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An Ubiquitin-like Motif in ASK1 Mediates its Association with and Inhibition of the Proteasome

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Abstract

Linear polyubiquitin is processed at LRLRGG sequences by deubiquitinating enzymes to make free monomeric ubiquitin. This LRLRGG ubiquitin-like motif is found in a limited number of mammalian non-ubiquitin proteins, including the MAP3K Apoptosis Signal-Regulating Kinase-1 (ASK1), which activates MAPK signaling pathways. The c-terminus of ASK1 binds to the 19S cap of the proteasome allowing ASK1 to phosphorylate and inhibit proteasomal activity. We investigated whether the ubiquitin-like sequence in the c-terminus of ASK1 mediates its association with and inhibition of the proteasome. To test this we generated ASK1 with substitutions or deletions in this ubiquitin-like domain and examined the activation of cellular signaling and the association of ASK1 with the 19S cap of the proteasome. We show that ASK1 mutants have reduced association with the 19S cap of the proteasome, reduced capacity to inhibit the proteasome, and diminished ability to inhibit TNF-induced NF-κB activation. Mutant forms of ASK1 also had reduced capacity to activate JNK signaling, suggesting that the ubiquitin-like motif in ASK1 is also important for coordinating the balance between JNK and NF-κB signaling. Together these results demonstrate that the ubiquitin-like sequence of ASK1 is important for binding to and inhibition of the proteasome, and for the coordinated activation of cellular NF-κB and JNK signaling.

Keywords

ASK1; JNK; NF-kappaB; proteasome; ubiquitin

1. Introduction

Apoptosis Signal-Regulating Kinase-1 (ASK1) is a mitogen-activated protein kinase kinase kinase family member (MAP3K5) that activates p38 and JNK pathways in response to intracellular and extracellular stimuli [1, 2, 3]. ASK1 activation can promote apoptosis or cellular survival depending on the initiating stimulus and the context of the cellular response [4, 5, 6]. ASK1 binds to the 19S cap of the proteasome where it phosphorylates and inhibits regulatory particle AAA ATPases (RPTs), resulting in proteasomal inhibition [7]. The mechanism of ASK1 association with the proteasome is not clear, but deletion of the c-terminal domain of ASK1 abrogates this interaction and its inhibition of proteasomal activity [7].

The c-terminal domain of ASK1 has a unique internal LRLRGG amino acid sequence that is also found in the sequence of unprocessed ubiquitin (Ub) and other ubiquitin-like proteins

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(UBLs) [8]. The processing and maturation of Ub and UBLs to generate free c-terminal glycines available for conjugation to target proteins is performed by ubiquitin specific proteases (USPs). The association of USPs with Ub and UBLs can depend on the LRLRGG residues of the substrate, suggesting that these residues can mediate association with ubiquitin binding proteins [9,10]. The proteasome has many ubiquitin-binding proteins in the 19S cap that mediate association with ubiquitinated substrates and their subsequent degradation [11, 12]. These ubiquitin-protein interactions are essential for the degradative function of the proteasome through removal of ubiquitin moieties from target proteins. Resident proteasome-associated deubquitinating enzymes, RPN11, USP14, and UCH37 cleave at the LRLRGG sequence removing polyubiquitin chains prior to degradation [13]. The presence of an LRLRGG sequence in the c-terminal proteasome-binding region of ASK1 led us to investigate whether this motif mediates ASK1 association with the proteasome. We assessed the role of the LRLRGG sequence of ASK1 on proteasomal degradation of a model substrate, and on TNF-induced activation of NF-κB, a proteasome dependent signaling pathway. A primary function of ASK1 is to activate MAPK pathways, including the JNK pathway [1]. Since JNK activation is regulated by NF-κB, we also tested the effects of ASK1 mutations on JNK activation [14].

Our findings show that mutations in the LRLRGG sequence of ASK1 reduce its association with the 19S cap of the proteasome. Consistent with this, these mutations reduce ASK1 inhibition of the proteasome, reduce ASK1 inhibition of TNF-induced NF-κB activation, and reduce activation of the JNK signaling pathway, without altering TRAF2 association or ASK1 kinase activity. These results further explain how ASK1 inhibits the proteasome and suggest new approaches to regulation of protein turnover in cells.

