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Report

Optineurin Negatively Regulates TNFα-Induced NF-κB Activation by Competing with NEMO for Ubiquitinated RIP

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Summary

NF-κB essential modulator (NEMO), the regulatory subunit of the IκB kinase (IKK) that activates NF-κB, is essential for NF-κB activation [1]. NEMO was recently found to contain a region that preferentially binds Lys (K)63-linked but not K48-linked polyubiquitin (polyUb) chains, and the ability of NEMO to bind to K63-linked polyUb RIP (receptor-interacting protein) is necessary for efficient tumor necrosis factor α (TNFα)-induced NF-κB activation [2, 3]. Optineurin is a homolog of NEMO, and mutations in the *optineurin* gene are found in a subset of patients with glaucoma [4], a neurodegenerative disease involving the loss of retinal ganglion cells [5]. Although optineurin shares considerable homology with NEMO, in resting cells, it is not present in the high-molecular-weight complex containing IKKα and IKKβ, and optineurin cannot substitute for NEMO in lipopolysaccharide (LPS)-induced NF-κB activation [6]. On the other hand, the overexpression of optineurin blocks the protective effect of E3-14.7K on cell death caused by the overexpression of TNFα receptor 1 (TNFR1) [7]. Here we show that optineurin has a K63-linked polyUb-binding region similar to that of NEMO, and like NEMO, it bound K63- but not K48-linked polyUb. Optineurin competitively antagonized NEMO's binding to polyUb RIP, and its overexpression inhibited TNFα-induced NF-κB activation. This competition occurs at physiologic protein levels because microRNA silencing of optineurin resulted in markedly enhanced TNFα-induced NF-κB activity. These results reveal a physiologic role for optineurin in dampening TNFα signaling, and this role might provide an explanation for its association with glaucoma.

Results and Discussion

Although optineurin cannot restore NF-κB activation in NF-κB essential modulator (NEMO)-deficient cells, the high degree of homology between the two proteins nevertheless suggests that it might modulate IκB kinase (IKK) activation. Therefore, we asked whether ectopically expressed optineurin can affect NF-κB activation. Because the overexpression of TRAF2 or RIP (receptor-interacting protein) alone can activate NF-κB [8, 9], an NF-κB reporter construct along with cDNA vectors containing TRAF2 or RIP in the absence or presence of optineurin were introduced into 293 cells. NF-κB induced by both TRAF2 (Figure 1A) and RIP (Figure 1B) was profoundly inhibited by the coexpression of optineurin. This inhibition was proximal to IKK because optineurin had no effect on NF-κB activation caused by a constitutively active IKKβ mutant (IKKβ-CA, S178E, S181E) [10] (Figure 1C). Therefore, optineurin's inhibitory effect is likely to be at an early stage in the signaling pathway leading to NF-κB activation, perhaps at the level of the signalosome assembled at or near the plasma membrane.

The K63-linked polyubiquitin (polyUb)-binding region of *NEMO* has recently been found to encompass a coiled-coil domain (CC2), a linker region, and a leucine zipper (LZ) [2, 3]. A BLAST search with a full-length *NEMO* sequence revealed that its overall homology with *optineurin* is 53%, and it is 64% in the area corresponding to the *NEMO* polyUb-binding region (Table S1 and Figure S4 in the Supplemental Data available online), prompting us to ask whether optineurin binds polyUb as well. NEMO binds to multiubiquitin arranged in a head-to-tail fashion in addition to K63-linked polyUb, most likely because of similar three-dimensional conformations [2, 11]. Therefore, ^{35}S-labeled in vitro-translated proteins were offered to recombinant GST-ubiquitin-coated beads (Figure 2A). As previously shown [2], GST-ubiquitin brought down NEMO, the efficacy increasing in proportion to the number of ubiquitin moieties. Notably, GST-ubiquitin brought down optineurin in a similar fashion. A direct comparison of NEMO and optineurin was made: 6His-tagged versions were used to pull down K63-linked polyUb (Figure 2B). Whereas both proteins brought down K63-linked polyUb, optineurin was clearly better than NEMO, implying that it has a higher binding affinity. Moreover, a mutation in the optineurin linker region analogous to one in NEMO that disrupts polyUb binding, D474N (D311N in NEMO), also abolished its binding to K63-linked polyUb. Both proteins had the same specificity in that they failed to bring down appreciable amounts of K48-linked polyUb ([2] and data not shown). Thus, optineurin and NEMO represent a family of polyUb-binding proteins with selectivity for chains with K63 linkages.

