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RELT family members activate p38 and induce apoptosis by a mechanism distinct from TNFR1

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Abstract

Receptor Expressed in Lymphoid Tissues (RELT) is a human Tumor Necrosis Factor Receptor (TNFR) family member that has two identified homologous binding partners, RELL1 and RELL2. This study sought to further understand the pattern of RELT expression, the functional role of RELT family members, and the mechanism of RELT-induced apoptosis. RELT protein expression was detected in the spleen, lymph node, brain, breast and peripheral blood leukocytes (PBLs). A smaller than expected size of RELT was observed in PBLs, suggesting a proteolytically cleaved form of RELT. RELL1 and RELL2 overexpression activated the p38 MAPK pathway more substantially than RELT in HEK-293 cells, and this activation of p38 by RELT family members was blocked by dominant-negative mutant forms of OSR1 or TRAF2, implicating these molecules in RELT family member signaling. RELT was previously shown to induce apoptosis in human epithelial cells despite lacking the characteristic death domain (DD) found in other TNFRs. Seven deletion mutants of RELT that lacked differing portions of the intracellular domain were created to assess whether RELT possesses a novel DD. None of the deletion mutants induced apoptosis as efficiently as full-length RELT, a result that is consistent with a novel DD being located at the carboxyl-terminus. Interestingly, induction of apoptotic morphology by RELT overexpression was not prevented when signaling by FADD or Caspase-8 was blocked, indicating RELT induces apoptosis by a pathway distinct from other death-inducing TNFRs such as TNFR1. Collectively, this study provides more insights into RELT expression, RELT family member function, and the mechanism of RELT-induced death.

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Keywords

RELT; RELL1; RELL2; TNFR; p38; apoptosis

Introduction

Tumor Necrosis Factor Receptor (TNFR) superfamily members are vitally important for a variety of processes including inflammation, apoptosis and development [1, 2]. The paradigm for transmembrane TNFR activation is through binding to its cognate external Tumor Necrosis Factor (TNF) ligand superfamily member, resulting in the activation of intracellular signal transduction pathways [3]. Many TNF and TNFR modulating therapeutics are currently in use for the treatment of diseases such as autoimmunity, inflammation and cancer [1, 4]. There are 19 distinct TNF ligands and 29 TNFRs currently identified in humans [1]. Receptor Expressed in Lymphoid Tissues (RELT) is a recently identified TNFR that is predominantly transcribed in lymphoid tissues. RELT was identified through its homology to OX40 in the extracellular domain (ECD) and the presence of two cysteine-rich domains (CRDs) characteristic of TNFRs [5]. RELT is an orphan receptor whose corresponding TNF ligand has not been identified despite a comprehensive study [6] and there exists a possibility that RELT does not bind to a TNF ligand. The extracellular domain (ECD) of RELT has been reported to stimulate T-cell proliferation in a CD3 dependent manner [5]. It is not clear whether RELT activates NF-κβ or binds TRAF1 based on conflicting reports, yet two studies reported that RELT does not bind TRAFs 2–6 [5, 7]. RELL1 and RELL2 are two homologous binding partners of RELT and are considered RELT family members, yet RELL1 and RELL2 contain truncated ECDs that lack the CRDs typical of TNFRs [8]. RELT binds PLSCR1 [9], and is phosphorylated by the closely related kinases OSR1 and SPAK [7, 8]. RELT activation of the p38 pathway requires phosphorylation by SPAK [7]. RELT family member overexpression induces cell death in human epithelial cells with features characteristic of an apoptotic pathway despite the absence of a characteristic death domain (DD) in RELT [10].

Potentially diverse roles for RELT family members in human health is supported by the identification of RELT and RELL1 in various recent non-biased screens and gene-profile analyses. Autoantibodies directed against RELT were identified as a potential tumor marker for the early detection of breast cancer [11]. RELT was observed to be expressed only in apoptotic cells, but not oncotic cells, in an acute pancreatitis model [12]. RELT expression doubled when tumor cells underwent the epithelial-mesenchymal transition critical for cancer development [13]. RELL1 was found to exhibit a circadian expression pattern in hepatocytes in a manner that is controlled post-transcriptionally by a microRNA [14]. RELL1 was identified as a gene associated with a higher rate of stillborn fetuses in pigs [15], is also implicated in the age of fertility in cattle [16], collectively suggesting that RELL1 may influence reproductive traits in humans. Finally, RELL1 has been associated with a higher incidence of *S. aureus* nasal carriage in a community-based sample of Mexican-Americans in a Texas community [17]. Our study sought to further understand RELT by examining its expression at the protein level, investigating the activation of p38 by

RELT family members, and examining the effects of RELT deletion mutants and extrinsic apoptotic pathway inhibitors on RELT-induced apoptosis.