2. Materials and methods

2.1. Expression plasmids

Wildtype (WT) murine ASK1 was cloned from C57BL/6 mouse kidney cDNA and ligated into the pENTR2B entry vector (Invitrogen, San Diego, CA). The ASK1 LRLRGGstop and LRLRAA mutant constructs were generated using the Quikchange II site-directed mutagenesis kit (Agilent, La Jolla, Ca). ASK1 with an N-terminal V5 tag was generated using the Gateway LR Clonase II enzyme kit (Invitrogen) by shuttling wildtype ASK1 in pENTR2B into pcDNA3.1/nV5 and pcDNA3.2 destination vectors (Invitrogen). The pNF-κB-TA-Luc and pRL-TK reporter plasmid were from Clontech (Palo Alto, CA). The PathDetect pFR-Luc and pFA-cjun Trans-reporter plasmids were from Agilent (La Jolla, CA). The GFPu expression plasmid was purchased from the American Type Culture Collection (Manassas, VA). All vectors were confirmed by sequencing through The University of Chicago Cancer Research Center DNA Sequencing Facility.

2.2. Cell culture and reagents

HEK293 cells (ATCC) were cultured (37°C, 5%CO_2) in complete Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum, Penicillin (100 μg/ml), streptomycin (100 U/ml) (Life Technologies, Rockville, MD). Recombinant human TNF-α was purchased from Peprotech (Rocky Hill, NJ). Hydrogen peroxide was purchased from Fisher Scientific (Pittsburgh, PA). SUC-LLVY-AMC and Rabbit Anti-RPT2 were purchased from Enzo Life Sciences (Farmingdale, NY). Goat anti-actin and rabbit polyclonal anti-HA antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); Monoclonal Mouse anti-V5 antibody was purchased from Invitrogen. Rabbit polyclonal phospho-MKK4 antibodies was purchased from Cell Signaling Technology (Beverly, MA).

2.3. Transfections, immunoprecipation, and immunoblot analysis

HEK 293 cells were transiently transfected as described [14]. Briefly, polyethylenimine (PEI) was mixed at a 1:1 ratio with plasmid dissolved in NaCl (150mM). PEI was added dropwise to DNA containing NaCl while vortexing. The DNA/PEI mixture was added to sub-confluent 6 well plates containing DMEM without serum or antibiotics. 24 hrs post-transfection the media was changed to complete DMEM. 48 hrs post-transfection cells were washed with 1X PBS and lysed in 1% Triton Lysis buffer (50mM Hepes, 150mM NaCl, 1.5mM $MgCl_2$, 1mM EGTA, 20mM N-Ethylmaleimide, 10mM 1,10 phenanthroline monohydrate, and 1X protease inhibitor cocktail (Roche, Basel, Switzerland). For immunoprecipitation, lysates were incubated (4°C, 18h) with anti-RPT2 antibody (Enzo Life Science), anti-HA (Santa cruz), and anti-V5 (Invitrogen) and purified on protein-G sepharose beads (Promega, Madison, WI). Lysates and immunoprecipitates were boiled in Laemmli Reducing Buffer, resolved by SDS-PAGE (NuPAGE Novex 4–12% Bis-Tris Polyacrylamide gel (Invitrogen)) and transferred to a Polyvinylidene Fluoride (PVDF) membrane. The membrane was incubated at room temperature with blocking buffer (LI-COR BioSciences, Lincoln, NE) diluted in PBS 1:1, incubated (4°C, 18h) with primary antibody, washed and incubated (20°C, 45 min) with fluorophore-conjugated secondary antibody, and imaged using IR Fluorescence Detection on the LI-COR Odyssey Infrared Imaging System (LI-COR). Densitometry of protein bands was quantified using the area under the curve method in Image J (http://rsbweb.nih.gov/ij).