The overlapping polyUb-binding selectivity of the two proteins raised the possibility that they might share binding partners. One such NEMO-binding protein in the tumor necrosis factor α (TNFα) signaling pathway is ubiquitinated RIP [2]. Lysate from HeLa cells that had been stimulated or not with TNFα were subjected to pull-down with similar amounts (Figure 3A, left panel) of GST-optineurin, GST-optineurin^{D474N}, or GST-NEMO (Figure 3A, right panel). None of the GST fusion proteins bound RIP in lysates from unstimulated cells. However, within 5 min of stimulation with TNFα, both GST-NEMO and GST-optineurin pulled down ubiquitinated, but not unmodified, RIP (Figure 3A, right panel). Furthermore, the polyUb-binding defective GST- optineurin^{D474N}

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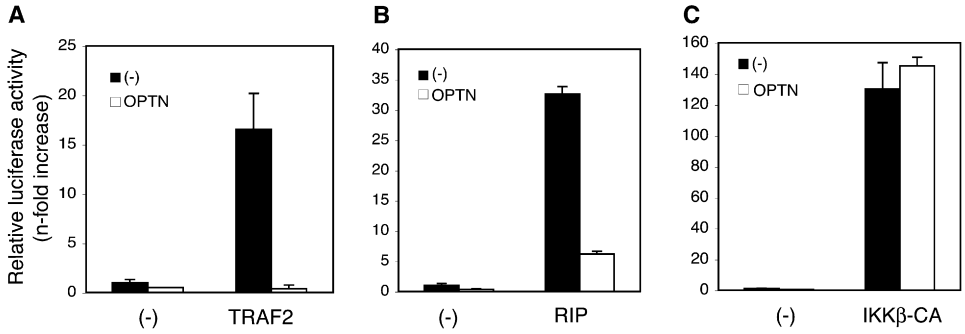

Figure 1. Optineurin Inhibits TRAF2- or RIP-Induced NF-κB Activation

(A) Vectors expressing wild-type optineurin and/or TRAF2 were cotransfected with NF-κB reporter and β-galactosidase plasmids into 293 cells and cultured for 20 hr. The cells were lysed, and luciferase activity was measured and normalized to β-galactosidase activity. Error bars represent standard error of the mean of triplicate samples.

(B) Vectors expressing wild-type optineurin and/or RIP with reporter constructs were cotransfected into 293 cells as above, and reporter assays were performed.

(C) Vectors expressing wild-type optineurin and/or constitutively active IKKβ (IKKβ-CA) with reporter constructs were cotransfected into 293 cells as above, and reporter assays were performed.

failed to bring down ubiquitinated RIP, indicating that recognition of polyUb chains is necessary for optineurin to bind RIP with high affinity.

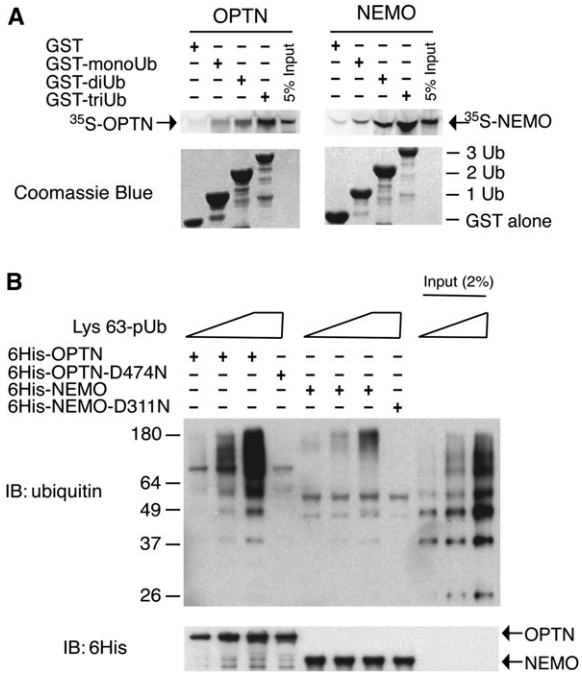

Figure 2. Optineurin Binds K63-Linked polyUb Chains

(A) In vitro-translated and ^{35}S-labeled optineurin or NEMO was incubated with glutathione-sepharose beads bound GST, GST-monoUb, GST-diUb, or GST-triUb for 2 hr at 4°C. The bound proteins were eluted and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized with a PhosphorImager. After exposure, the gels were stained with Coomassie blue to verify equal loading of GST fusion proteins.

