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A20 Ubiquitin Ligase-Mediated Polyubiquitination of RIP1 Inhibits Caspase-8 Cleavage and TRAIL-Induced Apoptosis in Glioblastoma

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Abstract

The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) apoptotic pathway has emerged as a cancer therapeutic target. However, clinical trials have proven that the vast majority of human cancers are resistant to TRAIL-targeted therapies. We show here that A20-mediated ubiquitination inhibits caspase-8 cleavage and TRAIL-induced apoptosis in glioblastoma through two signaling complexes. A20 is highly expressed in glioblastomas and, together with the death receptor 5 (DR5) and receptor-interacting protein 1 (RIP1), forms a plasma membrane bound preligand assembly complex (PLAC) under physiologic conditions. TRAIL treatment leads to the recruitment of caspase-8 to the PLAC for the assembly of a death-inducing signaling complex (DISC). In the DISC, the C-terminal Zinc finger (Znf) domain of A20 ubiquitin ligase mediates RIP1 ubiquitination through lysine (K)-63-linked polyubiquitin chains that bind the protease domain of caspase-8 and inhibits its dimerization, cleavage and the initiation of TRAIL-induced apoptosis in glioblastoma-derived cell lines and tumor-initiating cells.

Keywords
A20; apoptosis; caspase-8; TRAIL; ubiquitination

INTRODUCTION

The tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) (1, 2) executes the innate and adaptive immunity in the process of tumor immunosurveillance (3). The anticancer activity of TRAIL contributes to its ability to induce apoptosis through binding of the cancer cell surface death receptor 4 and 5 (DR4, DR5) and the recruitment of intracellular apoptosis-initiating caspase-8 through Fas-associated death domain (FADD) for the assembly of a death-inducing signaling complex (DISC) (4). Recombinant TRAIL and its agonistic DR4 and DR5 antibodies have been generated targeting this apoptotic pathway for cancer therapies (5). Clinical trials, however, have proved that cancers are resistant to the
TRAIL pathway-targeted therapies (6–8), thus suggesting the notion that cancer occurs in patients by escape from TRAIL-mediated immunosurveillance and are resistant to TRAIL-targeted therapies.

The dimerization and cleavage of caspase-8 in the DISC are the critical upstream events in TNF family ligand-induced apoptosis (9–12) and ubiquitination of proteins in the DISC regulates these biochemical processes (13). Ubiquitin (UB) is covalently attached to lysine residues of the substrate proteins through the catalytic reactions mediated by the UB-activating (E1), conjugating (E2) and ligase (E3) and removed by deubiquitinating (DUB) enzymes (14). UB has seven lysine (K) residues; each of them and its N-terminal methionine (M1) can be linked to the C-terminal glycine residue of another UB to form polyUB chains (15), which regulate TNFα-induced signal (13). Upon TNFα binding, TNF receptor 1 (TNFR1) recruits receptor-interacting protein 1 (RIP1), cellular inhibitor of apoptosis protein 1 and 2 (cIAP1 and cIAP2) and TNFR-associated factor 2 (TRAF2) for the assembly of TNFR1-associated complex I (16). TRAF2, cIAP1 and cIAP2 E3 ligases that activate nuclear factor-κB (NF-κB) through attaching polyUB chains to RIP1 (17) and binding of the polyUB chain to IκB kinase γ (IKKγ) (18). TRAF2 and RIP1 then detach from TNFR1 and recruit FADD and caspase-8 for the assembly of the cytoplasm complex II (19), where cylindromatosis (CYLD), a DUB removes the polyUB chains from RIP1 and promotes caspase-8 cleavage for TNFα-induced apoptosis (20).

Unlike TNFR1, however, DR4 and DR5 recruit FADD and caspase-8 in the assembly of a plasma membrane bound DISC where caspase-8 becomes dimerized and cleaved, initiating apoptosis (21, 22). Cullin 3 (CUL3), an E3 ligase adds K48 and K63-linked polyUB chains to caspase-8 and facilitates its dimerization and cleavage in the DISC, where A20 DUB removes the polyUB chains from caspase-8 (23). A20 (TNFα-induced protein 3; TNFAIP3) is well known for its anti-inflammatory activities (24) through its N-terminal ovarian tumor domain (OTU) that acts as a DUB and removes K63-linked polyUB chains from RIP1, TRAF6 and RIP2, thus restricting TNFR1, Toll-like receptor and nucleotide-binding oligomerization domain-induced NF-κB signal (25–27). In addition, A20 has a C-terminal Zinc finger (Znf) domain of an E3 ligase (28), but function of the Znf E3 ligase has yet to be established.

Here, we show that the Znf domain of A20 E3 ligase mediates RIP1 ubiquitination through a K63-linked polyUB chain that binds the caspase-8 protease p18 domain, blocks its dimerization and cleavage and thus inhibits TRAIL-induced apoptosis in human glioblastoma. Glioblastoma is the most common brain cancer for which there is no curative treatment. Recent studies have identified tumor-initiating cells (29) and shown that the tumor-initiating cells retain the original tumor genomic features (30), possess self-renewal and tumorigenic capacity (31) and are responsible for the tumor resistance to treatments (32). In this study, we further establish that A20 E3 ligase-mediated RIP1 ubiquitination inhibits caspase-8-initiated and TRAIL-induced apoptosis in the tumor-initiating cells isolated from glioblastomas surgically removed from patients.