Results and Discussion

Figure 1. RELT protein expression in human tissues

Western blots of human tissue extracts were utilized to assess RELT protein expression, as RELT expression has only been previously reported at the transcriptional level [5]. A band of the expected approximately 46 kDa protein was detected in the spleen, lymph node, brain, breast, kidney and PBLs (Fig. 1). The band detected in the human tissue extracts was the same size as recombinant RELT (data not shown). Elevated RELT protein expression in the spleen and lymph node are consistent with previous reports of RELT mRNA expression in lymphoid tissues [5]. Conversely, RELT protein expression was not detected in the bone marrow, despite previous detection of mRNA in the bone marrow [5]. Interestingly, although full-length RELT was observed in peripheral blood leukocytes (PBLs), the prominent band observed was approximately 30 kDa, suggesting that PBLs express a truncated form of endogenous RELT. A truncated form of recombinant RELT with an N-terminus corresponding to residue 131 of the ECD was reported [5], which would correspond to a protein of 31.8 kDa, in close agreement with the band observed in PBLs. A potential cleavage of the ECD of RELT in PBLs is of interest since the ECD of RELT has previously been reported to stimulate T lymphocytes. There have been reports of truncated forms of TNFRs possessing biological activity as either signaling molecules or decoy receptors [1, 3]. Alternatively, the 30 kDa form could result from an alternate transcription start site or an alternative mRNA splicing event, as has been reported for other TNFRs [18].

Figure 2. RELT family members activate the p38 pathway

This study examined the contribution of RELL1 and RELL2 to RELT activation of MAPK pathways. RELT has been reported to activate p38 in 293 cells [7] while the ability of RELT to activate NF-κβ is uncertain. [5, 7]. Luciferase reporter assays were used to measure the transactivation of promoters in response to transient transfection of expression plasmids as described in Materials and Methods. Preliminary results testing whether RELT activates NF-κβ were inconclusive (data not shown). Overexpression of RELL1 and RELL2 increased p38 activity approximately 3.7-fold and 2.8-fold respectively in comparison with the empty plasmid (Figs. 2B and 2C). The effect of RELT overexpression by itself, or co-expression of RELT with RELL1 or RELL2 had a negligible effect on p38 activation, despite detection of recombinant RELT protein (data not shown). Significantly enhanced p38 activation ($P<0.005$) was observed with co-expression of RELL1 and RELL2 together versus expression of either construct alone.

The ability of TRAF2 and a TRAF2 mutant lacking its N-terminal RING finger [19] to influence p38 activation by RELL1 and RELL2 was tested, as TRAF2 is a RELL2 binding protein [10]. Co-expression of the RING mutant with either RELL1 or RELL2 plasmids resulted in significantly less activation of p38 ($P<0.005$) in comparison to expression of the individual RELT members alone. Co-expression of TRAF2, a protein that is endogenously expressed in 293 cells, with RELL2, resulted in a modest increase in p38 activation (Fig.

2C). Interestingly, overexpression of TRAF2 with RELL1 resulted in significantly less activation of p38 ($P<0.05$) compared to RELL1 by itself (Fig. 2B). The differing effects of TRAF2 on p38 activation might be related to the differential binding of TRAF2 to RELL2, but not RELL1 [10]. These results suggest that signaling properties of RELT-RELL1 and RELT-RELL2 heterotrimeric complexes may not be identical. Likewise, RELL1 and RELL2 exhibited differing effects on RELT's ability to induce apoptosis in a previous study; RELL1 co-expression enhanced apoptosis induced by RELT, whereas RELL2 suppressed RELT-induced apoptosis [10].

We next tested the ability of RELL1 and RELL2 to activate p38 in the presence of either wild-type OSR1, a kinase that phosphorylates all 3 RELT family members, or the kinase inactive K46M mutant that is incapable of phosphorylation [8]. Overexpression of OSR1, a protein endogenously expressed in 293 cells, had no significant effect on the activation of p38 by RELL1 or RELL2. Co-expression of the K46M mutant significantly decreased p38 activation ($P<0.005$) by RELL1 or RELL2 (Figs. 2B, 2C). These results support a model in which the ability of RELL1 or RELL2 to activate p38 is dependent on phosphorylation by OSR1, similar to the previous report of p38 activation by RELT being dependent on phosphorylation by the closely related kinase SPAK [7].

Figure 3. RELT deletion mutants

A series of RELT deletion mutants were constructed to test whether RELT possesses a novel DD, as RELT overexpression is able to induce apoptosis [10] despite the absence of the characteristic DD found in other TNFRs [3]. Seven deletion mutants of RELT were created (Fig. 3A) and expression of the deletion mutant constructs in 293 cells produced proteins of the expected sizes (Fig. 3B). Interestingly, expression of RELT mutants lacking more than 60 intracellular amino acids proximal to the plasma membrane were only detectable when western blots were overexposed. These results suggest that regions of the ICD proximal to the plasma membrane (191-252) may be important for protein stability as proteins that are not able to properly fold in the rough endoplasmic reticulum are marked for destruction in the proteasome.