2.4. GFPu proteasomal inhibition assay

Proteasomal activity was measured by assessing the degradation of an uncleavable ubiquitin-tagged GFP (GFPu) in living cells, as described (15). The GFPu expression plasmid was transiently co-transfected with pcDNA3.1/nV5 ASK1 into HEK 293 cells. Following transfection, cells were treated as described, trypsinized and analyzed on a Becton-Dickinson FACSCalibur and gated on live (propidium $iodide^-$) GFP^+ cells to measure the mean fluorescence intensity of GFP as an index of proteasomal inhibition.

2.5. NF-κB and JNK activation assays

NF-κB activity: pNF-κB-TA-Luc was transiently co-transfected along with V5-ASK1 and Renilla-TK. At 36 hrs postransfection cells were serum starved (16hr) followed by treatment with TNF (20ng/ml, 5h). Following stimulation, cells were lysed in passive lysis buffer (Promega) and luciferase activity was assayed on a 96 well plate using the GloMax Multi Detection System (Promega). Levels were normalized to Renilla-TK.

JNK activity: pFR-Luc and pFA-cjun were transiently co-transfected along with V5-ASK1 and Renilla-TK. At 48 hrs post-transfection lysates were analyzed for luciferase activity and ASK1 expression as described above.

2.6. MKK4 In Vitro Kinase Assay

HEK 293 cells were transfected with 5ug ASK1 and empty vector control and were lysed and immunoprecipitated as described previously. Following the final triton-X lysis buffer wash, samples were washed twice with pre-chilled 50mM HEPES and all supernatant removed. Kinase buffer (20mM beta-glycerophosphate, 10mM $MgCl_2$, 20mM HEPES, 100mM NaCl, 10uM ATP) and 10ug of recombinant GST-MKK4 (Millipore, Billerica, MA) was added and the reaction incubated for 1hr at 30 degrees in a thermomixer at 1400 RPM. The reaction was terminated with 2X Laemli buffer containing 200mM DTT. Reactions were resolved by SDS PAGE, transferred to PVDF and immunoblotted for phospho-MKK4 (Cell signaling).

2.7. Statistics

All experiments were performed three times unless otherwise indicated. Results shown are the means and standard deviations of all samples taken. Statistical significance was determined using ANOVA with post-hoc Bonferroni

3. Results

3.1. LRLRGG-dependent ASK1 association and inhibition of the proteasome

The c-terminal domain of ASK1 binds to the 19S proteasomal cap, allowing ASK1 to phosphorylate and inhibit the proteasome [7]. We found that this region contains an LRLRGG sequence, and also additional homology to Ub and the Ub-like protein ISG-15 (Figure 1A). To analyze whether the LRLRGG sequence of ASK1 is important for binding to the proteasome, we used site-directed mutagenesis to generate ASK1 with either an LRLRAA sequence, or a premature stop codon (LRLRGGstop), mimicking ASK1 cleaved at the LRLRGG sequence. To measure association with the 19S proteasome, endogenous RPT2 (a component of the 19S proteasome) was immunoprecipitated and binding to wildtype (WT) ASK1 or mutant ASK1 was assessed. As reported in prior studies, WT ASK1 co-immunoprecipitated with RPT2 but mutating the LRLRGG sequence of ASK1 abrogated this association (Figure 1B) [7]. Similarly RPT2 co- immunoprecipitated with wildtype V5-ASK1 but not the mutant forms of ASK1 (Figure 1C). To test the effect of this abrograted association on proteasome function we utilized a non-cleavable ubiquitin-GFP fusion (GFPu) vector, which we co-transfected with ASK1 into HEK-293 cells stimulated with the ASK1-activating signal hydrogen peroxide. GFPu fluorescence, as measured by flow cytometry, correlates inversely with proteasome activity. Consistent with the association with RPT2, WT ASK1 inhibited the proteasome more efficiently than LRLRGG mutant forms of ASK1 (Figure 1D). Thus the c-terminal LRLRGG sequence of ASK1 mediates binding to and inhibition of the proteasome.