(B) Cobalt bead-bound 6His-optineurin, 6His-optineurin^{D474N}, 6His-NEMO, or 6His-NEMO^{D311N} was incubated with K63-linked polyUb (pUb). The beads were washed, and the proteins were eluted and resolved by SDS-PAGE; immunoblotting with 6His antibody and stripping and reblotting of the same membrane with anti-Ub followed. Two percent of the amount of input K63-linked polyUb used for each reaction is shown.

For determining whether optineurin binds polyubiquitinated RIP in vivo, HeLa cells were stimulated with TNFα and lysates were immunoprecipitated with anti-optineurin (Figure 3B). In this setting, a small amount of unmodified RIP coimmunoprecipitated with endogenous optineurin in the absence of stimulation. After stimulation, a large amount of ubiquitinated RIP was coimmunoprecipitated. Despite the fact that only a very small amount of RIP was polyubiquitinated in TNFα-stimulated cells (undetectable when whole-cell lysates were blotted) (Figure 3B), it was highly enriched in the material coimmunoprecipitated with optineurin, indicating that optineurin preferentially interacts with ubiquitin-modified RIP. The binding by optineurin to polyubiquitinated RIP is not cell-type dependent; similar results were obtained with lysates from TNFα-stimulated mouse embryonic fibroblasts (MEFs) (Figure S1).

Because NEMO and optineurin both bind to polyubiquitinated RIP, we asked whether they interacted with one another in the TNFα receptor (TNFR) signaling complex. HeLa cells were stimulated with TNFα, and lysates were immunoprecipitated with anti-optineurin or anti-NEMO (Figure 4A). Both NEMO and optineurin coimmunoprecipitated with polyubiquitinated RIP. Surprisingly, however, optineurin and NEMO were not found in the same complexes when immunoprecipitates of one were immunoblotted for the other. Therefore, although both optineurin and NEMO bind polyubiquitinated RIP, they do not bind the same molecules of RIP. This exclusivity of binding suggested that optineurin and NEMO might be competitive inhibitors of one another. For testing this, GST-NEMO was incubated with cell lysates from TNFα-stimulated HeLa cells, to which increasing amounts of soluble His-tagged optineurin was added (Figure 4B). The amount of polyubiquitinated RIP pulled down by GST-NEMO decreased as optineurin was titered into the lysate, and it was substantially reduced when an equivalent amount of optineurin was added and diminished further when more optineurin was present. For addressing the role of polyUb binding in the competition, the activity of wild-type optineurin and optineurin^{D474N} was compared; the latter failed to inhibit

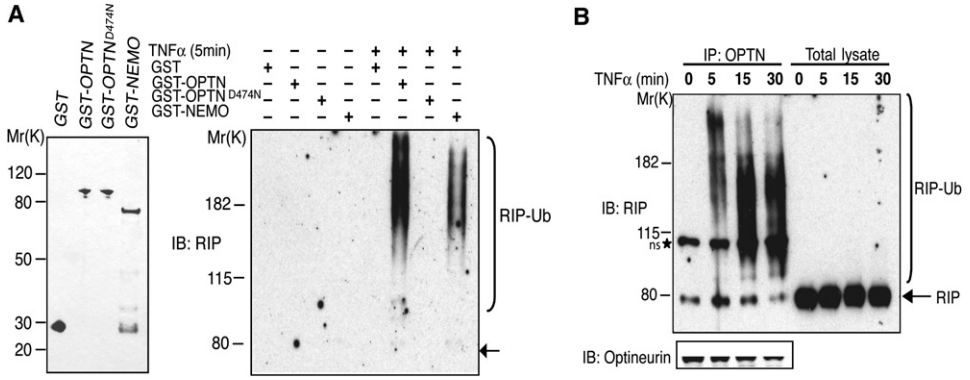

Figure 3. Optineurin Binds to Polyubiquitinated RIP

(A) Lysates of HeLa cells stimulated or not with 20 ng/ml TNFα for 5 min were incubated with glutathione Sepharose beads coated with GST, GST-optineurin, GST- optineurin^{D474N}, or GST-NEMO. The bound proteins were resolved by SDS-PAGE and immunoblotted with anti-RIP. The position of unmodified RIP in the cell lysate is indicated with an arrow. An equal amount of GST fusion proteins were used, as judged by Coomassie-blue staining (left panel).