RESULTS

The Preligand Assembly Complex (PLAC) Is Formed under Physiologic Conditions

In exploring the role of the UB enzymes A20, cIAP1/2, CUL3 and TRAF2 in glioblastoma, we first analyzed the expression of these proteins in glioblastoma tissues. Immunoblotting (IB) revealed that A20 was highly expressed in the tumors as compared to normal brains whereas CUL3, cIAP2 and TRAF2 were expressed consistently in the normal brain and tumor tissues and cIAP1 was seen in some tumors (Fig. 1A). To examine whether these enzymes regulate TRAIL signaling in glioblastoma cells, we analyzed their expression in...
TRAIL-sensitive (LN18, LN71, T98G, U343MG) and resistant glioblastoma cell lines (LN443, U87MG, U118MG, U138MG). The TRAIL sensitivity of these cell lines was defined in our earlier study (33) and confirmed by colony formation assay (Supplementary Fig. S1A). A20 was highly expressed in the resistant cell lines but barely detected in the sensitive cell lines and normal human astrocytes (34); in contrast, CUL3, cIAP1, cIAP2, RIP1 and TRAF2 were consistently expressed in the resistant and sensitive cell lines (Supplementary Fig. S1B).

The DISC was then isolated from each cell line through immunoprecipitation (IP) after the cells were treated with mixed Flag-TRAIL and Flag antibody. For the unstimulated control, the cells were lysed first and treated with Flag-TRAIL and Flag antibody. Taking this approach, we have shown that DR5 but not DR4 is the functional receptor that is expressed and interacts with Flag-TRAIL in glioblastoma cells (33). IB detected DR5, A20, TRAF2 and RIP1 in the DISC in the resistant lines but only DR5 and TRAF2 in the sensitive lines (Fig. 1B; Supplementary Fig. S2A). FADD and caspase-8 were recruited to the DISC in both resistant and sensitive cell lines; however, caspase-8 was cleaved only in the DISC isolated from the sensitive cells. In contrast, cIAP1 was not seen in the DISC from any of these cell lines whereas cIAP2 was detected only in the DISC of sensitive cell lines. CUL3 was detected in the DISC of H460, a TRAIL-sensitive lung cancer line as reported (23), but not in the DISC isolated from glioblastoma cell lines (Supplementary Fig. S2B). TRAIL stimulated the caspase-8 ubiquitination in H460, consistent with the report (23), but not in glioblastoma cell lines (Supplementary Fig. S2C).

To our surprise, A20, RIP1 and TRAF2 were seen in the unstimulated controls in the resistant cell lines whereas only TRAF2 was seen in the sensitive cell lines (Fig. 1B), suggesting that these proteins might interact with DR5 and form a complex prior to TRAIL treatment. To test this, we carried out size exclusion analysis and IB (19) and identified DR5, A20, RIP1 and TRAF2 in the ~669 kDa fractions from resistant lines, but only DR5 and TRAF2 in sensitive lines (Fig. 1C; Supplementary Fig. S3A). These proteins remained in the high molecular weight fractions in the cells after TRAIL treatment. In contrast, caspase-8 isoforms were eluted in the fractions corresponding to their monomeric molecular weights prior to TRAIL treatment but quickly shifted to high molecular weight fractions under TRAIL treatment. These results suggest the presence of a DR5-associated signaling complex that we named the preligand assembly complex (PLAC) under physiologic conditions. To confirm this, we isolated the PLAC from the pooled high and low molecular weight fractions through IP using Flag-TRAIL and Flag antibody and detected DR5, A20 and RIP1 in the high but not the low molecular weight fractions (Fig. 1D).

DR5 is a type I transmembrane protein and, to determine whether DR5-associated PLAC is plasma membrane bound, we conducted subcellular fractionation. IB detected DR5 mainly in the membrane fractions and RIP1, A20 and FADD in both the membrane and cytosol fractions of TRAIL-resistant cells (Fig. 1E). The DR5-associated complex was then isolated through IP using a DR5 antibody. IB identified DR5, A20, RIP1 and TRAF2 in the membrane but not cytosol fractions in the resistant but only DR5 and TRAF2 in the sensitive cells (Fig. 1F; Supplementary Fig. S3B). These data indicate that, although RIP1, A20 and TRAF2 are present in the membrane and cytosol fractions, the PLAC is formed as a membrane bound complex composed of DR5, A20, RIP1 and TRAF2 in resistant cells but only DR5 and TRAF2 in sensitive cells. TRAIL stimulates the recruitment of FADD and caspase-8 to the plasma membrane bound PLAC for the assembly of a plasma membrane bound DISC.
A20 Znf Domain of E3 Ligase Inhibits Caspase-8 Cleavage in the DISC

To determine whether A20, TRAF2 and RIP1 inhibit caspase-8 cleavage in the complex, we conducted knockdown experiments by transfecting in TRAIL-resistant cell lines with small interfering RNA (siRNA) specific to A20, RIP1 and TRAF2. Identical results were obtained using two different siRNA sequences specific to each gene, indicating no off-target effects of the sequences; thus, the data were presented with one of the siRNA sequences targeting each gene. The siRNA transected LN443 cells were treated with TRAIL. A significant apoptosis was observed in the cells transfected with A20 and RIP1 but not TRAF2 siRNA, as shown by cell death (Fig. 2A), caspase-8 enzymatic activity (Fig. 2B), phase contrast microscopy (Fig. 2C) and Annexin V assay (Supplementary Fig. S4A). These assays showed that the transfection of A20 or RIP1 siRNA alone did not cause apoptosis. The transected LN443 cells were then treated with TNFα, Fas ligand (FasL) and cisplatin; cell death assay showed that the A20 siRNA transfection did not affect the cell sensitivity to these agents (Supplementary Fig. S4B).

IB confirmed A20 and TRAF2 knockdown in the transfectants and revealed the cleavage of caspase-8 and RIP1 in the A20 but not in the TRAF2 siRNA transfected LN443 cells (Fig. 2D). Knockdown of A20 or RIP1 but not TRAF2 released the inhibition of caspase-8 cleavage in other resistant cell lines (Supplementary Fig. S5A). The DISC was isolated and IB identified caspase-8 cleavage in the DISC in the A20 and RIP1 siRNA transfected LN443 cells (Fig. 2E). The caspase-8 cleavage was abolished by the presence of z-IEDT, a caspase-8 inhibitor (Fig. 2F). To a long term effect, we introduced the short hairpin RNA (shRNA) through lentiviral infection into LN443 cells and the A20 siRNA transfection alone did not inhibit the colony formation of the cells (Supplementary SSB). The PLAC and DISC were isolated from the transfected LN443 cells; the non-cleaved RIP1 was detected in the PLAC whereas both the non-cleaved and cleaved RIP1 were not observed in the DISC in the A20 siRNA transfected cells (Supplementary Fig. S5C), suggesting that RIP1 is detached from the DISC in the A20 knockdown. These results indicate that both A20 and RIP1 are required for the inhibition of caspase-8 cleavage in the DISC.

