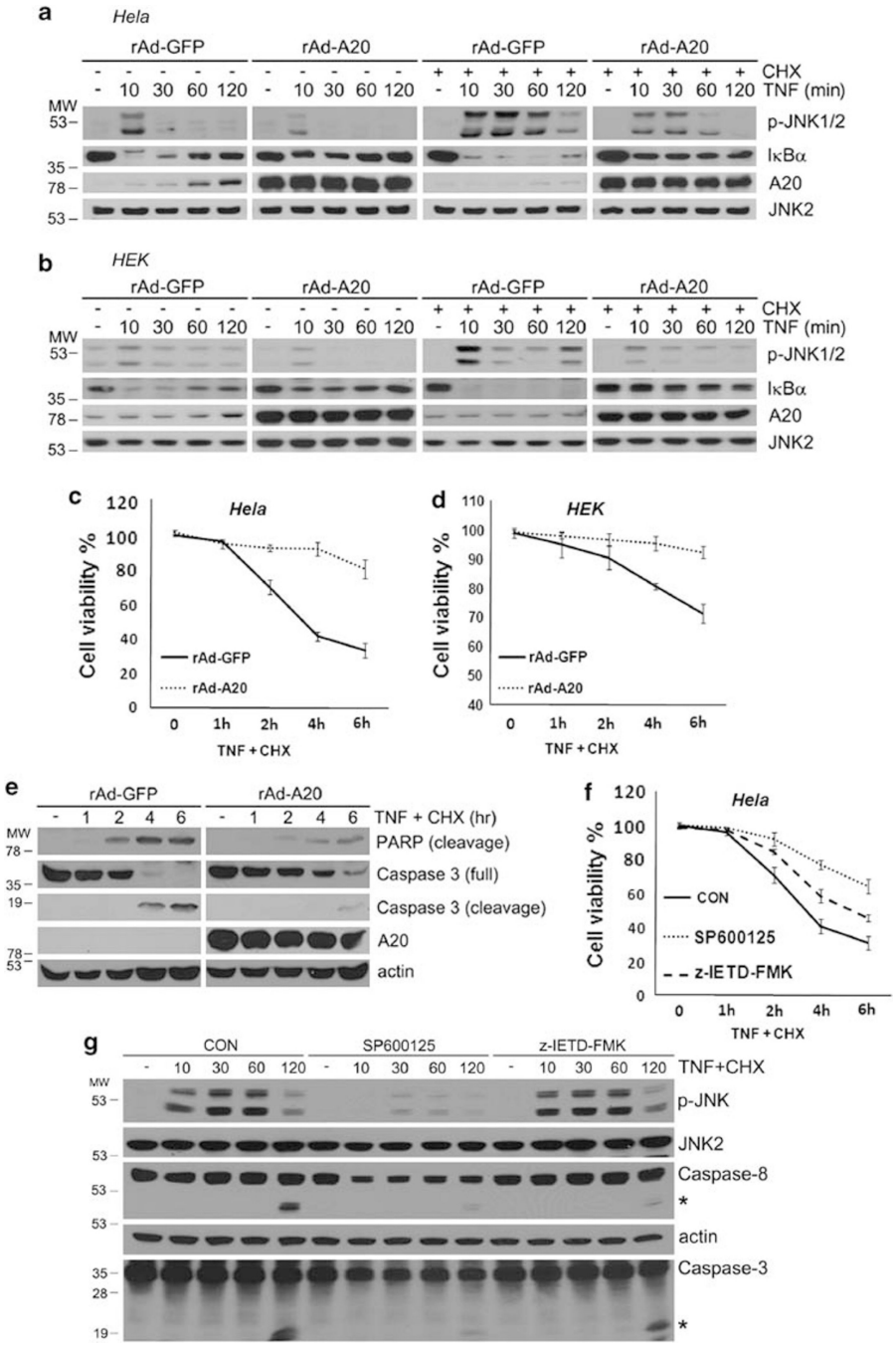

Figure 1.