Deletion mutants were tested for their ability to induce characteristics of cell death in 293 cells as previously described for full-length (FL) RELT [10]. Specific endpoints were the ability to induce apoptotic morphology, as measured by X-gal staining (Fig. 3C), and DNA fragmentation, as measured by TUNEL staining (Fig. 3D). All deletion mutants showed reduced cell death compared to FL RELT (Figs. 3C, 3D). Mutant E, which lacks only 10 amino acids from the carboxyl end, was not significantly different from full length RELT when apoptosis was assayed by TUNEL (Fig. 3D), while all other deletions showed a significant reduction in apoptosis. This is consistent with the presence of a novel DD in the carboxyl terminus of RELT. Alternatively, RELT-induced death may require multiple regions of RELT, through a folding mechanism for example. A more definitive experiment to test whether a novel DD exists at the carboxyl terminus will require creating constructs that encode for increasingly larger portions of the carboxyl terminus fused to mutant H, as the Δ252-430 deletion present in mutant H was the smallest RELT mutant construct that was stably expressed.

While our results using luciferase reporter assays suggest that RELT does not significantly enhance p38 activation (Fig. 2A), a previous report did show that RELT activates p38 in 293 cells [7]. To address this further we used a phospho-specific antibody for p38 to assess the ability of FL RELT and deletion mutants to activate p38 (Fig. 3E). RELT exhibited modest activation of p38 in comparison with sorbitol treatment (Fig. 3E) or OSR1 overexpression (data not shown). Mutant E appeared to activate p38 better than other mutants, consistent with a signaling domain being present at the carboxyl terminus. The lack of robust activation of p38 by RELT observed in this study compared with the previous report may be attributed to the use of different recombinant constructs combined with different p38 activity assays. Nevertheless, collectively there is agreement that RELT family members activate p38 in a manner that is dependent on phosphorylation by the closely related OSR1 and SPAK kinases (67% amino acid identity). The physiological relevance of this signaling needs to be explored further, for example by testing the dependence of p38 activation on RELT family member-induced death. Although 293 cells have been the primary focus of studying RELT by multiple laboratories due to their amenability to transient transfections, experiments conducted with endogenous RELT in hematopoietic cell lines would be more informative with regards to the physiological role of RELT activation of p38. Identification of the RELT ligand would be helpful in this regard.

Figure 4. RELT-induced death is independent of FADD and Caspase-8

The absence of an identifiable DD in RELT prompted us to examine whether RELT utilizes the extrinsic pathway of apoptosis. TNFR1 is a prototypical TNFR that activates the extrinsic pathway, a pathway characterized by binding of FADD to activated TNRF complexes, followed by subsequent recruitment and activation of Caspase-8, leading to apoptosis through activation of Caspase-3 [1]. Expression plasmids for dominant negative mutants of FADD and Caspase-8, as well as an expression plasmid for the caspase inhibitor CrmA, were co-transfected with either the expression plasmid for RELT or TNFR1. CrmA preferentially inhibits Caspase-8 and Caspase-6, while exhibiting minimal inhibition of Caspase-3 [20]. X-gal staining was used to detect apoptotic morphology as described in Materials and Methods. As shown in Fig. 4A, TNFR1-induced apoptosis was blocked by all 3 inhibitors of apoptosis ($P<0.005$). Conversely, induction of apoptosis by RELT was not blocked by the caspase or FADD inhibitors (Fig. 4A). Likewise, apoptosis induced by expression of either RELL1 or RELL2 was not blocked by the presence of CrmA (data not shown). These results suggest that RELT family members induce apoptosis by a pathway that is distinct from other death-inducing TNFR members such as TNFR1. RELT has previously been reported to induce the cleavage of Caspase-3 [10], therefore RELT appears to activate a novel pathway resulting in cleavage of Caspase-3 in a manner that is independent of FADD, Caspase-8 and Caspase-6. It will be of interest to explore the mechanism of RELT-induced death further and to elucidate the physiological significance of RELT-induced death. Collectively, the results in this report further our understanding of RELT expression at the protein level, activation of p38 by RELT family members, and identify RELT as a TNFR that induces apoptosis by a novel extrinsic pathway.

