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The Antiviral Enzyme, Viperin, Activates Protein Ubiquitination by the E3 Ubiquitin Ligase, TRAF6

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Abstract

Viperin is a broadly conserved radical SAM enzyme that synthesizes the antiviral nucleotide ddhCTP. In higher animals, viperin expression also accelerates the degradation of various cellular and viral proteins necessary for viral replication; however, the details of this process remain largely unknown. Here, we show that viperin activates a component of the protein ubiquitination machinery, which plays an important role in both protein degradation and immune signaling pathways. We demonstrate that viperin binds the E3 ubiquitin ligase, TRAF6, which catalyzes K63-linked ubiquitination associated with immune signaling pathways. Viperin activates ubiquitin transfer by TRAF6–2.5-fold and causes a significant increase in polyubiquitinated forms of TRAF6 that are important for mediating signal transduction. Our observations both imply a role for viperin as an agonist of immune signaling and suggest that viperin may activate other K48-linked E3-ligases involved in targeting proteins for proteasomal degradation.

Viperin (Virus Inhibitory Protein, Endoplasmic Reticulum-associated, Interferon iNducible), also denoted as cig5 and RSAD2 in humans,[1] is strongly induced by type I interferons as part of the innate immune response to viral infection.[2–4] Viperin is a member of the radical SAM enzyme superfamily and appears to be conserved in all 6 kingdoms of life,[5–7] hinting at its ancient and ubiquitous role in combatting viral infection. Notably, viperin is one of the very few radical SAM enzymes found in higher animals.[8] Viperin catalyzes the dehydration of CTP to form the antiviral nucleotide 3′-deoxy-3′,4′-didehydro-CTP (ddhCTP; Figure 1)[9] through a radical mechanism initiated by reductive cleavage of SAM.[7,10] The antiviral properties of this nucleotide against RNA viruses derive from its ability to act as a chain-terminating inhibitor of some, but not all, viral RNA-dependent RNA polymerases.[9]

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Supporting Information

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Experimental methods and supporting figures (PDF)

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Notes

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In addition to synthesizing ddhCTP, viperin interacts with a wide range of cellular and viral proteins.[11–16] This extensive network of protein–protein interactions remains poorly understood but constitutes an equally important aspect of the enzyme's antiviral properties. In many cases, it appears that viperin exerts its antiviral effects by facilitating the degradation of cellular and viral proteins important for viral replication.[11] A prevailing view is that viperin recruits the protein ubiquitination machinery to target proteins for proteasomal degradation (Figure 1).[11,17] However, the evidence for viperin promoting protein ubiquitination is indirect and is based largely on studies using proteins transfected in mammalian cell lines.[11,17] Thus, there's uncertainty about which components of the ubiquitination system viperin interacts with or whether other proteins may be required for viperin to engage the ubiquitination system. Therefore, to better understand viperin's role in promoting protein ubiquitination, we have examined viperin's interaction with the E3 ubiquitin ligase, TRAF6.[18,19]

The ubiquitination machinery comprises 3 enzymes:[20] E1 is responsible for the ATP-dependent activation of ubiquitin and transferring it as its C-terminal thioester to various ubiquitin conjugating enzymes (E2). E2 enzymes interact with a large set of E3 ubiquitin ligases, ~700 in humans, that recognize different protein targets for ubiquitination.[21]

Polyubiquitin chains may be constructed through isopeptide bonds to various ubiquitin lysine residues. K48-linked polyubiquitination marks proteins for degradation by the 26S proteasome,[20,22] whereas Lys-63-linked polyubiquitination is important in activating various components of signal transduction pathways that trigger the immune response.[23] E3 ligases, in particular, are highly regulated and may be activated or inhibited by a wide range of post-translational modifications and interactions with other proteins.[24]