TNF induces rapid expression of A20 and A20 acts as a negative regulator of JNK activation as well as apoptosis on TNF treatment. (**a**) HeLa and (**b**) HEK293 cells were infected with recombinant adeno-viruses expressing either control-GFP (rAd-GFP) or GFP-A20 (rAd-A20) at 200 PFU/cells for 2 h, and then replaced with DMEM containing 10% FBS. After 30 h, the cells were treated with either TNF (15 ng/ml) or TNF plus CHX (10 μg/ml) for indicated times. Whole cell extracts were subjected to immunoblotting with anti-phospho-JNK, IκBα, A20, and JNK2 antibodies. (**c**) HeLa and (**d**) HEK293 cells were infected with

either rAd-GFP or rAd-A20 as described in (**a**). After 30 h, the cells were treated with TNF (15 ng/ml) plus CHX (10 μg/ml) for indicated times and cell viability was determined by trypan blue exclusion assay. The results are presented as the mean ± S.E. from three independent experiments. (**e**) HeLa cells were infected with either rAd-GFP or rAd-A20 as described in (**a**). After 30 h, the cells were treated with TNF (15 ng/ml) plus CHX (10 μg/ml) for indicated times and immunoblotting was performed with anti-PARP, caspase-3, A20, and actin antibodies. (**f**, **g**) After 10 min pretreatment with a JNK-specific inhibitor (10 μM SP600125) and irreversible caspase-8 inhibitor (50 μM z-IETD-fmk), HeLa cells were treated with TNF (15 ng/ml) plus CHX (10 μg/ml) for indicated times. Cell viability (**f**) was determined as described in (**c**), and immunoblotting (**g**) was performed with anti-phospho-JNK, JNK2, caspase-8, caspase-3, and actin antibodies. The asterisk indicates cleaved product of caspase-8 and caspase-3 on TNF plus CHX treatment

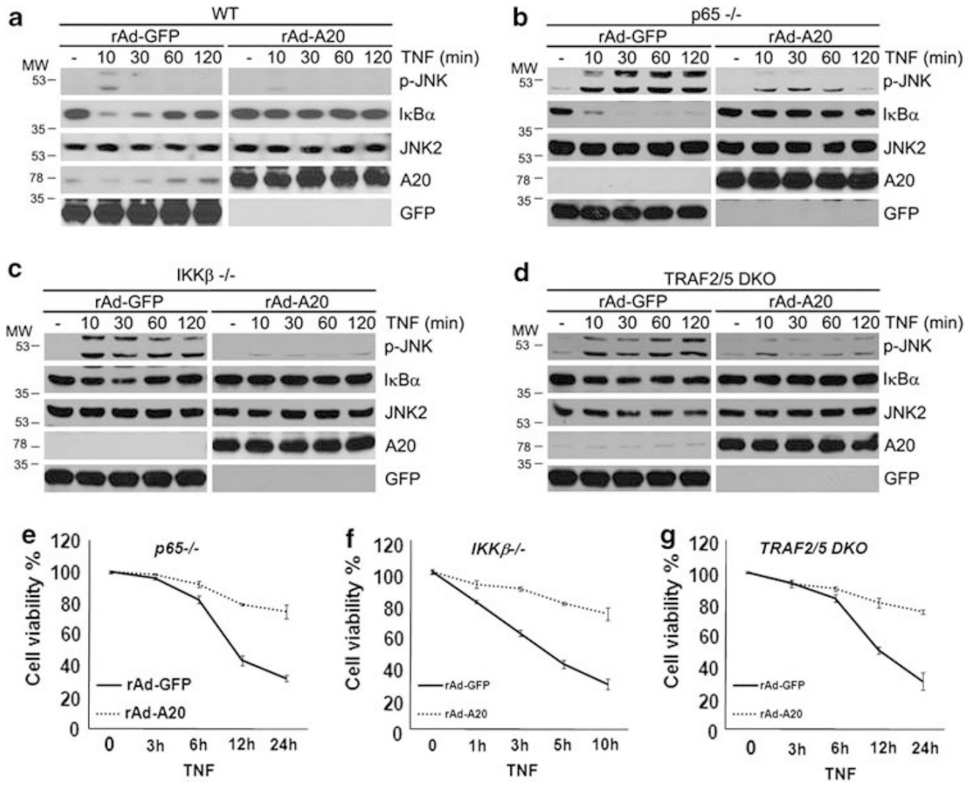

Figure 2.