(B) Lysates of HeLa cells stimulated with TNFα for the indicated times were immunoprecipitated with anti-optineurin. The cell lysate (5% of input) and immunoprecipitates were resolved by SDS-PAGE and immunoblotted with anti-RIP. Because RIP and optineurin have similar molecular weights, the amount of immunoprecipitated optineurin in this experiment was quantified in a gel loaded identically and run in parallel. "ns" indicates a nonspecific band.

the binding of NEMO to polyubiquitinated RIP (Figure 4C). Possible competition between NEMO and optineurin in vivo was assessed by looking for displacement of RIP from NEMO in cells expressing varying amounts of optineurin (Figure 4D). The amount of polyUb RIP that coimmunoprecipitated with NEMO in TNFα-stimulated cells decreased as the amount of optineurin increased. Consistent with its affinity for polyUb RIP, optineurin was recruited to the TNFR1 signaling complex in TNFα-stimulated wild-type cells but not in cells deficient in RIP (Figure 4E). Additionally, the introduction of optineurin resulted in a decrease in the amount of NEMO that coimmunoprecipitated with TNFR1 after TNFα stimulation, consistent with its ability to compete with NEMO in the signaling complex (Figure 4F). It is notable that the initial binding of both NEMO and optineurin to TNFR1 followed similar kinetics, first being detected between 1 and 5 min of stimulation. Furthermore, another component of the TNF proximal signaling pathway, TRADD (TNFR1-associated death domain), but not IKKα coimmunoprecipitated with optineurin after TNFα stimulation (Figure 4G). Taken together, these data support the notion that optineurin and NEMO compete with each other for polyUb RIP in the same TNFα-induced signaling complex.

Because the ability of NEMO to bind polyubiquitinated RIP is important for TNFα-induced NF-κB activation [2, 3], it was possible that competition between optineurin and NEMO could explain why optineurin inhibited TRAF2- or RIP-induced NF-κB activation. For addressing this, cDNA vectors containing wild-type optineurin or optineurin^{D474N} along with an NF-κB reporter were introduced into 293 cells that were stimulated or not with TNFα (Figure 5A). Optineurin had a substantial inhibitory effect on NF-κB activation after TNFα stimulation, whereas the optineurin mutant incapable of binding K63-linked polyUb did not. Overexpression of optineurin also prevented the TNFα-induced upregulation of endogenous IL-6, a gene product whose expression is dependent on NF-κB (Figure 5B). The transient expression of optineurin also prevented TNFα-induced IκBα degradation (Figure 5C). The observation that optineurin overexpression resulted in higher basal IκBα levels is consistent with the lower basal NF-κB activity observed in Figure 5A and Figure S2A. For the determination of whether optineurin inhibits NF-κB activation at physiologic levels, endogenous optineurin expression was stably reduced (knocked down) in 293 cells with microRNA (miRNA). Optineurin levels were markedly reduced in cells expressing *optineurin*-specific miRNA, compared to a nonspecific control (Figure 5D). Importantly, TNFα-induced NF-κB activity was greatly augmented when optineurin levels were reduced (Figure 5E). Consistent with the specific silencing of optineurin, transient re-expression in these cells of *optineurin* that lacked the 3′ untranslated region (UTR) targeted by the miRNA inhibited TNFα-induced NF-κB activation (Figure S2A). Although the silencing was not as complete, treatment with a mixture of small interfering RNAs (siRNAs) targeting different sequences also resulted in enhanced NF-κB activation (Figure S2B). Thus, at normal levels, optineurin strongly inhibits TNFα-signaled NF-κB activation.