A20 has an N-terminal OTU of DUB and a C-terminal Znf domain of E3 ligase (28). To test whether the OTU and Znf domain inhibits caspase-8 cleavage, we generated inactive OTU (C103A) and Znf4 (C624A, C627A) mutant (mt) from A20 wild type (wt) through point mutations (Supplementary Fig. S5D). A20 wt and inactive OTU and Znf4 mt plasmids were introduced through lentiviral infection into LN71, an A20-deficient TRAIL-sensitive cell line. Stable clones were established, the expression of A20 wt and mt proteins was verified by IB, and the stable clones were selected for expressing wt and mt A20 proteins at levels similar to the endogenous A20 in the resistant LN443 cells (Supplementary Fig. S6A). The clones were treated with TRAIL and assessed with the cell death (Fig. 3A), caspase-8 enzymatic activity (Fig. 3B) and Annexin V assay (Supplementary Fig. S6B), showing that the expression of A20 wt and OTU but not Znf4 mt inhibited TRAIL-induced apoptosis. These assays, together with colony formation assay (Supplementary Fig. S6C), showed that the expression of A20 wt, OTU or Znf4 mt alone did not cause apoptotic cell death. The PLAC and DISC were isolated from the clones and A20 wt and mt proteins were observed in the complexes. RIP1 was detected more in the TRAIL-resistant A20 wt and OTU mt expressing clones than in the TRAIL-sensitive empty vector and Znf mt expressing clones (Fig. 3C). The clones were treated with TRAIL and RIP1 cleavage was detected in the empty vector and Znf mt but not A20 wt and OTU mt expressing clones (Fig. 3D). These gain function studies suggest that Znf4 is responsible for caspase-8 inhibition in the DISC.
A20 E3 Ligase Mediates RIP1 K63-Linked Polyubiquitination in the DISC

The finding that both A20 Znf4 and RIP1 are required for caspase-8 inhibition suggests that A20 E3 ligase may inhibit caspase-8 through RIP1 ubiquitination. An in vitro ubiquitination assay has shown that A20 E3 ligase adds a K48-linked polyUB chain to RIP1 (28); however, an in vivo assay has revealed that RIP1 is ubiquitinated through K63-linked polyUB chain (17). To examine the A20 Znf domain, we repeated the in vitro ubiquitination assay using A20 OTU mt that contains the Znf domain in the presence of the K48-linked polyUB chain specific E2 enzyme UBCH5A and the K63-linked polyUB chain specific E2 enzyme UBC13 (28). Flag-RIP1 and His-myc-A20 OTU mt proteins were added to an in vitro ubiquitination assay consisting of ATP, biotin-UB, E1 and UBCH5A or UBC13 (Supplementary Fig. S7A). After reaction, proteins were separated and IB using avidin identified biotin-polyUB chains in the reaction in the presence of UBCH5A or UBC13 (Fig. 4A). To confirm this in vivo, HEK293T cells were co-transfected with His-myc-A20 OTU mt, Flag-RIP1 and HA-UB mts (17). HA-UB mts included the mts that contain only one lysine at K48 or K63 or those that have a single point mutation of R48 or R63 (Supplementary Fig. S7B). The transfected cells were lysed in 1% SDS denaturing buffer to dissociate proteins and then diluted ten times in non SDS-containing buffer. Flag-RIP1 was isolated through IP by the Flag antibody. IB using HA the antibody identified K48 and K63-linked polyUB chain-conjugated Flag-RIP1 (Fig. 4B). These assays suggest that the Znf domain can mediate RIP1 ubiquitination through either the K48 or K63-linked polyUB chain.

To determine how RIP1 is ubiquitinated in glioblastoma cells, we treated its cell lines with MG132, a 26S proteasome inhibitor. The K48-linked polyUB chain targets the substrates to the 26S proteasome for degradation; however, MG132 treatment did not affect the levels of RIP1 protein in the absence or presence of TRAIL (Supplementary Fig. S7C), suggesting that it is less likely that RIP1 is ubiquitinated by a K48-linked polyUB chain. To examine this further, U87MG cells were transfected with HA-UB mts, treated with TRAIL and lysed in a denaturing buffer. RIP1 was isolated through IP. IB using HA the antibody detected a polyUB chain-conjugated RIP1 more in the K63 and R48 mt than in the K48 and R63 UB mt transfected cells (Fig. 4C), suggesting that RIP1 is ubiquitinated with K63-linked polyUB chains in glioblastoma cells.

To localize the RIP1 ubiquitination, the PLAC and DISC were isolated from resistant cells. IB identified DR5 and RIP1 in the PLAC and DISC. IB was overexposed and revealed high molecular weight RIP1 species more in the DISC than in the PLAC (Fig. 4D), suggesting that TRAIL treatment enhances the RIP1 polyubiquitination in the DISC. To verify this, we carried out a double IP and isolated RIP1 from the PLAC and DISC using RIP1 antibody; the isolated RIP1 was examined by IB with a UB antibody, detecting the UB-conjugated RIP1 more in the DISC than in the PLAC (Fig. 4E). IB using an antibody specific to the K63-linked polyUB chain identified the polyUB chain-conjugated RIP1 more in the DISC than in the PLAC (Fig. 4F). To identify the A20 domains, we isolated the PLAC and DISC from A20 wt, OTU mt and Znf4 mt expressing clones. RIP1 was then isolated from the complexes and IB identified the K63-linked polyUB chain-conjugated RIP1 in the DISC of the A20 wt and OTU mt but not the Znf4 mt expressing clones (Fig. 4G). The data suggest that TRAIL stimulates the Znf E3 ligase-mediated RIP1 ubiquitination through K63-linked polyUB chains in glioblastoma cells.