A20 is an essential mediator on NF-κB-mediated suppression of JNK activation and apoptosis on TNF treatment. (**a**–**d**) Wild-type (WT), p65−/−, IKKβ−/−, and TRAF2/5 DKO MEFs were infected with either rAd-GFP or rAd-A20, as described in Figure 1a, and the cells were treated with TNF (15 ng/ml) for indicated times. Whole cell extracts were subjected to immunoblotting with anti-phospho-JNK, IκBα, A20, JNK2, and actin antibodies. (**e**–**g**) p65−/−, IKKβ−/−, and TRAF2/5 DKO MEFs were treated with TNF as indicated and the cell viability was determined by trypan blue exclusion assay. The results are presented as the mean ± S.E. from three independent experiments

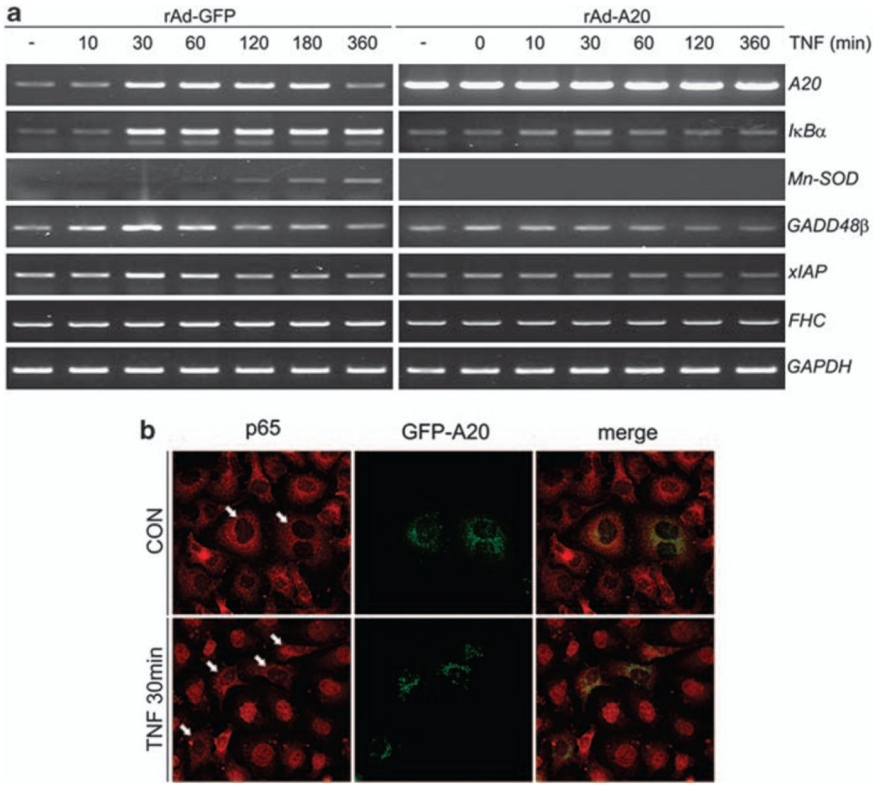

Figure 3.

Impaired expression of NF-κB-mediated inhibitors of JNK signaling by A20. (**a**) HeLa cells were infected with either rAd-GFP or rAd-A20 as described in Figure 1a. Total RNA was prepared at the indicated times after TNF treatment, and RT–PCR was performed with the primers specific to the human A20, IκBα, Mn-SOD, GADD45β, XIAP, FHC, and GAPDH. After PCR amplication, the products were separated by agarose gel electrophoresis and visualized using ethidium bromide staining. (**b**) HeLa cells were transfected with pEGFP-N1-A20, after which cells were either untreated (top) or treated with TNF (15 ng/ml) for 30 min (bottom). Cells were then analyzed by confocal microscopy with a rabbit anti-p65 antibody (red fluorescence). Green fluorescence indicates the expression of GFP-A20. Arrows indicate that the cells ectopically express A20 protein