TRAF6 (Tumor necrosis factor receptor-associated factor 6) is a member of the RING domain-containing E3 ligases.[25] TRAF6 functions with the heterodimeric Ubc13/Uev1A E2-conjugating enzyme to synthesize K63-linked ubiquitin chains. TRAF6-mediated protein ubiquitination is central to several important signal transduction pathways,[22,26] including activation of the NF-κB pathway and the MAPK signaling cascade. These pathways regulate such diverse biological processes as cell growth, oncogenesis, and immune and inflammatory responses.[26] TRAF6 substrates include interleukin-1 receptor-associated kinases (IRAKs) and NF-κB essential modulator (NEMO).[27,28] But TRAF6 also undergoes autoubiquitination specifically on Lys124[29] and these polyubiquitinated forms serve to recruit and assemble downstream kinases and associated factors into signaling complexes that ultimately activate NF-κB and MAPK pathways.[30,26]

Recently, viperin was shown *in cellullo* to interact with TRAF6 to promote K63-linked polyubiquitination of interleukin receptor-associated kinase 1 (IRAK1) as part of innate immune signaling in the Toll-like receptor-7 and 9 (TLR-7/9) pathways.[15,28] The viperin-TRAF6 interaction provides a unique opportunity to test whether viperin functions as an activator of protein ubiquitination in a well-defined biochemical system. We have reconstituted the TRAF6 autoubiquitination system *in vitro* using purified enzymes. This has allowed us to demonstrate that viperin does indeed activate the E3 ligase activity of

TRAF6, leading to a significant increase in the amount of polyubiquitinated TRAF6 species formed.

Full-length TRAF6 is a multidomain protein that forms large oligomers in the cell and has proven refractory to expression in *E. coli*. Therefore, to reconstitute the ubiquitination system *in vitro*, we used a truncated TRAF6 construct comprising the RING and first 3 zinc-finger domains[19] (designated TRAF6-N), which was previously shown to be functional and can be expressed and purified from *E. coli*.[19] A human viperin construct lacking the first 50 residues of the ER-localizing N-terminal amphipathic helix, designated viperin-ΔN50, was expressed and purified from *E. coli* and the [4Fe-4S] cluster reconstituted as described previously.[16] Preliminary pull-down experiments established that the truncated viperin-ΔN50 and TRAF6-N proteins form a stable complex with each other (Figure S1).

TRAF6 functions with the heterodimeric E2 ubiquitin-conjugating enzyme, Ubc13/Uev1A; this enzyme was expressed and purified from *E. coli*, as described previously.[31] The complete ubiquitination system was then reconstituted using commercially obtained E1 and ubiquitin. A typical assay comprised 0.1 μM E1, 2 μM Ubc13, 2 μM Uev1A, 2 μM TRAF6-N, and 2 μM viperin-ΔN50 in 20 mM Tris-HCl buffer pH 7.5, 150 mM NaCl, 2 mM DTT, 2 mM ATP, and 5 mM $MgCl_2$. Reactions were initiated by the addition of ubiquitin, 35 μM, and incubated at 37 °C. At various times, aliquots were removed and quenched by addition of SDS-PAGE loading buffer; samples were then analyzed by SDS-PAGE.

Initially, we examined the activity of TRAF6-N in the absence of viperin, with a typical experiment shown in Figure 2. Under these conditions, the formation of diubiquitin was clearly visible after 4 min and triubiquitin was visible as a faint band after 8 min. At longer times, the formation of polyubiquitin is evident as a faint smear of higher molecular weight material. Control experiments established that in the background, rate of ubiquitin ligation is negligible in the absence of TRAF6-N (Figure S2).

Quantification of the bands due to mono-, di-, and triubiquitin by imaging of Coomassie-stained gels (Figure 2) allowed the consumption of ubiquitin ligation to be quantified and the amount of ubiquitin incorporated into high molecular weight oligomers to be estimated. For the first 10 min of the reaction, the concentrations of di- and triubiquitin increased and then plateaued. In contrast, the concentration of monoubiquitin steadily decreased as more ubiquitin was incorporated into high molecular weight oligomers. After 20 min, ~80% of the ubiquitin was accounted for by mono-, di-, and triubiquitin, with only ~20% converted to high molecular weight oligomers.