K63-Linked PolyUB Chain Inhibits Caspase-8 Dimerization and Cleavage

We then examined whether ubiquitinated RIP1 binds to caspase-8 in resistant cells. LN443 cells were treated or untreated with TRAIL; caspase-8 was isolated with a caspase-8 antibody; and IB using a RIP1 antibody identified higher molecular weight RIP1 species
(Fig. 5A). The cells were then subjected to subcellular fractionation; caspase-8 was isolated from the membrane and cytosol fraction; IB detected polyUB species more in the membrane fraction (Supplementary Fig 8A). The data suggest that TRAIL stimulates the interaction of ubiquitinated RIP1 and caspase-8. Caspase-8 consists of two death effector domains (DEDs) and a protease domain composed of a p18 and p12 subunit. To determine whether ubiquitinated RIP1 binds to the caspase-8 protease domain and inhibits its cleavage, we carried out an UB-binding assay using Fv-caspase-8 (35), in which DEDs were replaced with Fv, a derivative of the FK506 binding protein (Supplementary Fig. S8B). LN443 cells were subjected to subcellular fractionation after treated with TRAIL. RIP1 was isolated from the cytosol and membrane fractions and then incubated with recombinant Fv-caspase-8. IB identified the binding of Fv-caspase-8 to the K63-linked polyUB chain-conjugated RIP1 in the membrane fractions (Fig. 5B).

To further define caspase-8 as an UB binding protein, we carried out a series of caspase-8 pull down assays. Fv-caspase-8 was bound to protein G-beads through the incubation of Fv-caspase-8, caspase-8 antibody and protein G-beads. The Fv-caspase-8 bound beads were incubated with recombinant mono-UB, M1-linked liner, K48- and K63-linked polyUB chains. Unbound UB proteins were washed off the beads and bound UB proteins were eluted. Fv-caspase-8 pulled down K63-linked polyUB chains (Fig. 5C). The experiment was repeated with Fv-caspase-8 p18 catalytic site mt (35, 36); the results show that the caspase-8 p18 catalytic site is not required for the interaction of caspase-8 and K63-linked polyUB chain (Supplementary S8C). To confirm this, we used a recombinant caspase-8 protease p18 subunit in a pull down assay. The protease p18 subunit was bound to protein G-beads with a caspase-8 antibody and caspase-8 p18-labelled beads were incubated with mono-UB and polyUB chains. The pull down assay showed that the protease p18 subunit-labeled beads mainly pulled down K63-linked polyUB chains. To determine whether K63-linked polyUB chain directly binds to Fv-caspase-8, we generated a nickel column bound with His-tagged K63-linked polyUB chains. Fv-caspase-8 was added to the His-K63-linked polyUB chain-labeled nickel column and the nickel column pull down assay identified the binding of Fv-caspase-8 to the His-K63-linked polyUB chains (Fig. 5D).

To define the role of the K63-linked polyUB chain in caspase-8 dimerization and cleavage, we used an in vitro Fv-caspase-8 dimerization and cleavage assay (35) in which Fv-caspase-8 is dimerized and cleaved by adding a synthetic Fv ligand, AP20187 (Supplementary Fig. S8D). Mono-UB and polyUB chains were added to the Fv-caspase-8 cleavage assay. After the reaction, the proteins were separated and examined by IB using caspase-8 and UB antibody. The results showed that the cleavage of Fv-caspase-8 in the presence of AP20187 was inhibited significantly by the K63-linked polyUB chains and slightly inhibited by the linear polyUB chain (Fig. 5E, Supplementary S8E). Taken together, these results suggest that the binding of K63-linked polyUB chain to the caspase-8 protease domain inhibits its dimerization and cleavage.

**PLAC and DISC Are Present in Glioblastoma Tissues and Tumor-Initiating Cells**

To validate the A20-mediated RIP1 ubiquitination in human glioblastomas, we utilized tumor tissues surgically removed from patients (Supplementary Fig. S9A) and tumor-initiating cells through CD133 sorting as reported (29, 32) (Supplementary Fig. S9B). The CD133+ cells were used soon after isolation to avoid prolonged culturing effects. IB showed that A20 and RIP1 were expressed in the CD133+ cells at levels similar to those seen in the matched parental tissues (Supplementary Fig. S9C), as recently reported (37). Size exclusion assay detected DR5, RIP1, A20 and TRAF2 in the high molecular weight fractions of the tissues (Fig. 6A) and matched CD133+ cells (Fig. 6B). Subcellular fractionation further revealed DR5, RIP1, A20 and TRAF2 in the membrane fractions of the tissues (Fig. 6C) and matched CD133+ cells (Fig. 6D). These results suggest that a membrane bound DR5-
associated PLAC is formed in glioblastoma tumor tissues and tumor-initiating cells. To confirm this in the tumor initiating cells, we isolated the PLAC from the pooled high and low molecular weight fractions of the CD133+ cells through IP using Flag-TRAIL and Flag antibody and detected A20 and RIP1 in the high but not the low molecular weight fractions (Fig. 6E).

To examine RIP1 ubiquitination in CD133+ cells, we propagated the cells in neurosphere culture conditions because the culture maintains the original cancer genomic features (30). The PLAC and DISC were isolated from the CD133+ cells through IP and IB identified DR5, RIP1 and A20 in the PLAC and DISC whereas FADD and caspase-8 were recruited to the DISC where caspase-8 was not cleaved (Fig. 6F). RIP1 was then isolated from the PLAC and DISC and IB identified K63-linked polyUB chain-conjugated RIP1 in the DISC but not the PLAC (Fig. 6G). Taken together, these results validate the PLAC and DISC models, as established from studies of TRAIL-resistant cell lines, in glioblastoma tissues and derived tumor-initiating cells and suggest that glioblastomas in patients are most likely resistant to TRAIL treatment.