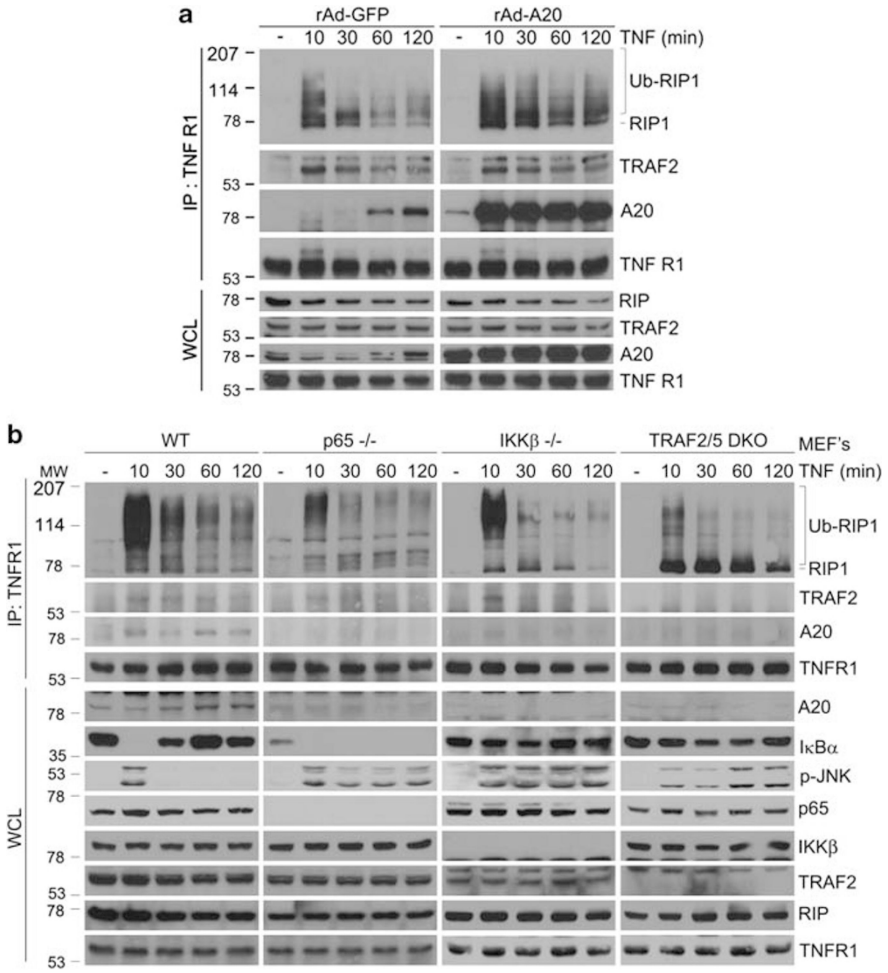

Figure 4.

A20 does not affect the TRAF2 recruitment into TNFR1 signaling complex. (**a**) HeLa cells were infected with either rAd-GFP or rAd-A20 as described in Figure 1a. After 30 h, the cells were treated with TNF (15 ng/ml) for indicated times, and cell extracts from each sample were subjected to immunoprecipitation with anti-TNFR1 antibody. Immunoprecipitates were analyzed by SDS–PAGE and immunoblotting with antibodies against RIP1, TRAF2, A20, and TNFR1. A total of 1% of the cell extract volume from each sample was used as input control. (**b**) WT, p65−/−, IKKβ−/−, and TRAF2/5 DKO MEFs were treated with TNF (15 ng/ml) for indicated times. Cell extracts were immunoprecipitated anti-TNFR1 antibody and immunoblotting was performed in (**a**)