3.2. Mutations in the LRLRGG sequence of ASK1 do not affect TRAF2 binding or in vitro kinase activity

To determine whether mutations in the LRLRGG sequence of ASK1 affect its other functions we measured whether these mutations altered association with known ASK1-binding partners or changed the kinase activity of ASK1. TRAF2 is a known ASK1 binding partner and complex formation is essential for ASK1 activation [17]. Immunoprecipitates of ASK1 and ASK1 mutants were analyzed for TRAF2 association. ASK1 and the two mutant forms of ASK1 associated equally well with endogenous TRAF2 (Figure 2A). To measure whether mutating the LRLRGG sequence of ASK1 would affect its kinase function, WT ASK1 and its mutant forms were immunoprecipitated from HEK293 cells and mixed with recombinant MKK4, a known ASK1 target substrate, in an *in vitro* kinase assay [1]. Both WT ASK1 and ASK1 with its LRLRGG sequence mutated were able to phosphorylate MKK4 *in vitro* (Figure 2B). Thus the general binding and function of ASK1 are not altered when the C-terminal LRLRGG sequence is mutated.

3.3. LRLRGG-dependent ASK1-induced NF-κB downregulation

As NF-κB activation is dependent on degradation of IκBα by a functioning proteasome, we investigated whether ASK1-dependent proteasomal inhibition could lead to decreased NF-κB activation [16]. To test this, WT and mutant forms of ASK1 were co-transfected with a luciferase reporter to measure NF-κB activation in the presence of TNF stimulation. WT ASK1 inhibited NF-κB activation, but the LRLRGG mutant forms of ASK1 displayed diminished capacity to inhibit NF-κB (Figure 3). Thus, the LRLRGG sequence of ASK1 that mediates binding of ASK1 to the proteasome is also important for blocking NF-κB activation.

3.4. LRLRGG-dependent ASK1-induced JNK activation

Since NF-κB activation blocks JNK signaling, mutant forms of ASK1 may be expected to have reduced capacity to induce JNK activation [14]. To test this we co-transfected WT and mutant forms of ASK1 with a c-jun luciferase reporter and measured JNK activation. Whereas wildtype ASK1 was able to activate JNK efficiently, mutant forms of ASK1 showed reduced levels of JNK activation (Figure 4). Thus the LRLRGG sequence of ASK1 is required for the full activation of the JNK signaling pathway.

4. Discussion

This study is the first to show that proteasomal binding and inhibition by ASK1 is mediated by an Ub-like sequence in the c-terminal domain of ASK1, suggesting that this sequence mediates a previously reported ASK1 association with, and inhibition of, the proteasome [7]. Since a complete reduction in binding and inhibition by the LRLRGG mutants was not evident, additional residues outside the LRLRGG sequence of ASK1 that mediate binding may exist. Indeed we found additional homology between ASK1 and Ub and ISG-15 N-terminal of the LRLRGG sequence. Structural prediction software (PHYRE), does not predict an UB-like fold in this region of ASK1 and the crystal structure of this region of ASK1 has not yet been resolved. There is a predicted Sterile Alpha Motif (SAM) domain in this region, which might explain the association of ASK1 with other SAM domain-containing proteins. Although SAM domains have not been implicated in proteasomal association, we cannot exclude the possibility of an indirect association between ASK1 and the proteasome mediated by this putative SAM domain. The LRLRGG sequence of ASK1 mediates its association with USP9X, a deubiquitinating enzyme that does not contain a SAM domain, suggesting that the LRLRGG domain of ASK1 may mediate SAM-domain independent associations with proteins involved in the regulation of ubiquitination [8]. In our search of the genome we found a small subset of proteins that contain this LRLRGG sequence: CD163, CD177 and MAP3K15. The functional role this sequence plays in these proteins has not been examined but given that the LRLRGG sequence of ASK1 mediates its association with USP9X and the proteasome, it is conceivable that the LRLRGG sequence in these other proteins are functional regulatory sequences.