Materials and Methods

Reagents

The human embryonic kidney 293 (293) cell line was purchased from ATCC. The mammalian expression plasmid constructs for RELT, RELL1, RELL2, RFRV mutant of RELT [8] and MACH 360 C/S [21] were described previously. Expression constructs for TRAF2, TRAF2-RING, CrmA and Fadd DN were a kind gift from D. Goeddel. The MEKK3 plasmid was a kind gift from G. Johnson. The human tissue extracts, monoclonal anti-RELT (C-6), p38 alpha/beta (A-12) and GADPH antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The RELT polyclonal antibody (AF1385) was purchased from R&D systems (Minneapolis MN). The phospho-p38 (D3F9), mouse anti-DYKDDDDK (equivalent to FLAG) tag and rabbit anti-HA antibody were purchased from Cell Signaling Technology, Inc. (Danvers, MA). IR-800 and IR-680 secondary antibodies were purchased from LI-COR Biosciences (Lincoln, NE). TUNEL staining kit was purchased from Roche Diagnostics (Mannheim, Germany). The CHOP luciferase trans-reporting system and QuickChange site-directed mutagenesis kit were purchased from Stratagene (La Jolla, CA).

Western blots

Tissue extracts were analyzed by western blotting utilizing a monoclonal RELT antibody. For detection of RELT deletion constructs or p38 activation, 293 cells ($\approx 5 \times 10^6$) were transfected with 10 μg of empty vector plasmid (V), or expression plasmids for either FL RELT or the indicated deletion mutants. RELT expression was detected using a polyclonal RELT antibody. For detecting p38 activation, protein lysates were created from 293 cells a day after being transfected with the indicated plasmids, or alternatively from 293 cells treated with 0.7 M sorbitol as indicated. Detection of phosphorylated p38 used an antibody that recognizes all phosphorylated isoforms of p38 (D3F9). Detection of both phosphorylated and unphosphorylated alpha/beta p38 used a monoclonal antibody (A-12).

Luciferase assay

The detection of p38 activity was indirectly determined by measuring CHOP activity utilizing the Pathdetect in vivo Signal Transduction Pathway trans reporting systems protocol from Stratagene. 293 cells ($\approx 1 \times 10^5$) were transfected with 2.0 μg of the indicated expression plasmids, 0.5 μg of pFR-luciferase reporter plasmid, 0.05 μg of the trans-activating plasmid pFA-CHOP and 0.2 μg of RSV-β-galactosidase luciferase reporter plasmid. Empty vector control was added as appropriate to ensure transfection of equal amounts of DNA. Luciferase assays were performed 16 hours after transfection as described previously [22]. Each value represents an average of 6 individual data points from 3 separate experiments performed in duplicate. A two-tailed student's t-test was utilized to determine statistical significance.

Creation of deletion mutants

For deletion mutants B, D, E, F and G, restriction digests and subsequent ligations into pcDNA3 were utilized to create the indicated constructs. Degenerate PCR primers and the

Quickchange site-directed mutagenesis kit (La Jolla, CA) were utilized to introduce premature stop codons for the creation of the H and I mutants. The DNA sequences of all constructs were confirmed. Western blotting was utilized to confirm the expected sizes of the constructs.

Apoptotic assays

For all apoptotic assays, a minimum of 5 viewing fields were quantified for each individual data point. Each experiment was performed a minimum of 3 separate times. A two-tailed student's t-test was utilized to determine statistical significance. Morphology staining: 293 cells (~1×10^5) were transfected with 1.0 μg of the indicated expression plasmids together with 0.1 μg of an expression plasmid for the β-galactosidase gene. Cells were stained with X-gal 48 hours after transfection as described previously [10]. Results are expressed as the percentage of β-galactosidase positive cells that are undergoing cell death based on observed morphology as observed under x20 magnification. TUNEL staining: 293 cells (~5×10^4) were transfected with 0.5 μg of an expression plasmid for Green Fluorescent Protein (GFP) together with 0.5 μg of the indicated expression plasmid and assayed for DNA fragmentation 48 hours later utilizing TUNEL staining as described previously [10]. Results are expressed as the percentage of cells that are both TUNEL and GFP positive versus cells that are solely GFP positive as viewed under x40 magnification.

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- RELT protein is expressed in lymphoid tissues; truncated RELT is observed in PBLs
- RELT family members activate p38
- RELT-induced apoptosis is not blocked by FADD or Caspase-8 inhibitors