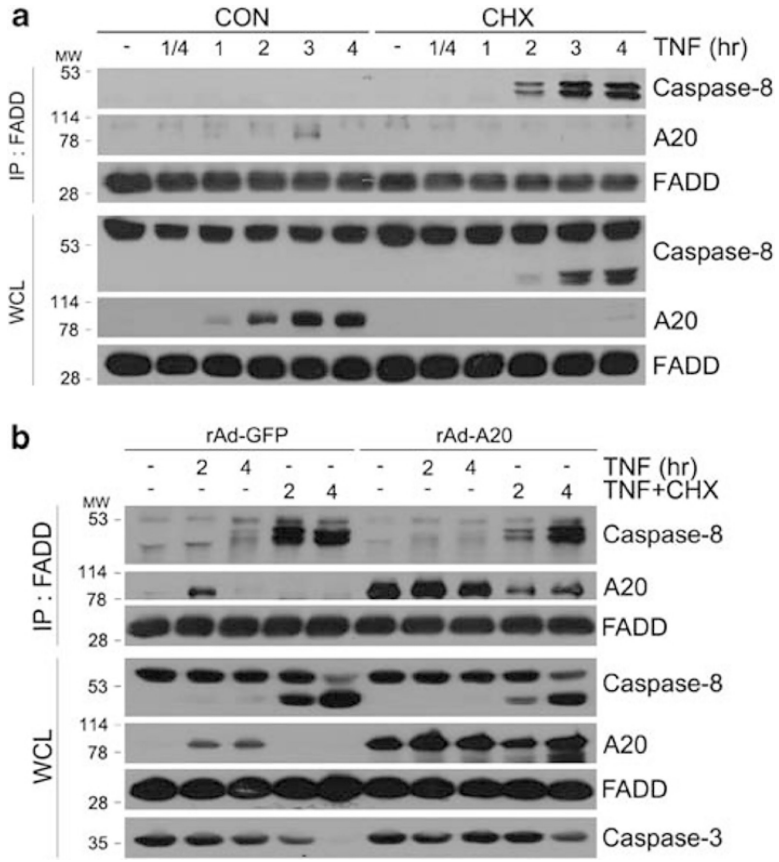

Figure 5.

A20 is recruited to complex II and partly suppresses the interaction of caspase-8 and FADD. (**a**) HeLa cells were treated with TNF (15 ng/ml) for indicated times in the absence or presence of CHX (10 μg/ml). Cells were lysed and immunoprecipitated with anti-FADD antibody and immunoblotting was performed with antibodies against caspase-8, A20, and FADD. A total of 1% of the cell extract volume from each sample was used as input control. (**b**) HeLa cells were infected with either rAd-GFP or rAd-A20 as described in Figure 1a and were treated with TNF (15 ng/ml) or TNF plus CHX (10 μg/ml) for indicated times. Cells were lysed and immunoprecipitated with anti-FADD antibody and immunoblotting was performed in (**a**)