Most studies of optineurin have focused on its binding partners and, to a lesser extent, possible functional properties. Optineurin interacts with adenovirus E3-14.7K [7], Huntington [12], RAB8 [13], transcription factor IIIA [14], and myosin VI [15]. siRNA studies have suggested that optineurin plays a role in Golgi integrity and the exocytosis of vesicular-stomatitis-virus G protein [15]. Optineurin normally resides in the cytoplasm, but it has recently been shown to shuttle to the nucleus in NIH 3T3 cells stimulated with H_2O_2, although the biological significance of this is not yet known. Despite the enumeration of these individual activities, how optineurin might regulate cell functions is unclear, and its role in glaucoma is unexplained.

The finding that optineurin, like NEMO, has a predilection for binding K63-linked polyUb chains led to the observation that it is a competitive antagonist of NEMO that at physiologic levels dampens NF-κB activation in

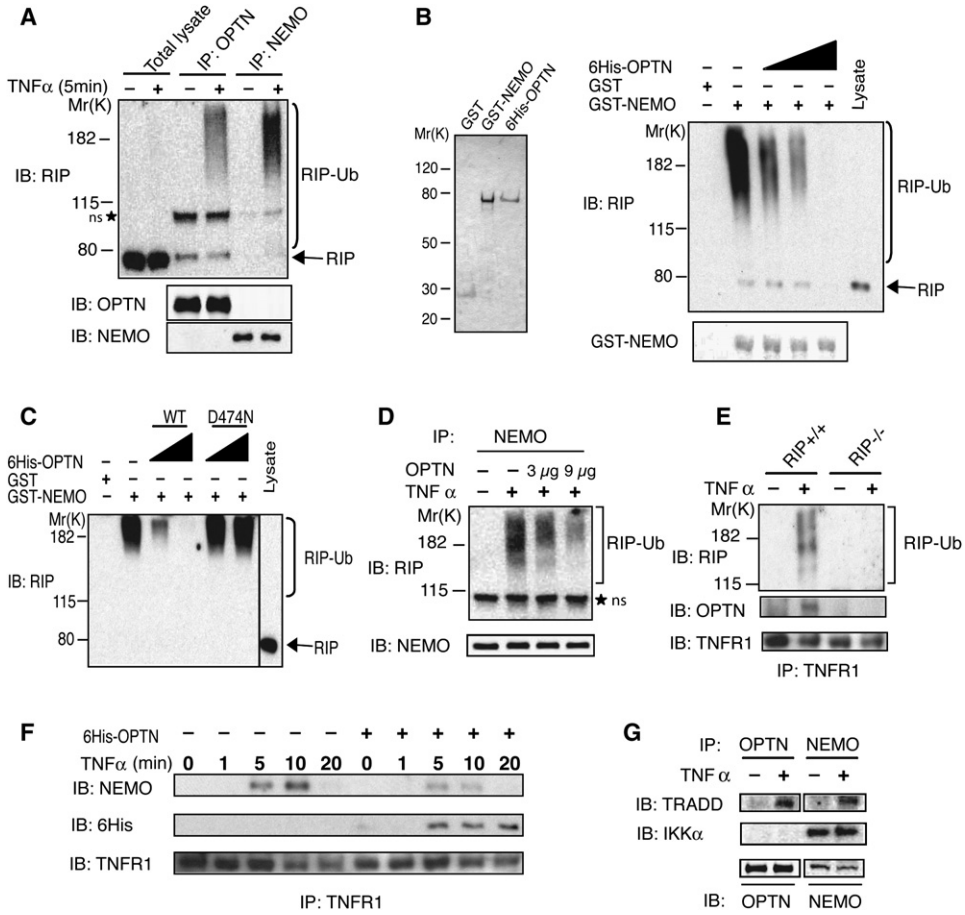

Figure 4. Optineurin Competes with NEMO to Bind to Polyubiquitinated RIP

(A) Lysates of HeLa cells stimulated or not with 20 ng/ml TNFα for 5 min were immunoprecipitated with anti-optineurin or anti-NEMO. The cell lysates (5% of input) and immunoprecipitates were resolved by SDS-PAGE and immunoblotted with anti-RIP, anti-optineurin, and anti-NEMO. "ns" indicates a nonspecific band.