**A20 E3 Ligase Inhibits TRAIL-Induced Apoptosis in the Tumor-Initiating Cells**

To confirm that the CD133+ cells are tumor-initiating cells (32), we established the self-renewal ability by neurosphere formation, the differentiation ability by cell differentiation assay and the tumorigenic ability by mouse brain xenograft formation (Supplementary Fig. S10A, B, C). Once identified as the tumor-initiating cells, CD133+ cells were treated with 100 ng/ml TRAIL for 24 h. An approximately 20% rate of cell death was detected in EH 091112 and 100113 cells but no cell death was seen in EH 091217 and 091106 cells (Fig. 7A). Next, we transfected A20 siRNA in CD133+ cells and confirmed the A20 knockdown by IB (Fig. 7B). This showed that transfection of the A20 siRNA alone had no significant effects on the cells survival (Fig. 7A) and self-renewal ability (Supplementary Fig. S11A). The A20 siRNA transfected CD133+ cells were treated with 100 ng/ml TRAIL and TRAIL-induced apoptosis was shown by a significant increase in the enzymatic activities of caspase-8 (Fig. 7C) and caspase-3/-7 (Supplementary Fig. S11B). To confirm that the Znf domain of E3 ligase inhibits caspase-8-initiated apoptosis, we examined the negative dominant effect of inactive OTU and Znf4 mt on TRAIL-induced apoptosis in CD133+ cells. A20 wt, OTU and Znf4 mt were introduced into CD133+ cells through lentiviral infection. The expression of A20 Znf4 mt but not A20 wt and OTU mt in the cells enhanced TRAIL-induced apoptosis, as made evident by cell death and caspase activity (Fig. 7D). These results suggest that the Znf E3 ligase is responsible for caspase-8 inhibition and TRAIL resistance in the tumor-initiating cells of human glioblastomas.

**DISCUSSION**

Recent advances have generated novel cancer therapeutics targeting the TRAIL apoptotic pathway; however, clinical trials have proved that cancers are resistant to these treatments. The results presented here reveal a molecular mechanism by which A20 E3 ligase-mediated RIP1 polyubiquitination inhibits caspase-8 dimerization and cleavage and TRAIL-induced apoptosis in glioblastoma cells through two signaling complexes (Fig. 7E). A20 and RIP1 are highly expressed in glioblastomas, in which they interact with the transmembrane DR5 and form a plasma membrane bound PLAC under physiologic conditions. TRAIL treatment leads to the DR5-mediated recruitment of FADD and caspase-8 to the PLAC for the formation of the DISC, where A20 E3 ligase mediates RIP1 polyubiquitination through K63-linked polyUB chains that bind the caspase-8 protease domain. This in turn blocks caspase-8 dimerization and cleavage and inhibits TRAIL-induced apoptosis in glioblastoma cells.
This two-complex model is compatible with the recent reports that ubiquitination regulates the TRAIL pathway. TRAIL-induced apoptosis requires caspase-8 polyubiquitination in that caspase-8 is a substrate protein of CUL3 E3 ligase and caspase-8 ubiquitination facilitates UB binding protein p62-mediated cleavage of caspase-8 in TRAIL-sensitive cell lines (23). As a complement to this apoptosis model, our study establishes the molecular model of TRAIL resistance. Here, we identify caspase-8 as an UB binding protein and show that RIP1 K63-linked polyUB chain binds to the caspase-8 protease domain and inhibits its dimerization and cleavage. The findings that A20 and RIP1 are highly expressed and present in the PLAC and DISC in resistant cells are in line with the recent report that the content and status of proteins in a cell determine whether or not cell death or cell survival occur under TRAIL treatment (38).

The results reported here provide a novel model that reconciles the disparity in earlier studies. Size exclusion assay has identified TNFR1 in fractions corresponding to its monomeric molecular weight, leading to the notion that the TNFR1-associated complex I is formed after TNFα stimulation for TNFα-induced NF-κB signaling whereas the cytoplasmic complex II is formed through the recruitment of FADD and caspase-8 after complex I is detached from TNFR1 for TNFα-induced apoptosis (20). Distinguished from this TNFα model, we report here a two-complex TRAIL signaling model where the PLAC is formed spontaneously under physiologic conditions and TRAIL treatment stimulates the recruitment of FADD and caspase-8 to the PLAC for the formation of the DISC leading to either cell death or cell survival, depending on the composition and status of proteins in the complexes. A20 and RIP1 are associated with DR5 in the spontaneously formed PLAC in TRAIL-resistant cells and TRAIL stimulates A20 E3 ligase-based RIP1 ubiquitination through a K63-linked polyUB chain that binds to caspase-8 and inhibits its dimerization, cleavage and initiation of TRAIL-induced apoptosis. Once apoptosis is inhibited by A20-mediated RIP1 ubiquitination, the further recruitment to the DISC of IKKγ (17), cellular FADD-like interleukin-1β-converting enzyme-inhibitory protein and phosphorprotein enriched in diabetes leads to the activation of NF-κB (33, 39) and extracellular signal-regulated kinase 1/2 (40, 41) for the cell growth (42).

The results presented here establish the role of A20 E3 ligase in the inhibition of TRAIL-induced apoptosis. A20 has an N-terminal OTU DUB and a C-terminal E3 ligase (28). An in vitro ubiquitination assay suggests that A20 E3 ligase mediates RIP1 K48-linked ubiquitination for its degradation and thus inhibits TNFα-induced NF-κB signaling (28). In contrast, in vivo studies indicate that RIP1 is ubiquitinated through a K63-linked polyUB (17) and IKKγ binds to either K63 or M1-linked polyUB chains for NF-κB activation (18). Further studies have confirmed that the A20 can remove K63-linked polyUB chain from RIP1, RIP2 and TRAF6 (25–27). On the other hand, A20 has been shown to inhibit TNFα-induced apoptosis in mouse hepatocytes (24), IKKγ deficient Jurkat cells (43) and glioblastoma-derived CD133+ cells (37) through unknown mechanisms. We show here that A20 E3 ligase is able to mediate RIP1 ubiquitination through K48 and K63-linked polyUB chains in vitro and in vivo in the presence of polyUB chain specific E2. In glioblastoma cells, however, A20 E3 ligase mediates RIP1 ubiquitination through a K63-linked polyUB chain that binds to the caspase-8 protease and inhibits TRAIL-induced apoptosis.