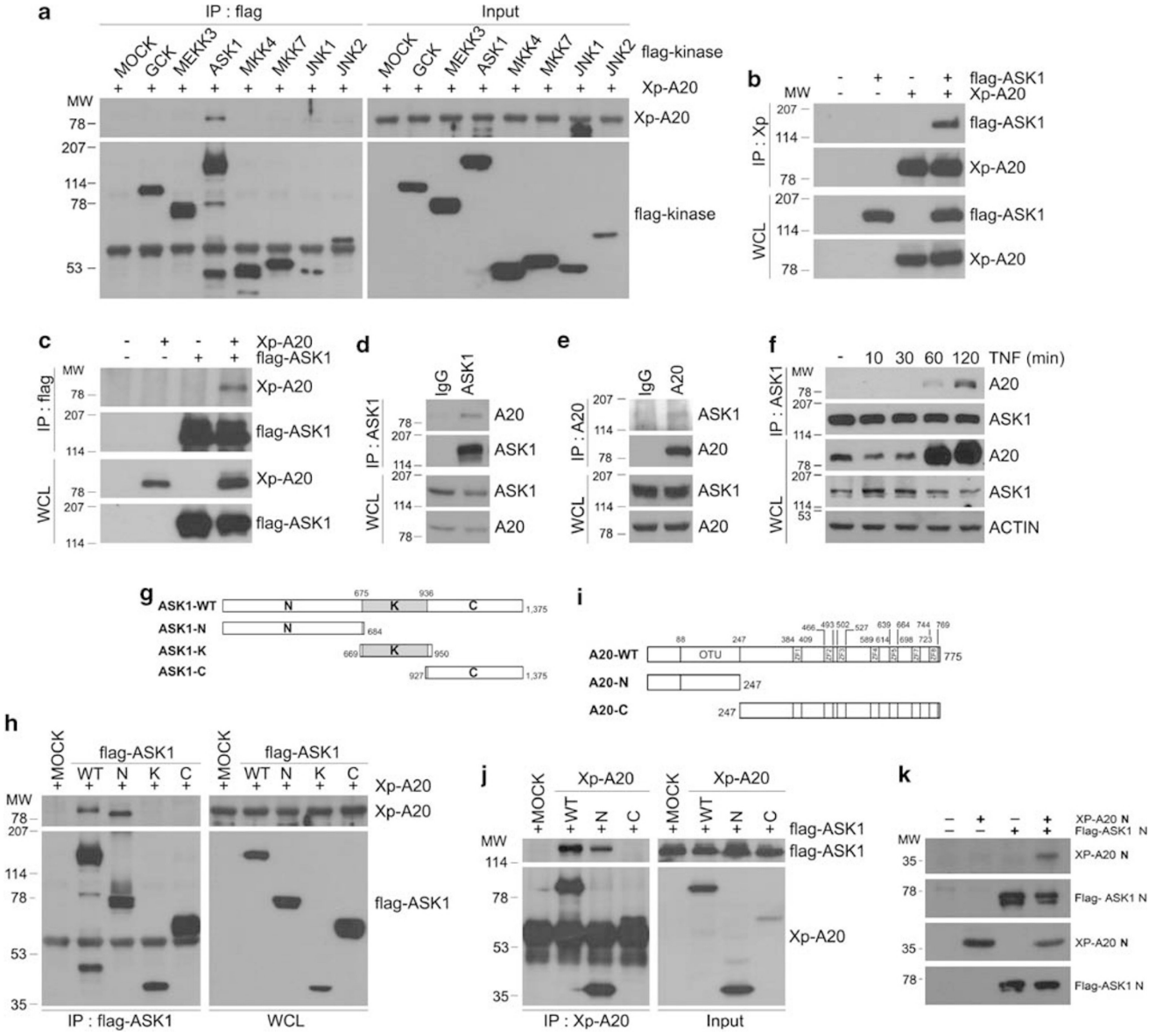

Figure 6.

A20 physically interacts with MAP3K, ASK1 in the JNK signaling cascade. (**a**) HEK293 cells were co-transfected with flag-tagged kinases (GCK, MEKK3, ASK1, MKK4, MKK7, JNK1, or JNK2) and Xp-tagged A20 expression plasmids. After immunoprecipitation with anti-flag antibody, co-immunoprecipitated proteins were detected by immunoblotting with anti-Xp antibody (top row). Expression levels of the transfected proteins in the immunoprecipitates and total cell lysates were analyzed by immunoblotting with anti-flag antibody (bottom row). (**b**) HEK293 cells were co-transfected with flag-tagged ASK1 and Xp-tagged A20 plasmids, as indicated. After immunoprecipitation with anti-Xp antibody, co-immunoprecipitated ASK1 was detected by immunoblotting with anti-flag antibody. The efficiency of immunoprecipitants and expression levels of the transfected proteins were analyzed by immunoblotting with the indicated antibodies. (**c**) Similar experiments as described in (**b**), with IP using anti-flag-antibody. (**d**) HEK293 cell extracts were

immunoprecipitated with antibodies against ASK1 or normal IgG, and the resulting immunoprecipitates were analyzed by immunoblotting with anti-ASK1 and anti-A20 antibodies. (**e**) Similar experiments as described in (**d**), with IP using anti-A20 antibody. (**f**) HeLa cells were treated with TNF (15 ng/ml) for indicated times. After immunoprecipitation with anti-ASK1 antibody, co-immunoprecipitated A20 was detected by immunoblotting with anti-A20 antibody (top row). Expression levels of A20 and ASK1 in the total lysates were analyzed by immunoblotting with the indicated antibodies. (**g**, **i**) Schematic diagram of ASK1 and A20 domains for their expression constructs (see the text for details). (**h**, **j**, **k**) Various truncated forms of ASK1 and A20 were transfected in HEK293 cells. A20-ASK1 association was determined by immunoprecipitation and followed by immunoblotting with the indicated antibodies (top row). The efficiency of immunoprecipitants (**h**, **j**, bottom, left) and expression levels of the transfected proteins in the total lysates (**h**, **j**, bottom, right) were analyzed by immunoblotting with anti-flag and anti-Xp antibodies, respectively