(B and C) Lysates of HeLa cells stimulated with TNFα were incubated with glutathione Sepharose beads coated with GST or GST-NEMO in the presence of increasing amounts of His-tagged optineurin or optineurin^{D474N} in solution. The relative amount of 6xHis-optineurin to GST-NEMO in (B) was 1:1, 10:1, 100:1 and in (C) was 1:1 and 10:1. The bound proteins were eluted and resolved by SDS-PAGE and immunoblotted with anti-RIP. Equal amount of GST-NEMO were used for each pull-down, as judged by Ponceau S staining of the same membrane.

(D) Forty hours after transfection of 293 cells with the indicated amounts of optineurin plasmid, the cells were stimulated with TNFα, lysed, and immunoprecipitated with anti-NEMO. The immunoprecipitates were immunoblotted with anti-RIP or anti-NEMO. "ns" indicates a nonspecific band.

(E) Lysates of *RIP*$^{+/+}$ and *RIP*$^{-/-}$ MEFs were stimulated with mouse TNFα for 10 min, immunoprecipitated with anti-TNFR1, and immunoblotted with anti-RIP and anti-optineurin.

(F) MEFs were infected with virus encoding 6His-OPTN and after 48 hr were stimulated with mouse TNFα for the indicated times. Lysates were immunoprecipitated with anti-TNFR1 and immunoblotted with anti-NEMO, anti-6His, and anti-TNFR1.

(G) Lysates of HeLa cells stimulated with TNFα were immunoprecipitated with anti-optineurin or anti-NEMO and immunoblotted with antibodies to the indicated molecules.

response to TNFα. The simultaneous binding of optineurin and NEMO to the same signaling complex with similar kinetics indicates that optineurin attenuates signaling from the beginning and therefore elevates the threshold of activation for NF-κB. *Optineurin* is a regulated gene, its expression being induced by TNFα as well as type 1 and type 2 interferons (IFNs) [6, 7]. It is noteworthy, therefore, that the pretreatment of cells with TNFα for 18 hr, a time sufficient to allow optineurin upregulation [6], has been found to reduce subsequent TNFα-induced NF-κB activation [16]. Furthermore, pretreatment with IFN-γ strongly inhibits NF-κB activation by RANKL (receptor activator of NF-κB ligand) [17], a member of the TNF superfamily. IFNs also sensitize tumor cells to TNFα- or TRAIL (TNF-related apoptosis-inducing ligand)-induced apoptosis by inhibiting NF-κB activation [18–21]. The present results suggest that the upregulation of optineurin is the cause of cytokine crosstolerance between IFNs and TNF family members such as TNFα, RANKL, and TRAIL.

Glaucoma is a neurodegenerative disease involving the loss of retinal ganglion cells [5]. Optineurin mutations were initially reported to account for 16.7% of normal-tension primary open-angle glaucoma [4], although subsequent studies have found the degree of association in different populations to be considerably less [22–24]. The association is autosomal dominant, consistent with, although not proof of, a gain-of-function

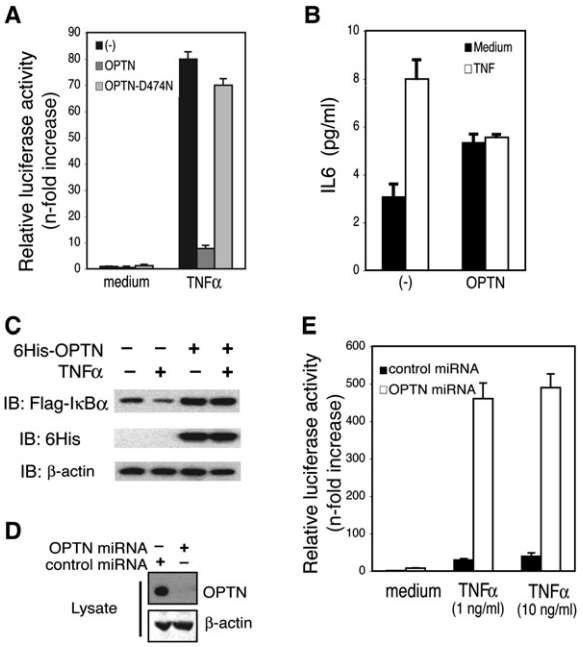

Figure 5. Optineurin Inhibits NF-κB Activation Induced by TNFα

(A) Vectors containing wild-type *optineurin* or *optineurin*^{D474N} were cotransfected with NF-κB and β-galactosidase reporter plasmids into 293 cells for 20 hr. After 5 hr of incubation with TNFα (20 ng/ml), the cells were lysed, and luciferase activity was measured and normalized to β-galactosidase activity. Error bars represent standard error of the mean of triplicate samples.