Somatic mutations in TNFAIP3, the gene encoding the A20 protein, have been identified in lymphomas (44, 45). The mutations are clustered in the A20 Znf6 and Znf7 domains and result in loss of A20 function. Reconstitution of wild type TNFAIP3 in the TNFAIP3 mutated lymphoma cells induces cell death (44). However, it is unclear how the transfected TNFAIP3 triggers apoptosis in the lymphoma cells. These results suggest that TNFAIP3 may act as a tumor suppressor gene in lymphomas (46). In contrast to the lymphomas, genomic analysis of glioblastomas by the Cancer Genome Atlas (TCGA) (47) has failed to
identify TNFAIP3 mutations. However, the REpository for Molecular BRAin Neoplasia DaTa (REMBRANDT), sponsored by the National Cancer Institute indicates TNFAIP3 mRNA overexpression in human glioblastomas, keeping in line with our finding that A20 protein is highly expressed in these tumors and inhibits TRAIL-induced apoptosis in the tumor-initiating cells. It appears that A20 plays a different role in lymphocytic tumors as opposed to solid tumors, consistent with the original report that it restricts NF-κB signaling in lymphocytes but protects hepatocytes from apoptotic insult in mice (24). In conclusion, the data presented here suggest that A20 E3 ligase acts as an oncogene that inhibits TRAIL-induced apoptosis. Targeting of the A20-mediated RIP1 ubiquitination process may therefore lead to the development of combination therapies that can eliminate TRAIL resistance in tumor-initiating cells and enhance the therapeutic efficacy of TRAIL-targeted therapies in human glioblastomas.

METHODS

Human Glioblastoma Tissues, CD133+ Cells, Cell Lines and Normal Human Astrocytes

The glioblastoma tissues were collected in accordance with protocols approved by the Emory University Institutional Review Boards. CD133 cells were sorted from dissociated tumors using CD133 antibody-labeled magnetic microbeads (Miltenyi Biotec) based on prior reports (32) and labeled with EH (Emory Hospital) and numbers. Glioblastoma cell lines (33) and normal human astrocytes were reported (34) and no authentication was done by the authors since.

Generation of A20 wt and mt Clones through Lentiviral Infection

The pcDNA3.1/myc-his A20 WT, OTU and Znf4 mt were generated through the site directed-mutagenesis (Genscript). Lentiviral vector pLenti6.3/V5-DEST was inserted with A20 wt, OTU and Znf4 mt and introduced into LN71 cells through lentiviral infection in the presence of polybrene (2 µg/ml). The transfectants were grown in blasticidin-containing selection medium and single cell clones were expanded and examined by IB for the stable expression of the A20 protein.

RNA Interference, Cell Death and Caspase Activity Assay

The siRNA specific to TNFAIP3 (CCGAGCTGTTCCACTTGTAA; CAGATGTATGGCTAACCGGAA), RIP1, TRAF2 and scramble siRNA (Qiagen) were transfected using HiPerfect Transfection (Qiagen) for 72 hr. The transfectants were treated or untreated with TRAIL (PeproTech, Inc.) and examined by Cell Titer-Glo® Luminescent Cell Viability assay for cell death and Caspase-Glo® 8 and Glo® 3/7 kits (Promega) for the caspase activities. Lentiviral scramble shRNA (SHC002) and A20 shRNA (NM_006290.2-635s1c1: 5′-CCGGCACTGGAAGAAATACATACATATCTCGAGATATGTATTTCTCAGTGT-3′ (Sigma Mission RNAi).

Size Exclusion Chromatography

Cell lines and CD133+ cells (10⁸), treated or untreated with 100 ng/ml TRAIL for 5min, were lysed in CHAPS-containing lysis buffer (14mM CHAPS, 150mM NaCl, and 20mM Tris-Hcl (pH 7.4) plus complete protease inhibitors (Sigma) and 1mM PMSF. Lysates were filtered through a 0.45 micron filter and loaded onto superdex-200 HR16/60 column. Proteins were eluted at 1ml/min. Fractions (1ml) were analyzed by western blotting for DR5, RIP1, A20, TRAF2 and caspase-8. The apparent molecular weight was evaluated after column calibration with standard proteins (GE Healthcare): thyroglobulin (669kDa), ferritin (440kDa), adolase (158kDa), Conalbumin (75kDa) and ovalbumin (43kDa).
**Subcellular Fractionation**

Subcellular fractionation experiments were performed using the ProteoExtract subcellular proteome extraction kit (Calbiochem) with 5 x 10^6 cells per sample. For RIP ubiquitination, the cytosol and membrane fractions were treated with 1% SDS and boiled for 10 min to separate proteins in the complexes and then diluted 10 x 0.1% SDS. RIP1 was immunoprecipitated using 2 μg of RIP1 polyclonal antibody (Santa Cruz) for 4 hrs, washed 5 times: 2 times for 10 min in lysis buffer plus 1M NaCl and 3 times for 10 min in lysis buffer.