We then repeated the reaction with the addition of viperin-ΔN50 in a 1:1 ratio with TRAF6-N (2 μM of each enzyme). The addition of viperin markedly altered the kinetics of ubiquitination (Figure 3). In this case, there was an initial rapid increase in the amount of diubiquitin formed, which then decayed to a steady state level. Notably, ubiquitin was converted to high molecular weight species much more rapidly when viperin was bound to TRAF6-N. High molecular weight ubiquitin oligomers accounted for ~70% of the ubiquitin pool while mono-, di- and triubiquitin comprised only ~30%.

Control experiments established that TRAF6-N activation is specific to viperin, as proteins such as bovine serum albumin had no effect on TRAF6-N activity (Figure S2). Furthermore, the Fe–S cluster of viperin appears to be important for TRAF6 activation, as a viperin-ΔN50–C83A mutant that is unable to bind the Fe–S cluster did not activate TRAF6-N (Figure S3). These results clearly demonstrate that viperin activates TRAF6-N and promotes the formation of longer polyubiquitin chains that are considered to be important mediators of signaling in the MAPK and NF-kB pathways.[26,30]

TRAF6 is known to autoubiquitinate Lys124,[30] a process that is important for its role in signal transduction.[30] To examine whether viperin promotes TRAF6 autoubiquitination, we probed gels with antibodies against ubiquitin and the N-terminal domain of TRAF6 (Figure 4). Immunoblotting with antiubiquitin antibody confirmed identity of the di- and triubiquitin bands and, as expected, strongly stained the high molecular weight material evident in Coomassie-stained gels. The high molecular weight material also cross-reacted with anti-TRAF6 antibodies demonstrating that it represents autoubiquitinated forms of TRAF6-N. When probed with antiviperin antibodies, only the viperin band was cross-reactive. This result demonstrates that although viperin promotes TRAF6 polyubiquitination, it is not itself a substrate for ubiquitination.

Although the time course for ubiquitin ligation is complex, at early time points, the major reaction catalyzed by TRAF6-N is the formation of diubiquitin

$$\mathrm{E2{\sim}Ub + Ub \rightarrow Ub - Ub + E2}$$

The ubiquitin-charged E2 functions as a substrate for TRAF6-N, which is then rapidly replenished through the action of E1 so that the steady state concentration of E2 ~ Ub remains constant. This simplification allowed us to quantify the ubiquitin ligase activity of TRAF6-N and compare its activity when complexed with viperin. Preliminary experiments established that the rate of ubiquitin consumption was linear with TRAF6-N concentration (Figure S4). Under these conditions, the apparent turnover number for diubiquitin formation by TRAF6-N was k_{app} = 0.47 ± 0.06 min^{-1}, whereas in the presence of viperin, k_{app} = 1.25 ± 0.08 min^{-1} representing a ~ 2.5-fold rate enhancement (Figure S5).

TRAF6 is one of the better studied members of this class E3 ligases, in part due to the important role it plays in NF-kB and MAPK signaling.[22,26] However, to our knowledge, quantitative measurements of rate at which TRAF6 catalyzes ubiquitin transfer have not been previously been reported. The kinetics of ubiquitin transfer catalyzed by various other E3 ligases have been quite extensively investigated, with k_{cat} ranging from several per second, e.g., the RING-E3 ligase SCF^{Cdc432} and HECT-E3 ligase E6AP,[33] to several per minute, e.g., the RING-E3 ligase San1.[34] Compared with those of these E3 ligases, the rate of ubiquitin ligation catalyzed by TRAF6-N is relatively slow, but we note that ubiquitination rates are also dependent on the protein substrate and may accelerate as the polyubiquitin chain is extended.[32] Furthermore, TRAF6-N lacks the C-terminal TRAF domain through which TRAF6 binds many of its protein substrates and which may also influence the ligase activity of the enzyme.