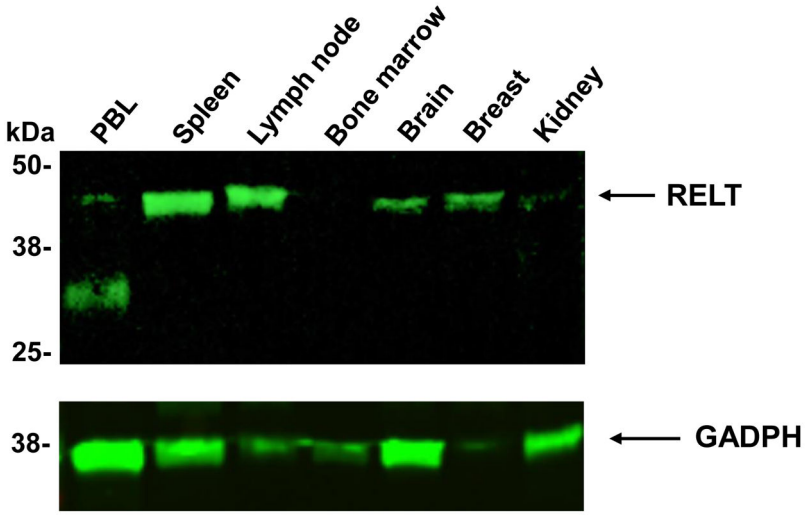


Figure 1. RELT protein expression in human tissues

RELT expression was detected in human tissue extracts by western blotting as described in Materials and