**Immunoprecipitation and Immunoblotting**

To isolate the DISC, 1 x 10^7 cells were incubated with mixed 500 ng/ml Flag-TRAIL and 1,500 ng/ml Flag antibody for 15 to 90 min at 37°C or 3 hr at 4°C for detection of CUL3. After wash, the cells were lysed for 30 min on ice in DISC IP lysis buffer (30 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100). Cell lysates were incubated with protein G-linked agarose overnight at 4°C. The beads were washed and eluted with 150 ng/μl of 3X Flag peptide (Sigma) (33). To isolate the PLAC, the cells were lysed first and then incubated with mixed Flag-TRAIL and Flag antibody as described above. The PLAC, DISC, size exclusion samples, subcellular fractions and lysates from cell lines, CD133+ cells, and tissues were examined by IB as described (33) using antibodies specific to CUL3 (BD Biosciences), DR5 (ProSci Inc), TRAF2 (H249), UB (P4D1), myc (Santa Cruz Biotech), RIP1, FADD (BD Biosciences), K63-linked polyUB chain (Biomol International and Millipore), caspase-8 (MBL), DFF45 (StressGen), HA (Covance), and His tag (Novagen).

**In Vitro and in Vivo Ubiquitination**

Flag-RIP1 and myc-A20 wt proteins were generated through TNT® Quick Coupled Transcription/Translation Systems (Promega). After reaction, a Flag-RIP1 was mixed with Flag antibody-labeled agarose beads and eluted using Flag peptide (Sigma-Aldrich) and concentrated through Amicon Ultra-0.5 Centrifugal Filters (Millipore). Myc-A20 wt protein was isolated through IP using myc-agarose beads. In vitro ubiquitination was performed in a 20 μl reaction volume containing 2 μg N-terminal biotinylated UB, 5 μg of UB, 200 ng E1, 400 ng UBC13 (Boston Biochem) or UBCH5A (Calbiochem), 2 μl 10X reaction buffer, and 1× Mg-ATP (Boston Biochem). After 1 hr incubation at 30°C, reactions were terminated by adding SDS loading buffer and examined by IB. For in vivo ubiquitination, U87MG cells were transfected with plasmids encoding HA-UB and mts for 24 hr and treated or untreated with 100 ng/ml TRAIL for 1.5 hr. Cell lysates were heated at 95°C for 10 min in 1% SDS to dissociate proteins and diluted ten times in non-SDS-containing buffer. RIP1 was isolated by RIP1 antibody (Santa Cruz) through IP and examined by IB using HA (Covance) and RIP1 antibody (BD Biosciences).

**Caspase-8 Binding, Dimerization and Cleavage Assay**

In vivo caspase-8 binding to UB was examined as follows: LN443 cells were treated or not with 100 ng/ml TRAIL for 1.5 and 3 hr and subjected to subcellular fractionation. RIP1 was isolated from the cytosol and membrane fraction under denaturing conditions, then incubated with Fv-caspase-8 for 1 hr and examined by IB using antibodies to caspase-8 and K63-linked polyUB chain. In vitro caspase-8 binding to UB was examined in the following two experiments. In the first experiment, Fv-caspase-8 labeled or caspase-8 protease p18 domain labeled agarose beads were incubated with recombinant UB (Boston Biochem) for 1 hr at 4°C in UB binding buffer (50 mM Tris, 150 mM NaCl, 0.2% Triton x-100, 1 mM EDTA, 0.5 mM DTT). After washes, the agarose beads were precipitated by centrifugation. In the second experiment, Fv-caspase-8 or caspase-8 protease p18 subunit was incubated with His-K63-linked polyUB chain bound Nickel agarose beads (Qiagen). In both experiments, unbound proteins were washed off the beads with binding buffer and bound proteins were...
eluted by 1% SDS-containing binding buffer and examined by IB using UB and caspase-8 antibody. The caspase-8 dimerization and cleavage assay was carried out as described (35, 36).

**Statistical Analysis**

All values are expressed as mean ± SD. Statistical significance was assessed by unpaired Student’s t-test, one way ANOVA followed by Dunnett test, and two ways ANOVA followed by Bonferroni test.

**SIGNIFICANCE**

These results indentify A20 E3 ligase as a therapeutic target whose inhibition can overcome TRAIL resistance in glioblastoma and thus have an impact on ongoing clinical trials of TRAIL-targeted combination cancer therapies.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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**REFERENCES**


30. Lee J, Kotliarov S, Kotliarov Y, Li A, Su Q, Donin NM, et al. Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of...
primary tumors than do serum-cultured cell lines. Cancer Cell. 2006; 9:391–403. [PubMed: 16697959]