A role for viperin in immune signaling was initially suggested through studies on TRAF6-catalyzed polyubiquitination of IRAK1 in mouse cell-lines lacking viperin.[28] More recently, our studies in HEK 293T cells demonstrated that cotransfection of viperin with TRAF6 significantly increased the polyubiquitination of IRAK1.[15] However, these studies left open the possibility that viperin activated TRAF6 indirectly through additional unknown factor(s). Reconstituting the ubiquitination system *in vitro* with purified enzymes has allowed us to unambiguously demonstrate viperin's role in activating TRAF6. Viperin both speeds up the rate of ubiquitin consumption and increases the formation of high molecular weight autoubiquitinated forms of TRAF6 that mediate downstream signaling. Although the ~2.5-fold activation of TRAF6 by viperin is relatively modest, this level of amplification may be appropriate to modulating transcription of the various genes needed to establish the antiviral response.

Here, we have shown that viperin interacts with the N-terminal RING-domain of TRAF6, whereas it is known that the C-terminal TRAF domain (lacking in TRAF6-N) mediates TRAF6's interactions with most other protein substrates.[24] This modular arrangement suggests that activation of TRAF6 E3-ligase activity by viperin may enhance polyubiquitination of other target proteins, which would be consistent with our observation that viperin stimulates TRAF6-catalyzed polyubiquitination of IRAK1.[15] These observations provide further support the idea, for which there is extensive but indirect evidence in the literature, that viperin, more broadly, activates other K48-linked E3 ligases to increase proteasomal degradation of specific proteins in response to viral infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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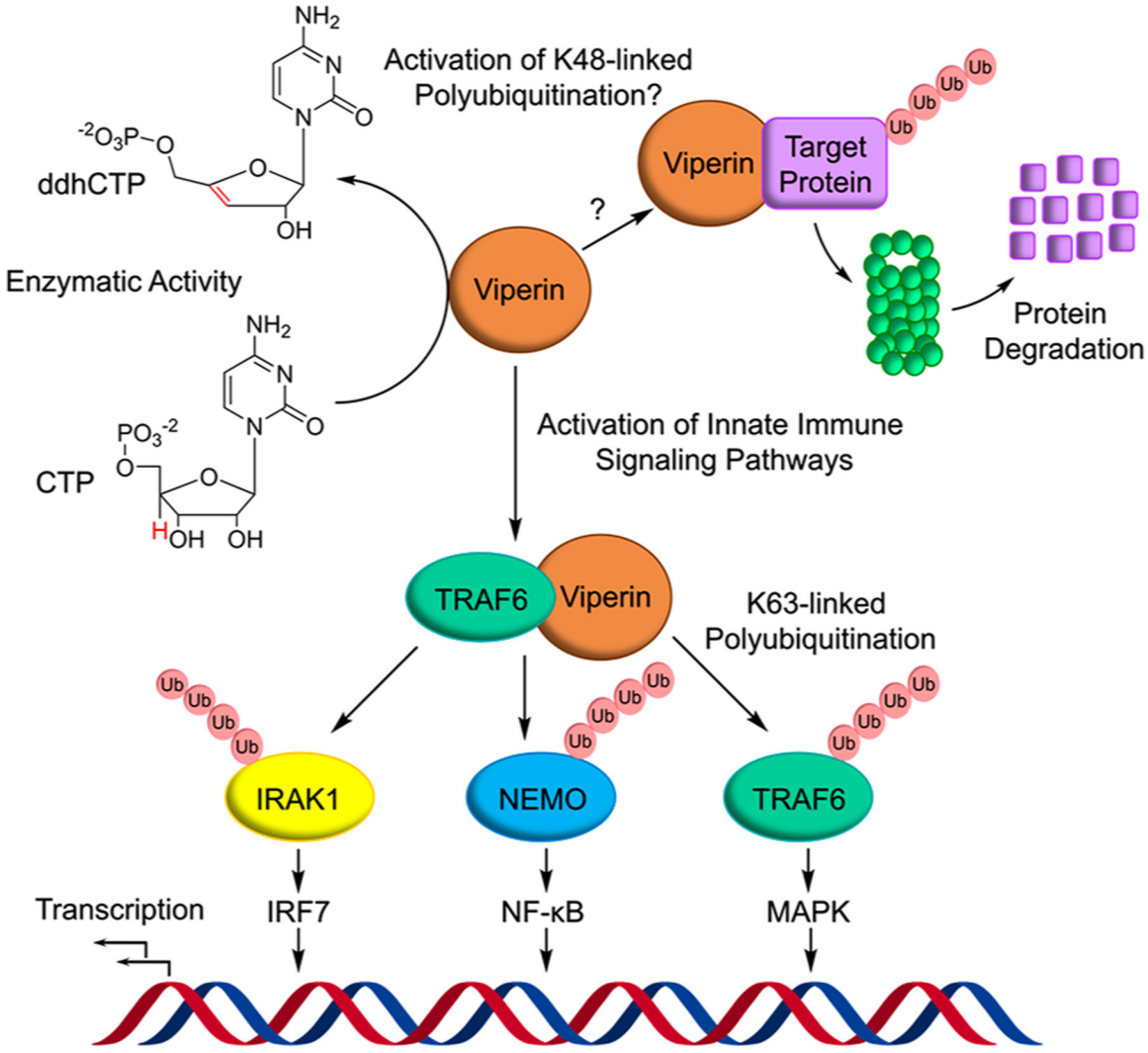