We found that ASK1 with a synthetically produced “cleavage” at the LRLRGG site (LRLRGGstop) was markedly inefficient in proteasomal inhibition and activation of JNK signaling, when compared to WT. Proteins such as poly-ubiquitin and ISG-15 are cleaved at internal LRLRGG sequences in order to produce mature, fully functioning proteins [9,18]. In tandem with the LRLRGG sequence, ASK1 contains additional homology to ubiquitin and ISG-15 suggesting that endogenous ubiquitin-specific proteases might cleave ASK1 at this site. In addition, pathogens such as SARS coronavirus produce ubiquitin-specific proteases that cleave internal LXGG sites in their own precursor proteins, as well as linear Ub chains and ISG15 precursor proteins [19,24]. The cleavage of ASK1 in the c-terminal domain has been demonstrated in several cell lines but the precise sites of this cleavage or the proteases involved have not been determined [25]. The potential cleavage of this LRLRGG sequence of ASK1 by pathogen encoded or endogenous USPs may profoundly alter cellular signals in response to stress or inflammatory cytokines. Our “cleaved” LRLRGGstop mutant had reduced capacity to bind to and inhibit the proteasome, suggesting that cleavage of WT ASK1 at the LRLRGG site might represent a mechanism for reversal or control of ASK1 mediated proteasome inhibition. Cleavage could interrupt ASK1’s ability to bind to and inhibit the proteasome, leading to elevated NF-κB and reduced JNK activation and resulting in a skewed cellular response toward inflammation and away from apoptosis.

Although it has been reported that ASK1 inhibits NF-κB activation, this is the first report that ASK1's LRLRGG sequence plays an important role in this process [26]. Our data suggest that ASK1 inhibition of NF-κB may occur indirectly through inhibition of the proteasome. Stress response ligands such as TNF activate both NF-κB and MAPK signaling pathways. TNF-induced JNK activation is limited by concurrent NF-κB activation to prevent apoptosis in some cell types [14, 27, 28]. As activation of NF-κB inhibits JNK signaling, reduced inhibition of the proteasome and resulting increased NF-κB activation may explain why mutant forms of ASK1 display decreased capacity to induce JNK signaling. Importantly, we have shown that mutations in the LRLRGG sequence of ASK1 don't alter TRAF2 binding or ASK1 kinase activity, but do alter the ability of ASK1 to activate JNK. Our data provide an alternative method of ASK1-dependent JNK regulation. It is possible that in order to completely activate JNK signaling, ASK1 must both phosphorylate the MAPK pathway and block the NF-κB pathway. The crosstalk between activating JNK and inhibiting NF-κB may serve to finely tune the pro-survival or pro-death functions of ASK1. The regulation of NF-κB activation by ASK1 is complex and likely involves other mechanisms, including the ability of ASK1 to bind and inhibit TAK1-TRAF6 interactions that mediate TLR or IL-1 induced NF-κB activation [26]. Whether ASK1 inhibits TNF-induced NF-κB through a similar mechanism such as the interruption of TRAF2/5 association with TAK1 is not known. The N-terminal domain of ASK1 is sufficient for disrupting TAK1 activation of NF-κB and so this may be an additional component of NF-κB regulation that is distinct from the regulation of the proteasome, which requires the c-terminal domain of ASK1.

The LRLRGG sequence of ASK1 mediates association of ASK1 with the deubiquitinating enzyme USP9X. Similar to other USP9X-associating proteins, ASK1 association with USP9X facilitates deubiquitination and stabilization of ASK1 [8]. ASK1 is ubiquitinated by cIAP and TNFAIP3, two ligases involved in regulation of the TNF signaling to activate NF-κB, MAPK and apoptosis. The association and deubiquitination of ASK1 by USP9X requires activation of ASK1, whereas the degradation of ASK1 by cIAP or TNFAIP3 may occur independent of ASK1 activation [29, 30]. We found that ASK1 inhibition of the proteasome was enhanced dramatically by ASK1 activation by hydrogen peroxide. It may be the case that inactive ASK1 is degraded by the proteasome whereas active ASK1 is stabilized, binds to and inhibits the proteasome, and blocks NF-κB activation. This inhibition of the NF-κB pathway may be required to enhance the activation of MAPK signaling and thus the propensity of TNF to activate apoptosis versus inflammation in the context of cellular stress.