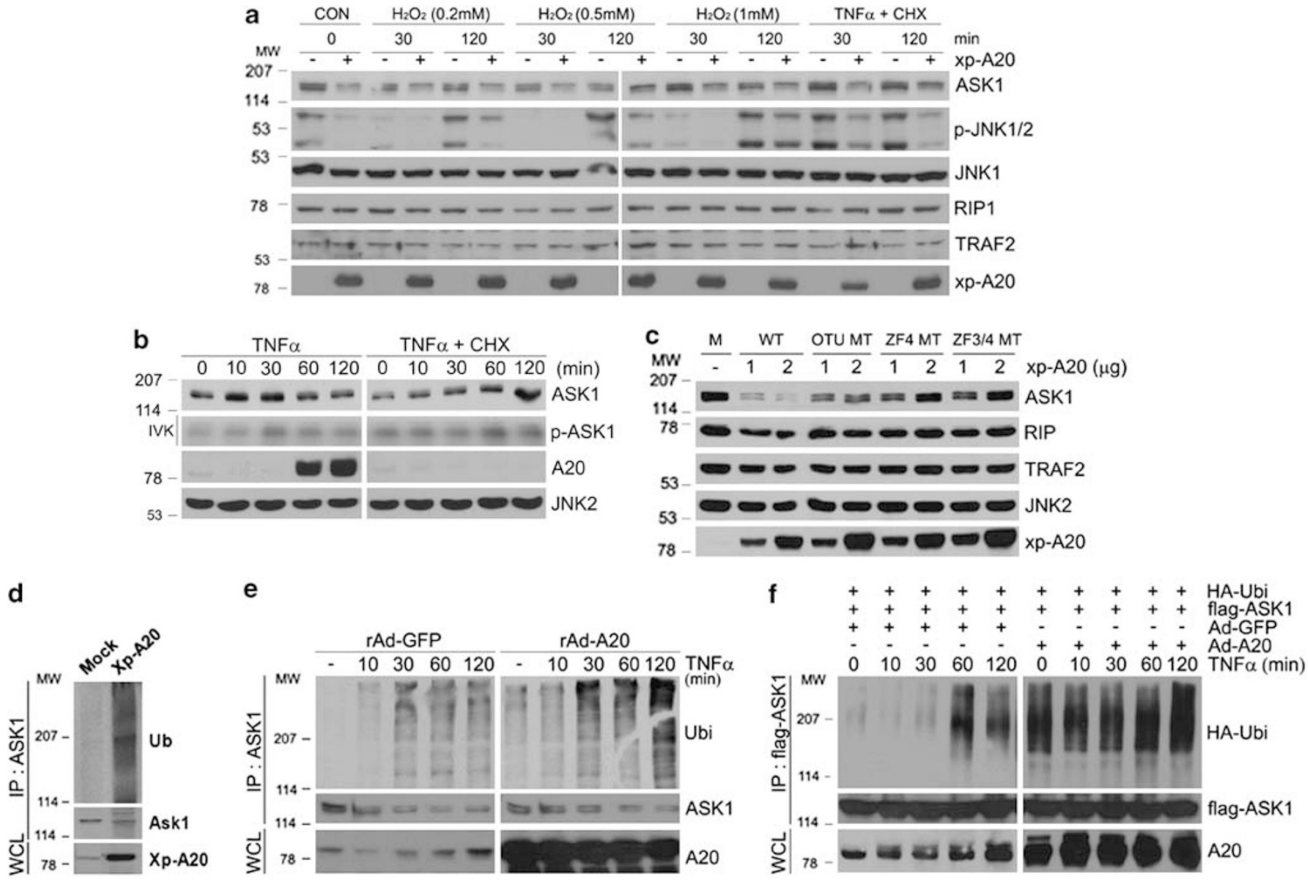

Figure 7.