Figure 1.

Overview of viperin's interactions with the protein ubiquitination machinery and the E3-ligase, TRAF6.

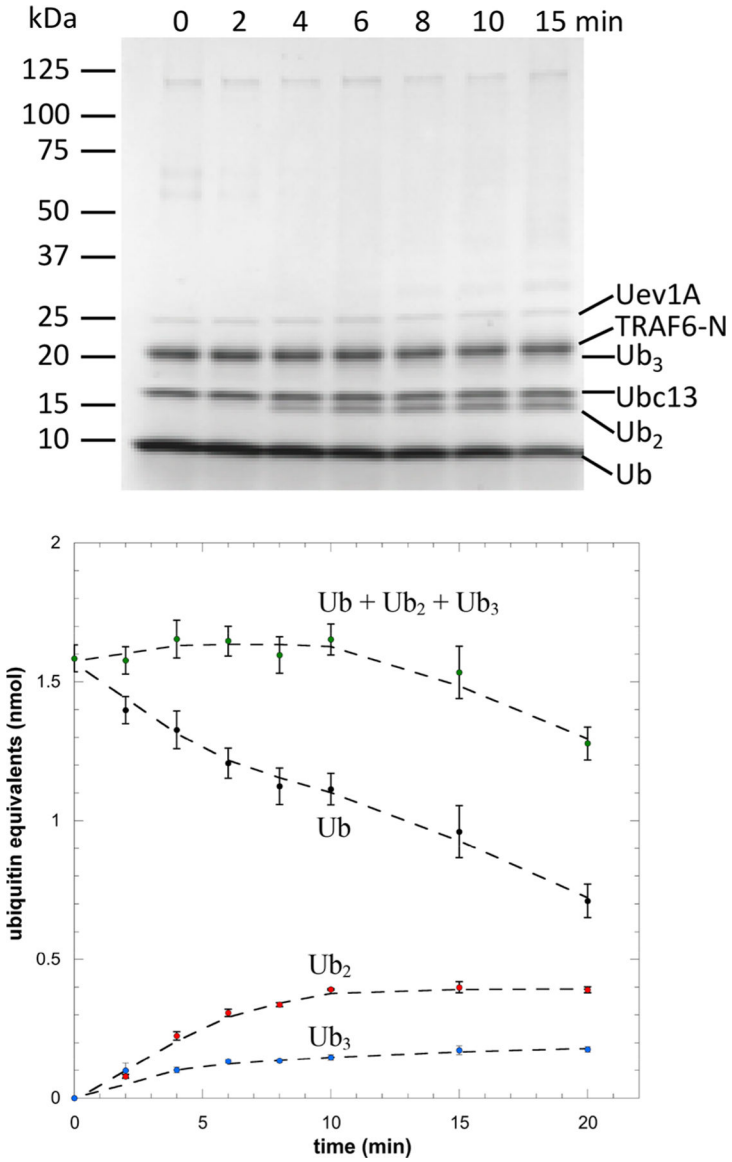

Figure 2.

Kinetics of ubiquitin ligation catalyzed by TRAF6-N. *Top*: Representative Coomassie-stained gel