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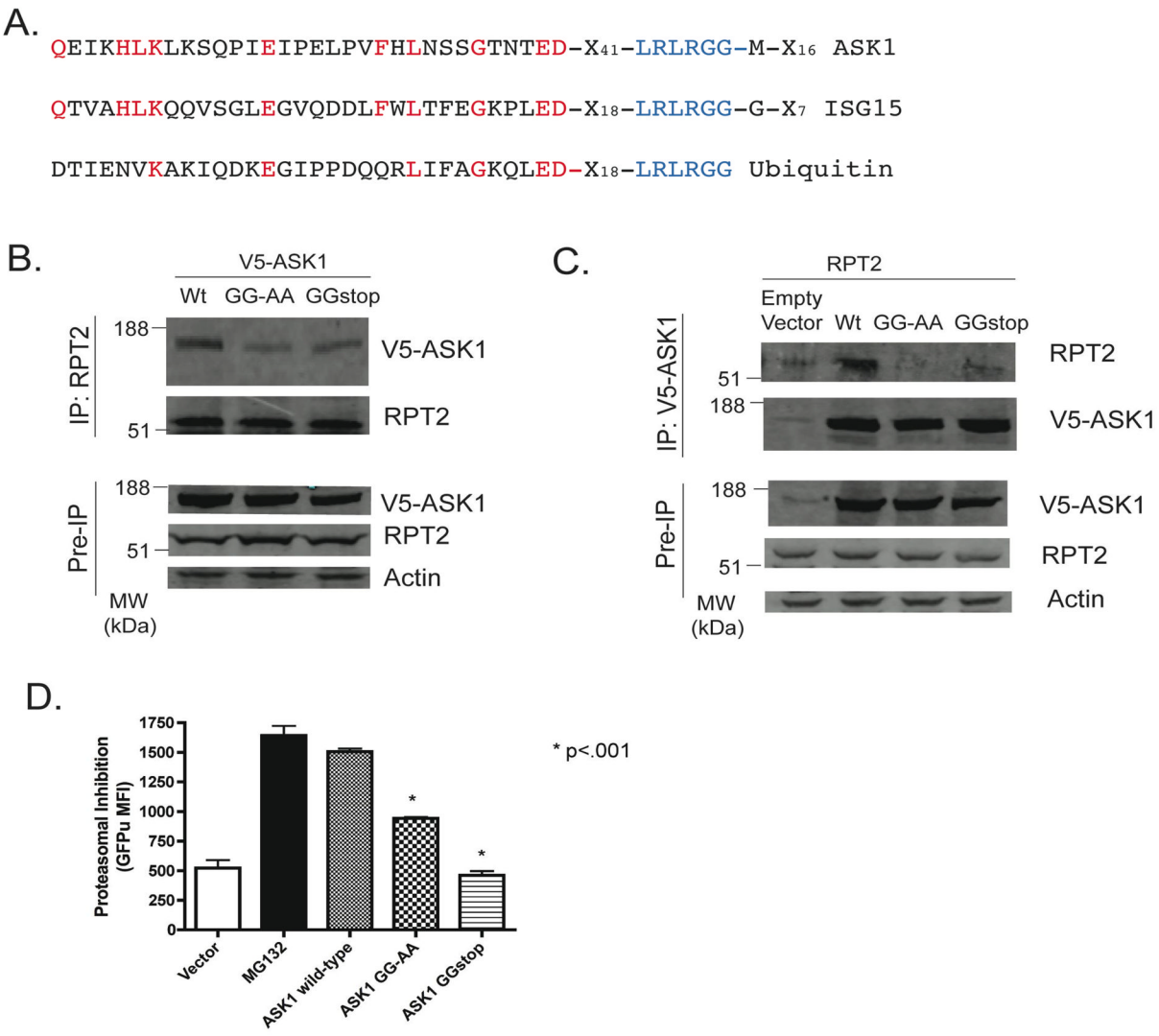

Fig 1.