A20 reduces the stability and promotes the degradation of ASK1 through the ubiquitination process. (**a**) Control empty vector or Xp-tagged A20 constructs were transfected into HEK293 cells, followed by treatment with various concentrations of H2O2 or TNF (15 ng/ml) plus CHX (10 μg/ml) for indicated times. Whole cell extracts were subjected to immunoblotting with anti-ASK1, phospho-JNK, JNK1, RIP1, and TRAF2 antibodies. Expression levels of the transfected A20 were analyzed by immunoblotting with anti-Xp antibody. (**b**) HeLa cells were treated with either TNF (15 ng/ml) or TNF (15 ng/ml) plus CHX (10 μg/ml) for indicated times. Whole cell extracts were immunoprecipitated with anti-ASK1 antibody and autophosphorylation of ASK1 was examined by immune complex kinase assay (second row). The same extracts were subjected to immunoblotting with anti-ASK1, A20, and JNK antibodies (top and bottom two rows). (**c**) HEK293 cells were transfected with indicated plasmids for 30 h, and whole cell extracts were subjected to immunoblotting with anti-ASK1, RIP1, TRAF2, and JNK antibodies. Expression levels of the transfected proteins were analyzed as in (**a**). (**d**) HEK293 cells were transfected with control empty vector or Xp-tagged A20 for 30 h. Cells were lysed and ASK1 was immunoprecipitated with anti-ASK1 followed by immunoblotting with anti-Ub antibody (top). (**e**) HEK293 cells were infected with either rAd-GFP or rAd-A20 as described in Figure 1a, and the cells were treated with TNF for the indicated times. Cells were lysed and ASK1 ubiquitination was examined as described in (**d**). (**f**) HEK293 cells expressing with either rAd-GFP or rAd-A20 were transfected with flag-ASK1 and HA-Ub. After 30 h, the

cells were treated with TNF for the indicated times and immunoprecipitated with anti-flag antibody followed by immunoblotting with anti-HA antibody (top)

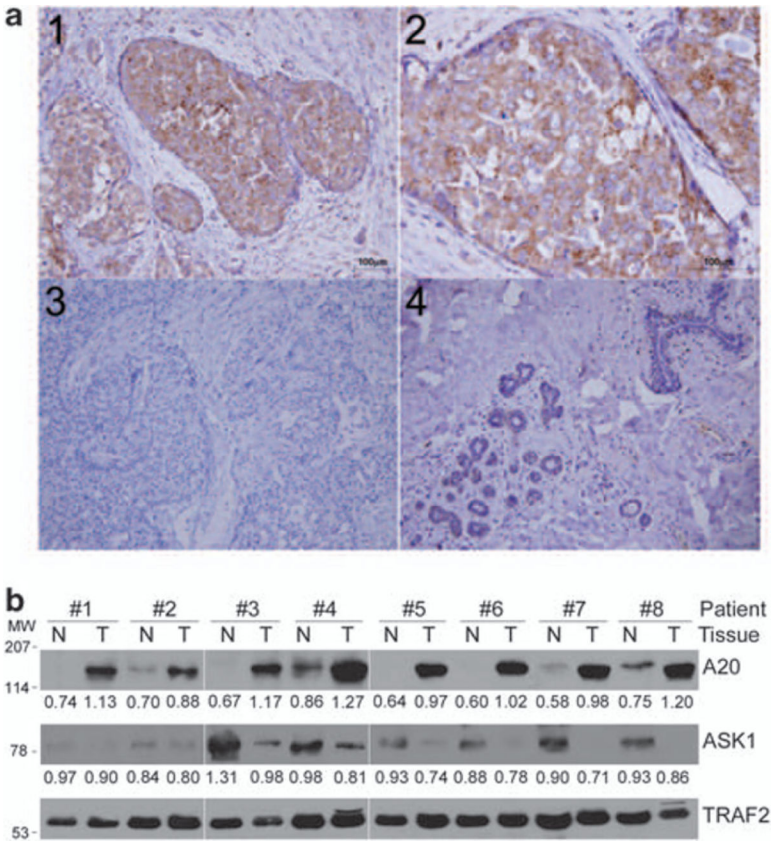

Figure 8.

An inverse correlation of expression levels between A20 and ASK1 in human breast cancer tissues. (**a**) (1, 2) Immunohistochemical staining of A20 in invasive ductal breast cancer. A20 was highly expressed in malignant cancer tissue compared with faintly stained normal breast tissue. (3, 4) Negative control with mouse IgG1 isotype control serum. Original magnification, 1, 3, and 4,200x; 2,400x. (**b**) Normal and cancer tissue extracts from eight patients who underwent surgery for malignant breast cancer were prepared as described in Materials and Methods, and expression levels of A20 and ASK1 were analyzed by immunoblotting with anti-A20 and anti-ASK1 antibodies, respectively. Relative densities of A20 and ASK1 were obtained by densitometry for corresponding TRAF2 at each lane