showing consumption of ubiquitin and formation of ubiquitin oligomers. *Bottom:* Quantification of mono-, di- and triubiquitin; after 20 min, only a small fraction of the ubiquitin is converted to larger oligomers.

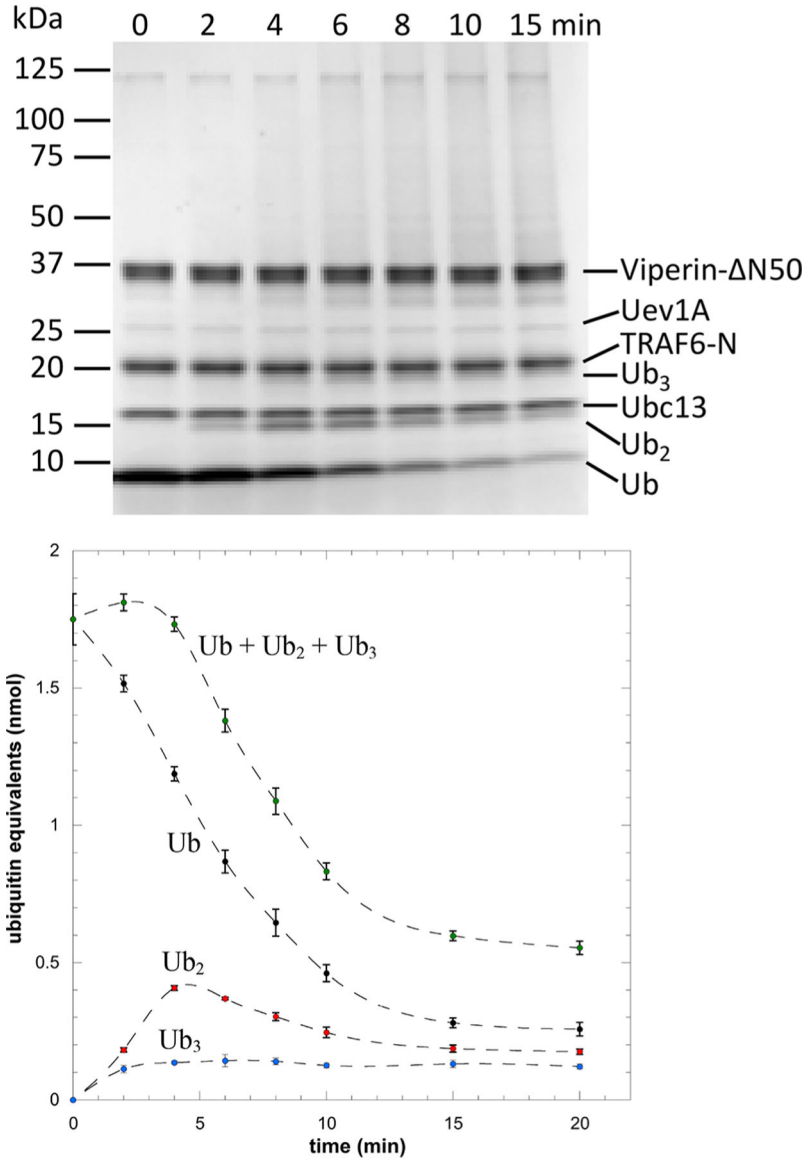

Figure 3.