Methods. The blot was reprobed with an anti-GADPH primary antibody for a loading control (PBL, peripheral blood leukocyte).

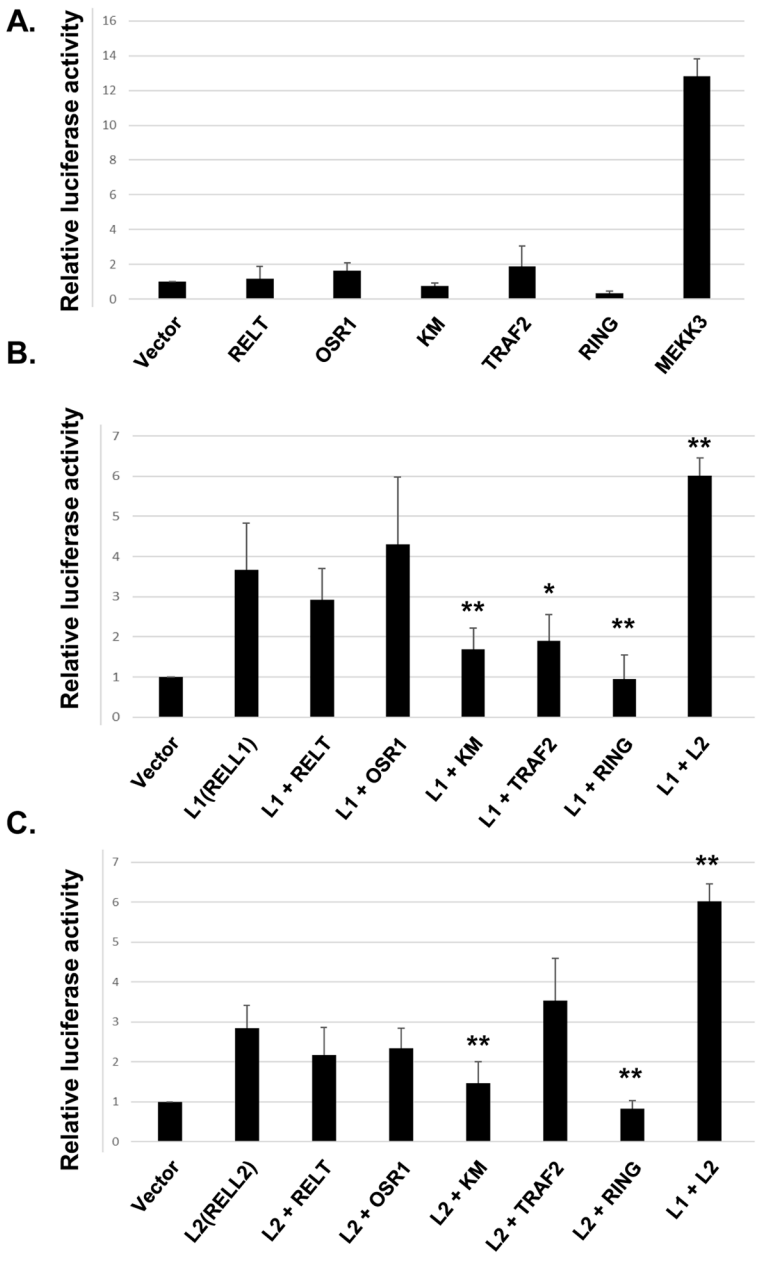
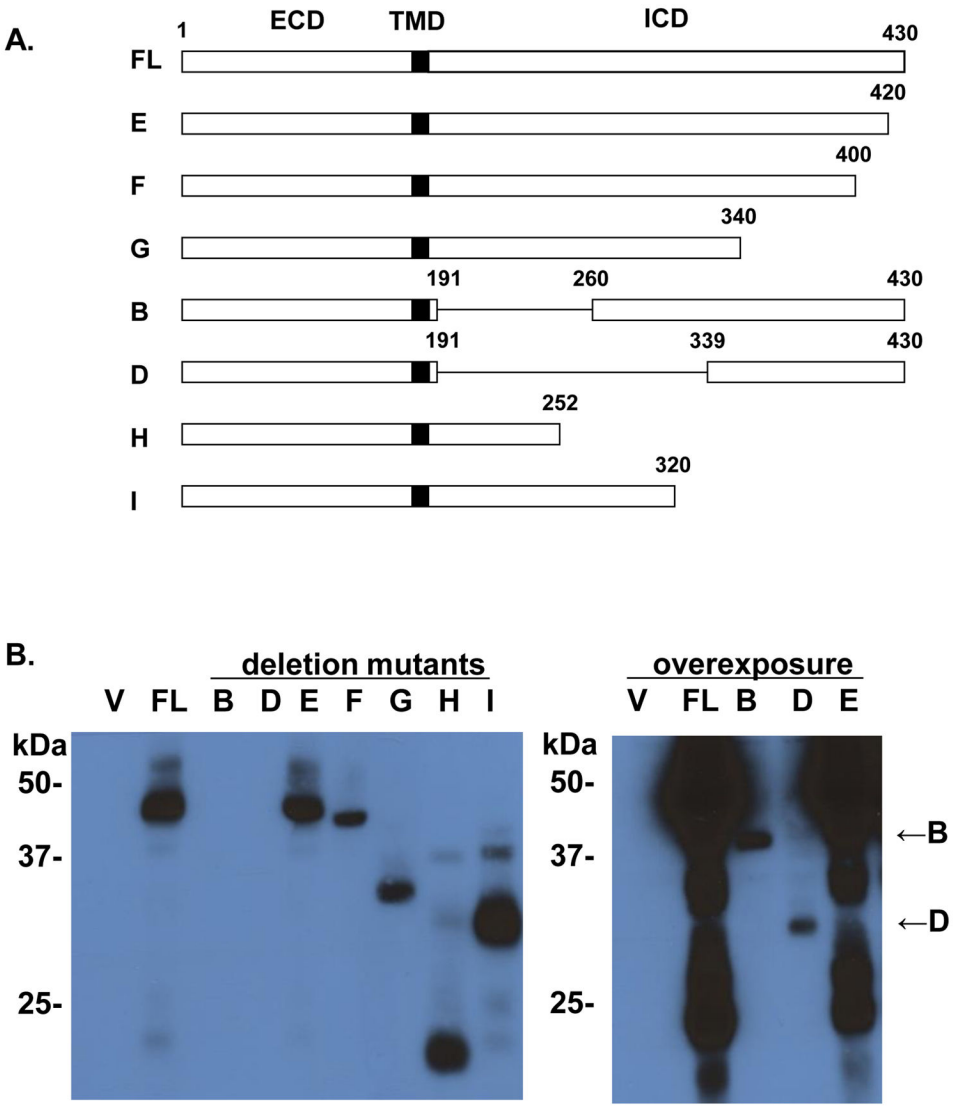