LRLRGG-Dependent ASK1 binding and inhibition of the proteasome. A. Sequence homology between human ASK1, ISG-15, and Ubiquitin. B. HEK 293 cells were transfected with either Wildtype WT V5-ASK1, V5-ASK1 with LRLRGG mutated to LRLRAA, or V5-ASK1 with a stop codon introduced after the LRLRGG sequence. Endogenous RPT2 was immunoprecipitated and immuno-blotted for V5. C. V5-ASK1 was immunoprecipitated and immuno-blotted for endogenous RPT2. D. HEK 293 cells were transfected with ASK1 and mutants along with a degron containing vector GFPu, stimulated with 1mM H_2O_2 overnight, and PI-negative GFPu fluorescence was measured using flow cytometry. adjustment using GraphPad Prism software (La Jolla, CA) and significance was inferred with *p*-values less than 0.05.

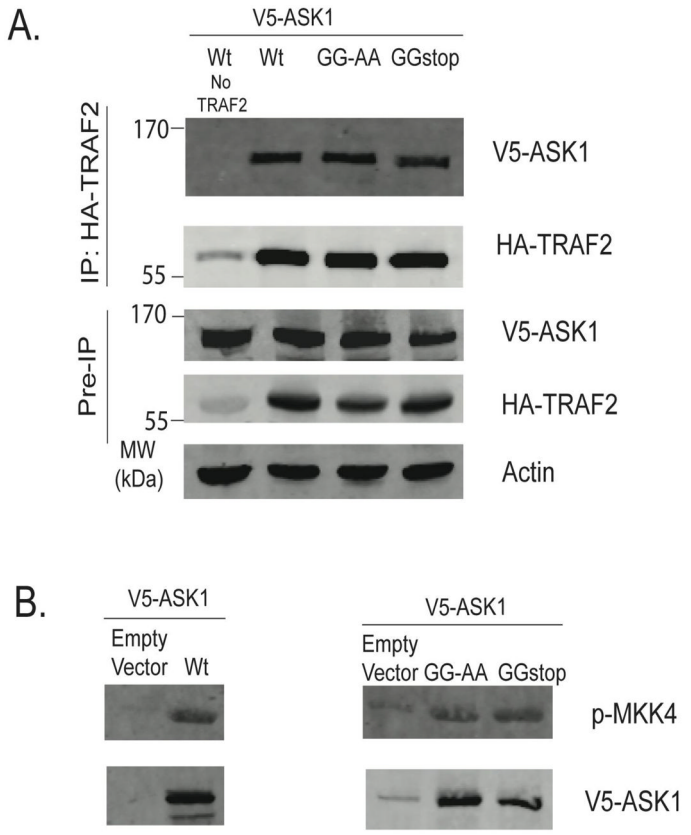

Fig 2.

Mutations in the LRLRGG sequence do not impair ASK1 kinase activity or association with TRAF2. A. HEK 293 cells were transfected with ASK1 or mutants as described, and V5 immunoprecipitates were assessed for ASK1 kinase activity in vitro by assessing the phosphorylation of recombinant MKK4 by immunoblotting for phospho-MKK4. B. HEK 293 cells were transfected with ASK1 or mutants as described along with HA-TRAF2, and HA immunoprecipitates were assessed for association with ASK1 by immunoblotting for V5.