(B) Twenty-four hours after transfection with an optineurin expression vector, 293 cells were stimulated with TNFα and IL-6 was measured 20 hr later.

(C) 293 cells were transfected with 6His-optineurin and Flag-IκBα expression vectors. After 20 hr, the cells were stimulated or not for 15 min with TNFα, lysed, and immunoblotted with anti-Flag, anti-6His, and anti-actin.

(D) Optineurin levels were stably reduced in 293 cells by the transfection of a plasmid containing miRNA targeting optineurin, and blasticidin selection for 2 weeks followed. A miRNA plasmid containing a nontargeting sequence was used as a negative control. Knockdown efficiency was verified by the immunoblotting of the whole-cell lysate with anti-optineurin.

(E) NF-κB reporter and β-galactosidase reporter plasmids were cotransfected into optineurin knockdown and control cells for 20 hr. After 5 hr of incubation with TNFα (1 or 10 ng/ml), the cells were lysed, and luciferase activity was measured and normalized to β-galactosidase activity.

mutation. It has been postulated that neuron loss resulting from glutamate receptor excitotoxicity is a common mechanism for glaucoma and other neurodegenerative disorders such as Parkinson's, Alzheimer's, and multiple sclerosis [25], and a significant association between glaucoma and other neurodegenerative diseases has been reported [26]. TNFα upregulates the expression of the receptor for α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPAR), which represents one major excitatory subgroup of glutamate receptors, in neuronal cells [27] and synergizes with TNFα to enhance excitotoxic cell death, both responses being dependent on NF-κB activity [28]. The upregulated expression of TNFα by astrocytes is observed in glaucomatous optic nerve heads [29], and, interestingly, the inhibition of NF-κB activation in neuronal cells ameliorates the pathology of murine experimental allergic encephalomyelitis [30]. We have found that the overexpression of optineurin dramatically inhibits TNFα-induced NF-κB activation in a neuronal cell line (data not shown), consistent with a model in which the reduction of functional optineurin might render neural cells hypersensitive to TNFα- and AMPA-induced cell death. It should be noted that two mutations in optineurin in particular are likely to be disease causing. One is truncation resulting from the insertion of a premature stop codon and the loss of the polyUb-binding domain [4]. As expected, this mutation (optineurin^{trunc}) rendered optineurin unable to bind K63-linked polyUb or polyubiquitinated RIP (unpublished data). The other mutation is a single amino acid substitution at residue 50 (optineurin^{E50K}) [31, 4]. This mutation is far from the polyUb-binding domain, and in fact we found that recombinant optineurin containing this mutation bound polyubiquitinated RIP and inhibited TNFα-induced NF-κB activation like wild-type optineurin (unpublished data). It should be noted, however, that optineurin^{E50K} protein levels were found to be markedly reduced in cells from glaucoma patients [4], raising the possibility that it is the reduction in expression, rather than altered function, that contributes to the development of glaucoma.

NEMO-mediated NF-κB activation is involved in many signaling pathways, including that of the T cell receptor (TCR) [32], B cell receptor (BCR) [33], receptors for proinflammatory cytokines and interleukins [34], and Toll-like receptors [1]. The ability of optineurin to inhibit NEMO-dependent NF-κB activation raises the possibility that optineurin possibly plays roles in these signaling pathways as well. In fact, the overexpression of optineurin resulted in the inhibition of IL-1-induced NF-κB activation (unpublished data). The close relationship between activation of NF-κB, inflammation, and neoplasia is well established [35]. By inhibiting IKK and NF-κB activation, optineurin might therefore have regulatory functions in chronic inflammation and cancer development.

Supplemental Data

Experimental Procedures, four figures, and one table are available at http://www.current-biology.com/cgi/content/full/17/16/1438/DC1/.

Acknowledgments

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