Figure 2. RELT family members activate p38

A. HEK-293 cells were transfected with the indicated expression plasmids and assayed for p38 indirectly through measurement of CHOP mediated luciferase expression as described in Materials and Methods. Data are normalized to empty vector. Significant values of $P<0.05$ (*) and $P<0.005$ (**) are indicated. *A.* Transfection of empty vector plasmid or expression plasmids for RELT, OSR1, OSR1 K46M (KM), TRAF2, TRAF2 RING mutant (RING) and MEKK3 as indicated. *B.* Transfection of empty vector plasmid or co-transfection of expression plasmids for RELL1 (L1) with the indicated plasmid. *C.* Same as in B, except that co-transfection of expression plasmid for RELL2 (L2) with the indicated plasmid.



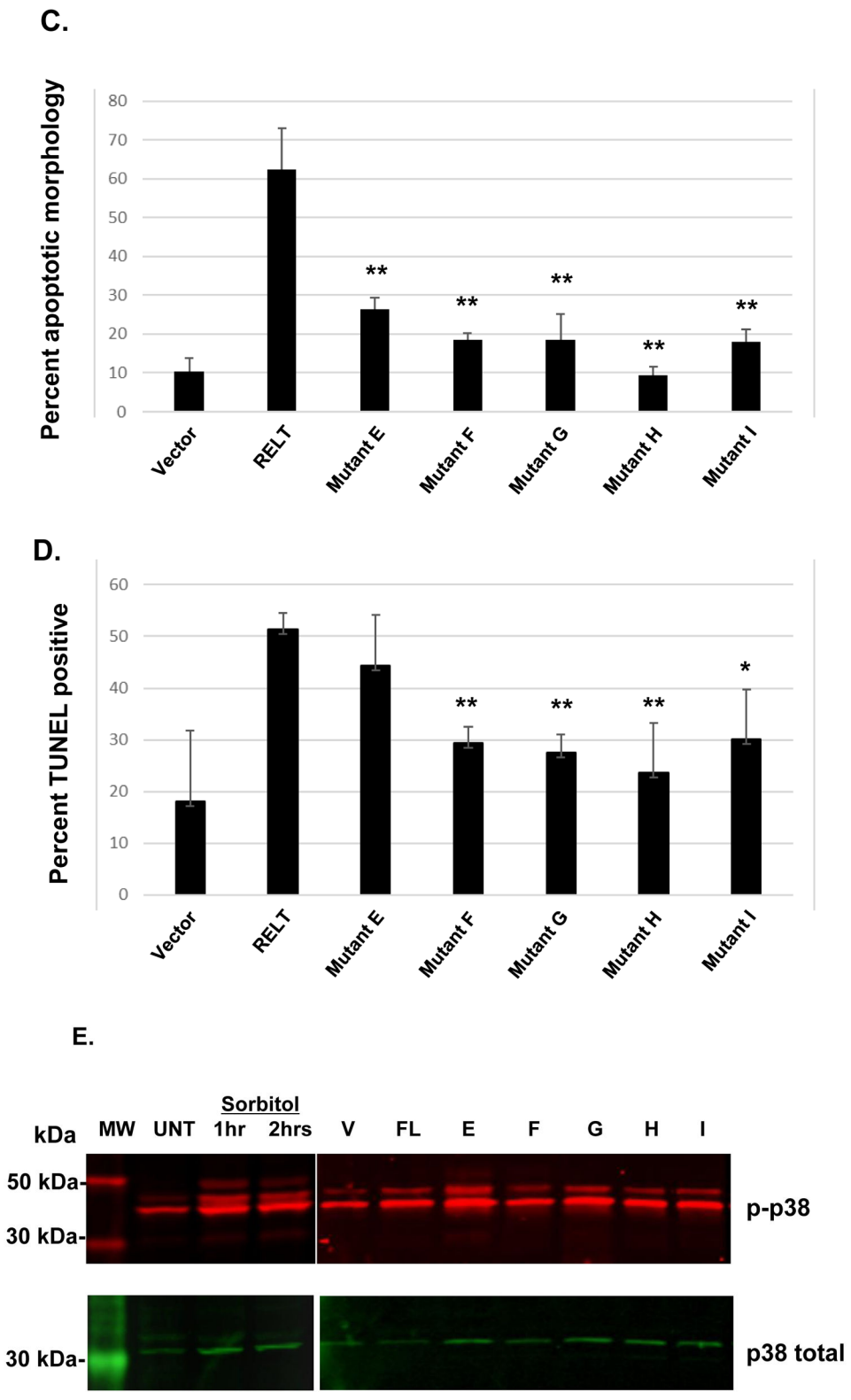


Figure 3. RELT deletion mutants

A. Map of deletion mutants of RELT constructed utilizing site-directed mutagenesis. The predicted ECD, TMD highlighted in black and ICD of each construct are indicated. Mutants E, F, G, H and I were designed to possess a truncated carboxyl-terminal end as indicated. Mutant B was designed to contain an internal Δ192-259 deletion and mutant D was designed to contain a Δ252-420 deletion*. B. Western blot of RELT mutant expression*. 293 cells were transfected with the indicated expression plasmids and protein lysates were analyzed by western blotting as described in Materials and Methods. Two images of the same representative blot are shown and overexposure of the representative blot is indicated. *C. Ability of RELT mutants to induce apoptotic morphology*. 293 cells were transfected with the indicated expression plasmids together with an expression plasmid for the β-galactosidase gene. Cells were stained with X-gal as described in Materials and Methods. Results are expressed as the percentage of β-galactosidase positive cells that are undergoing cell death based on morphology. Significant values of $P<0.005$ (**) are indicated. *D. Ability of RELT mutants to induce DNA fragmentation*. 293 cells were transfected with expression plasmid for GFP together with the indicated expression plasmid and assayed for DNA fragmentation as described in Materials and Methods. Results are expressed as the percentage of cells that are both TUNEL and GFP positive versus cells that are solely GFP positive. Significant values of $P<0.05$ (*) and $P<0.005$ (**) are indicated. E. *Ability of RELT mutants to activate p38*. 293 cells were either left untreated (UNT), treated with sorbitol for 1 or 2 hours (hr), or transfected with the indicated expression plasmids: empty vector (V), FL RELT, or for the indicated RELT deletion mutants. Protein lysates were collected and analyzed by western blotting for phosphorylated p-38 (top panel - red) as well as alpha and beta isoforms of p38 regardless of phosphorylated state (bottom panel - green) as described in Materials and Methods.