Figure 1.
TRAIL-induced formation from the PLAC to the DISC. A, Normal brain and glioblastoma tissues were examined by IB using antibodies as indicated (left). The molecular weights were indicated to the right of the panels. Actin was used as a loading control. B, DR5-associated PLAC and DISC were isolated from LN443 and LN71 cells treated with mixed Flag-TRAIL and Flag antibody for 15 min and analyzed by IB with cell lysates as controls. C, Size exclusion fractions from LN443 and LN71 cells untreated or treated with 100ng/ml TRAIL for 15 min were analyzed by IB. The elution position of molecular weight markers in kDa are indicated at the top of the panels. D, The PLAC was isolated from the pooled high molecular weight fraction 42–50 and low molecular weight fraction 62–70 of LN443 cells and examined by IB. The input was included, showing the protein loading. E, Subcellular cytosol (Cytos), membrane (memb), and nuclear (Nucl) fractions from LN443 were examined by IB with antibodies to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), c-Jun and epidermal growth factor receptor (EGFR) as loading controls, respectively, for cytosol, nuclear and membrane fraction. F, Subcellular cytosol and membrane fractions from LN443 and LN71 were subjected to IP using a DR5 antibody with IgG included as a negative control and examined by IB for the presence of the proteins as indicated (left).
Figure 2. A20 inhibits caspase-8 cleavage and TRAIL-induced apoptosis. A, TRAIL-resistant LN443 cells were transfected with A20, TRAF2 and scrambled siRNA, treated with TRAIL for 24 hr, and examined for cell death (points: means; bars: SE; n=6; ***, p < 0.001). B, The transfected cells were treated with 100ng/ml TRAIL and examined by caspase-8 enzymatic activity assay (points: means; bars: SE; n=6; ***, p < 0.001; NS, no significance). C, Apoptotic cell death was observed under phase contrast microscopy in A20 siRNA but not scrambled and TRAF2 siRNA transfected cells. D, LN443 cells were transfected with siRNA, treated with 100 ng/ml TRAIL and examined by IB for the knockdown of the proteins and the cleavage of caspase-8 and RIP1. Non-transfected TRAIL-sensitive U343MG cells were included as the control for caspase-8 and RIP1 cleavage. E, The PLAC and the DISC were isolated from the LN443 cells transfected with siRNA and examined by IB for caspase-8 cleavage. F, LN443 cells transfected with A20 and RIP1 siRNA were treated or not with 100 ng/ml TRAIL in the absence or presence of the caspase-8 inhibitor z-IEDT and examined by IB for caspase-8 cleavage.
Figure 3.
A20 E3 ligase inhibits caspase-8 cleavage. A, LN71 stable clones expressing A20 wt, OTU and Znf mt and empty vector were treated with 100 ng/ml TRAIL for 24 hr and examined for cell death (points: means; bars: SE; n=6; ***, p < 0.001). B, LN71 stable clones were treated with 100 ng/ml TRAIL in the absence or presence of z-IEDT for caspase-8 activity (points: means; bars: SE; n=6; ***, p < 0.001). C, The PLAC and DISC were isolated from LN71 stable clones through IP using Flag-TRAIL and Flag antibody and examined by IB for the presence of the proteins as indicated (left). D, LN71 stable clones were treated with TRAIL for the times indicated (top) and examined by IB for A20 expression and cleavage of RIP1.
Figure 4.
A20 E3 ligase mediates RIP1 K63-linked polyubiquitination. **A**, *In vitro* ubiquitination was carried out in a reaction consisting of the components as indicated (top) with ubiquitinated RIP1 detected by an avidin antibody on IB. **B**, *In vivo* ubiquitination was performed by transfecting HEK293T cells with the vectors encoding Flag-RIP1, A20 and HA-UB mts and detecting ubiquitinated RIP1 by IB using myc and HA antibody. **C**, *In vivo* ubiquitination in U87MG cells were conducted by transfecting the cells with the vectors encoding HA-UB and mts, treating the transfectants with 100 ng/ml TRAIL for 1.5 hr, isolating RIP1 under denaturing condition and detecting ubiquitinated RIP1 by IB using antibodies to HA and RIP1. The number represents the quantification of the density. **D**, The PLAC and DISC were isolated from LN443 cells through IP using Flag-TRAIL and Flag antibody. Ubiquitinated RIP1 was detected by overexposure of IB using a RIP1 antibody. **E**, RIP1 was purified through IP under denaturing conditions from the PLAC and DISC as in (D) and ubiquitinated RIP1 was detected by IB using an UB antibody. **F**, RIP1 isolated from LN443 cells as in (E) was examined by IB using antibodies specific to K63-linked polyUB chain and RIP1. **G**, The PLAC and DISC were isolated from LN71 clones expressing A20 wt, OTU and Znf mt through IP as in (F) and examined by IB using antibodies specific to K63-linked polyUB chain and RIP1.
Figure 5.
K63-linked UB chain binds to caspase-8 and inhibits its cleavage. A, Caspase-8 was purified using its antibody through IP from LN443 cells after treatment with 100 ng/ml TRAIL and examined by IB for UB-conjugated RIP1. B, In vivo Fv-caspase-8 binding assay was carried out in LN443 cells. The cell line was either treated or not with 100 ng/ml TRAIL for the times indicated (top) and then subjected to subcellular fractionation. RIP1 was isolated through IP and caspase-8 binding to the RIP1 K63-linked polyUB chain was detected by IB. C, In vitro caspase-8 binding to UB was examined by Fv-caspase-8 (left) and caspase-8 protease p18 subunit pull down (right), in which Fv-caspase-8 and p18 subunit bound beads were incubated with the mono-UB, K63, K48-linked and linear polyUB protein. Unbound (U) were washed off the beads and bound (B) proteins were eluted and identified by IB. * indicates a non-specific (NS) band. D, Nickel column pull down assay was carried out by incubating a His-K63 polyUB chain labeled nickel column and Fv-caspase-8 in various concentrations as indicated (top). The column was washed and the caspase-8 and His-K63 polyUB chain were eluted and examined by IB. E, In vitro caspase-8 cleavage assay was performed by incubating mono-UB and polyUB chains with Fv-caspase-8 for 2 hr, then adding Fv ligand AP20187 and detecting caspase-8 cleavage in the reactions by IB.
Figure 6.
The PLAC and DISC are identified in glioblastomas. **A–D**, Glioblastoma tumor tissues and derived CD133+ cells (EH 091112) were subjected to size exclusion assay (**A, B**) and subcellular fractionation (**C, D**) and then examined by IB for the presence of the proteins as indicated (left). **E**, The PLAC was isolated using Flag-TRAIL from the pooled high molecular weight fraction 42–50 and low molecular weight fraction 62–70 of CD133+ cells (EH 091112) and examined by IB. The input was included to show the protein loading. **F–G**, The PLAC and DISC were isolated from CD133+ cells (EH 091112, 091106) through IP using Flag-TRAIL and Flag antibody and examined by IB for the presence of the proteins (**F**) and the RIP1 ubiquitination (**G**).
A20 protects the tumor-initiating cells from TRAIL-induced apoptosis. **A**–**C**, CD133+ cells (EH 091112, 100113, 091217 and 091106) were transfected or not with A20 and scrambled siRNA, treated or not with 100 ng/ml TRAIL, and examined for cell death (**A**), IB (**B**) and caspase-8 activity (**C**). **D**, EH 091112 cells were transfected with the lentiviral vectors encoding the A20 wt, OTU and Znf4 mt, treated or untreated with 100 ng/ml TRAIL and examined for cell death and caspase-8 activity. Each of the experiments was repeated eight times (**points**: means; **bars**: SE; n=6; ***, p < 0.001). **E**, A two-complex model of A20 E3 ligase-mediated inhibition of caspase-8 through RIP1 polyubiquitination.