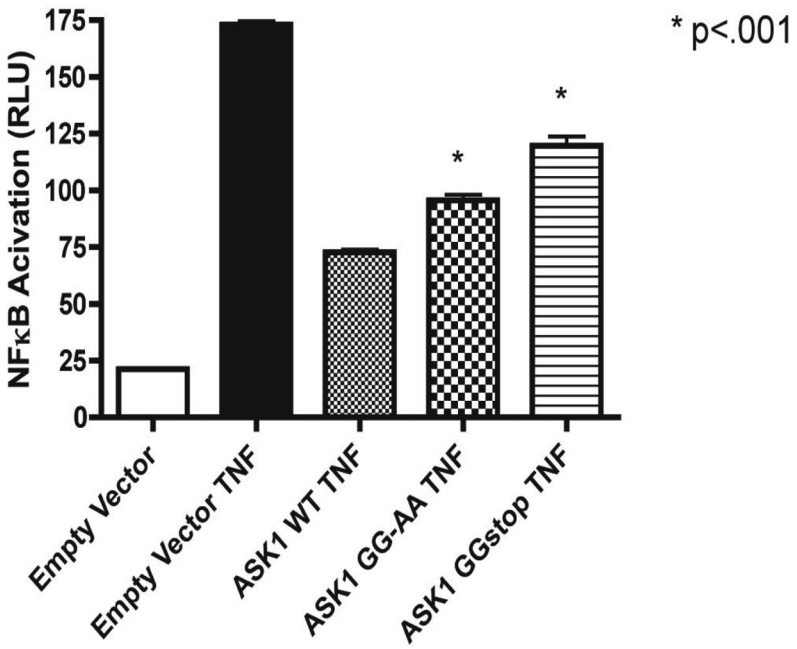

Fig. 3.

Mutations in the LRLRGG motif decrease inhibition of NF-kB activation by ASK1. HEK 293 cells were transfected with ASK1 or mutants as described, HEK 293 cells were transfected with either 5ug of Wildtype ASK1, ASK1 with LRLRGG mutated to LRLRAA, or ASK1 with a stop codon introduced after the LRLRGG sequence along with a NF-κB luciferase reporter plasmid and Renilla-TK as a control. Cells were treated with TNF (20ng/ml; 12 h) and lysates were assayed for NF-kB activity.

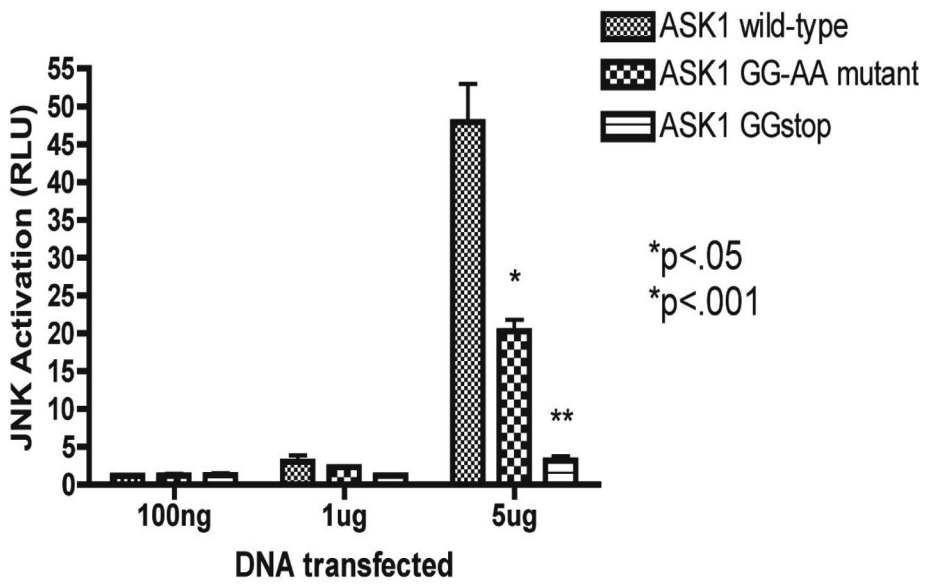

Fig. 4.

Mutations in the LRLRGG motif decrease ASK1-induced JNK activation. HEK 293 cells were transfected with ASK1 or mutants as described. HEK 293 cells were transfected with 100ng, 1ug and 5ug of Wildtype ASK1, ASK1 with LRLRGG mutated to LRLRAA, or ASK1 with a stop codon introduced after the LRLRGG sequence along with a c-jun luciferase reporter plasmid and Renilla-TK as a control, and lysates were assessed for jun activation by measuring luminescence.