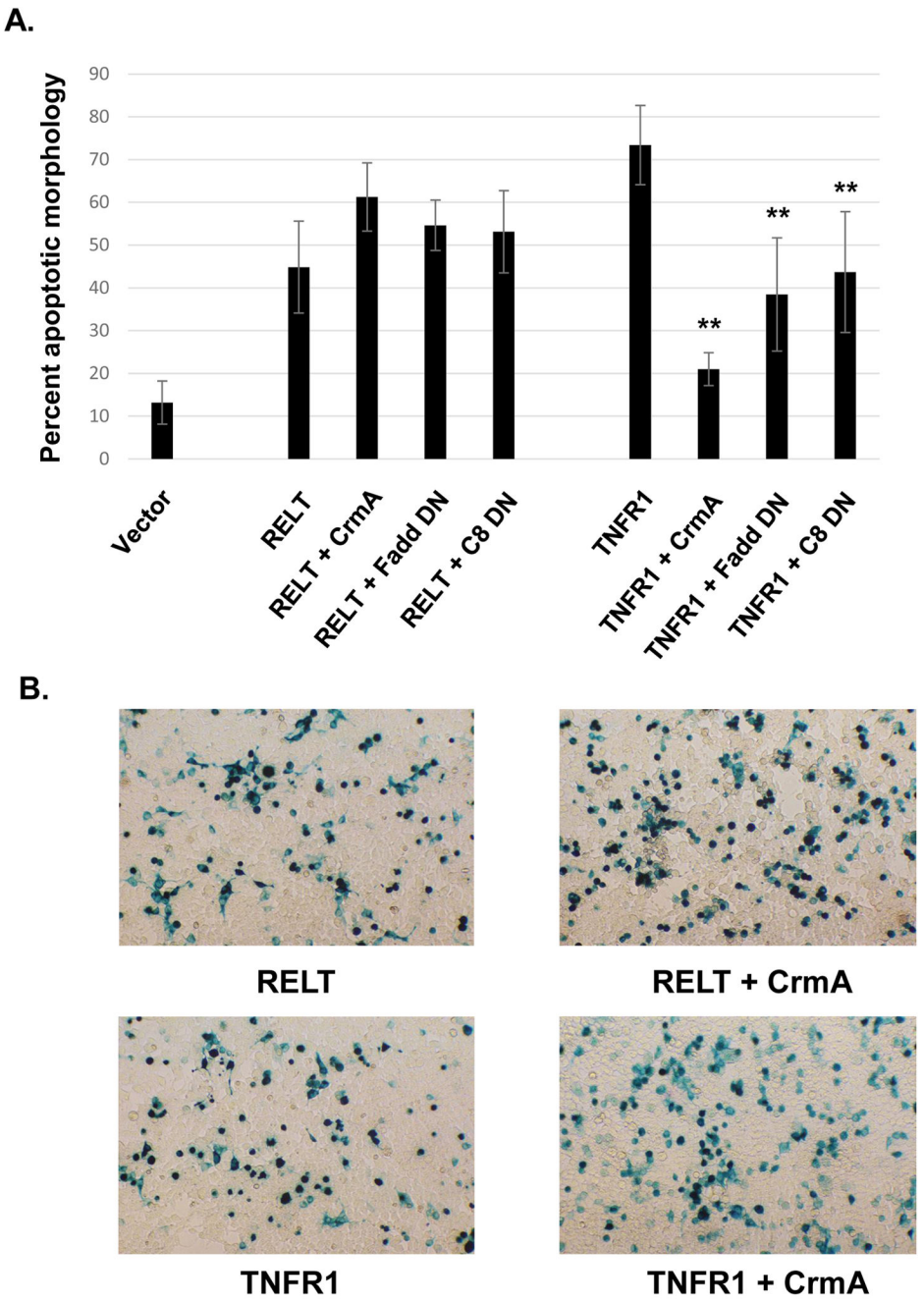


Figure 4. RELT-induced death is independent of FADD and Caspase-8

293 cells were transfected with RELT or TNFR1 in combination with the indicated inhibitor of apoptosis: CrmA, the Fadd dominant negative mutant (FaddDN) or a Caspase-8 C360S dominant negative mutant (C8 mutant). Additionally, each well was transfected with an expression plasmid for β-galactosidase. Cells were stained with X-gal 48 hours after transfection as described in Materials and Methods. Results are expressed as the percentage of β-galactosidase positive cells that are undergoing cell death based on morphology.

Significant values of $P<0.005$ (**) are indicated. B. Representative bright-field images under x20 magnification of cells transfected with the indicated expression plasmids.