Activation of TRAF6-N by viperin. *Top*: Representative Coomassie-stained gel showing consumption of ubiquitin and formation of ubiquitin oligomers, note the smear of high M_r species at longer times. *Bottom:* Quantification of mono-, di-, and triubiquitin; these oligomers are rapidly depleted as they are converted to higher M_r species. (Experiments were performed with 1:1 molar ratio of TRAF6-N to viperin.)

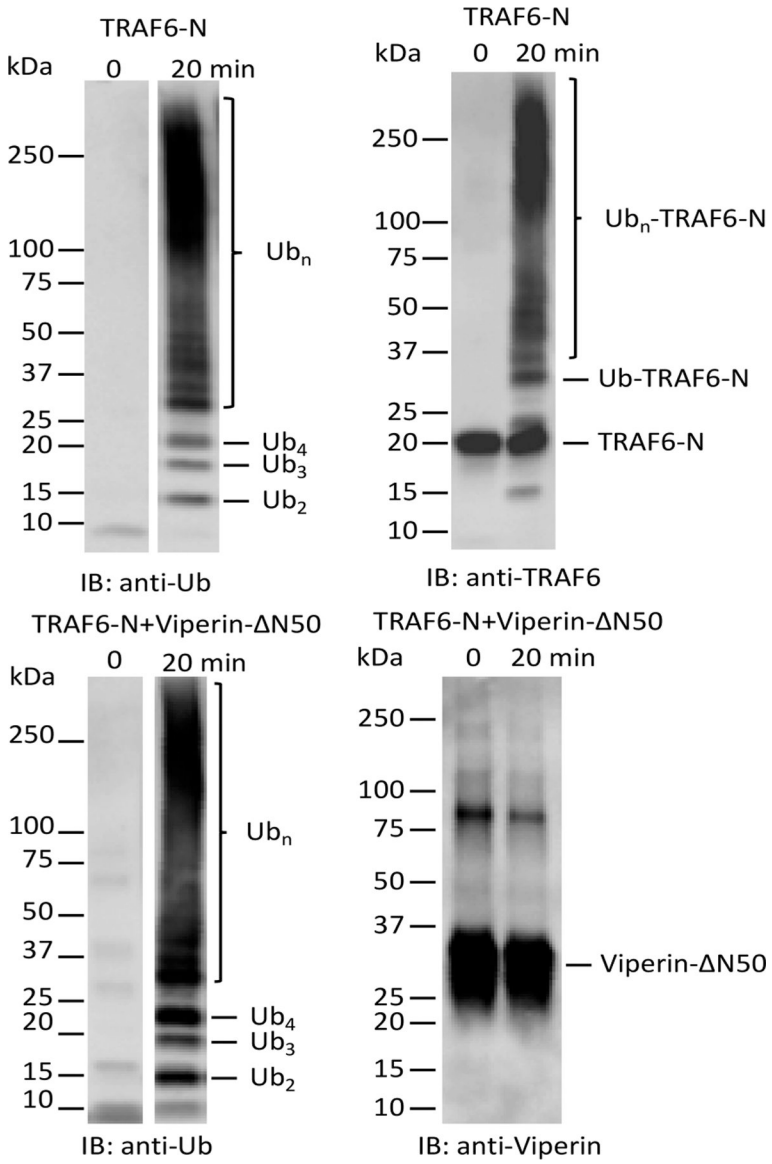

Figure 4.

Immunoblot analysis of ubiquitination reactions. *Top*: Staining for ubiquitin (left) and TRAF6 (right) in reactions containing TRAF6-N. *Bottom:* Staining for ubiquitin (left) and viperin (right) in reactions containing TRAF6-N and viperin. (Note: the polyclonal antiubiquitin antibody used in staining recognizes monoubiquitin very poorly; both $t = 0$ and $t = 20$ min lanes contain similar amounts of ubiquitin.).