Optineurin Increases Cell Survival and Translocates to the Nucleus in a Rab8-dependent Manner upon an Apoptotic Stimulus*

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In glaucoma the retinal ganglion cells of the retina die through the induction of apoptosis leading to excavation of the optic nerve and blindness. Mutations in the optineurin (optic neuropathy inducing) protein were found associated with an adult form of glaucoma. To date, the role of optineurin in the neurodegeneration process that occurs during glaucoma is still unknown. We now report that in response to an apoptotic stimulus, optineurin changes subcellular localization and translocates to the Golgi from the nucleus. This translocation is dependent on the GTPase activity of Rab8, an interactor of optineurin. Furthermore, we demonstrate that the overexpression of optineurin protects cells from H2O2-induced cell death and blocks cytochrome c release from the mitochondria. A mutated form of optineurin, E50K, identified in normal tension glaucoma patients loses its ability to translocate to the nucleus and when overexpressed compromises the mitochondrial membrane integrity resulting in cells that are less fit to survive under stress conditions. The correlation between optineurin function and cell survival will be key to begin to understand retinal ganglion cell biology and signaling and to design general “survival” strategies to treat a disease of such a complex etiology as glaucoma.

Mutations in the human optineurin (optic neuropathy inducing protein) gene, OPTN, are associated with adult-onset glaucoma (1). Glaucoma is a heterogeneous group of neuropathies that are characterized by the progressive loss of retinal ganglion cells (RGC),4 excavation of the optic nerve, and characteristic visual field changes (2, 3). It is one of the leading causes of irreversible blindness affecting ~67 million people worldwide (4). Glaucoma is genetically heterogeneous and is often associated with an increase in intraocular pressure (IOP) (5, 6). RGCs are categorized into open angle, closed angle, and congenital glaucoma based on the mechanism by which the aqueous outflow is impeded in the anterior chamber of the eye (7). Risks for developing the disease, in addition to elevated IOP, include myopia, race, and age. The most common form of glaucoma is the autosomal dominant primary open angle glaucoma (8). Sequence alterations in OPTN were found in 16.7% of families with hereditary primary open angle glaucoma. Interestingly, this gene is mainly mutated in families with normal IOP (1, 9) thereby suffering a form of glaucoma known as normal tension glaucoma (NTG).

Optineurin is 577 amino acids long and contains one bZIP, two leucine zippers, and one zinc finger domain. To date the function of optineurin is not known, although the protein was partially characterized (10). Phorbol esters and cytokines regulate the expression of optineurin that is phosphorylated following phorbol 12-myristate 13-acetate stimulation (11). Its characterization also involved the identification of several proteins with diverse functions that bind to optineurin such as transcription factor IIIA (12) and E3-14.7 K, an adenovirus protein involved in the TNFα signaling pathway (10). The interaction of optineurin with E3-14.7 K inhibits the protective effect of E3-14.7 K on TNFα-induced apoptosis, an effect that is independent of other viral proteins (10). Optineurin also binds Rab8, a member of the small GTPase family known to be involved in vesicular transport (13, 14). The activated form of Rab8 interacts with the amino-terminal region of optineurin, whereas a dominant negative GDP-bound Rab8 has much lower affinity (13). The carboxyl-terminal region of optineurin binds to Huntington (15). When co-expressed, optineurin and Huntington enhanced the recruitment of Huntington to Rab8-positive vesicular structures (13). Optineurin has also been shown recently to link myosin VI and to be involved in the organization of the Golgi apparatus and in exocytosis (16).

In this study we demonstrate that optineurin is expressed in RGC, and in response to an apoptotic stimulus optineurin changes its subcellular localization from the Golgi to the nucleus. The translocation of optineurin to the nucleus is dependent on the GTPase activity of Rab8. We also investigated whether optineurin can induce cell survival, and we observed that cells overexpressing optineurin are more resistant to H2O2-induced cell death. Furthermore, the overexpression of optineurin blocks cytochrome c release from the mitochondria. The mutated form of optineurin identified in normal tension glaucoma patients loses its ability to translocate to the nucleus and when overexpressed compromises mitochondrial membrane integrity resulting in cells that are less fit to survive under stress conditions.

MATERIALS AND METHODS

Expression Plasmids—Full-length Optn cDNA was amplified from mouse retina mRNA (5′CCG GAA TTC ATG GTT CAC CCC AGC CTG3′ and 5′CCG GAA TTC CAC ACA ACA AGA TGC CCA CT3′) and cloned into pBS-SK. To generate HA-Optn, the Optn cDNA was PCR-amplified from the pBS construct with a proofreading polymerase and placed in-frame with the HA epitope of the pcDNA3 plasmid (Invitrogen). The Myc-Optn was generated by placing the Optn cDNA in-frame with the c-Myc epitope 5′ into a modified pMT21 vector. The c-Myc/GFP-Optn was generated by placing Optn cDNA 3′ in-frame with the c-Myc/GFP tag into the plasmid pcDNA3 (Invitrogen). The
artificially deleted Optn1 cDNA constructs were created using PCR fragments cloned 3’ in-frame with the HA epitope into plasmid pcdNA3 (Invitrogen).

The Rab8 plasmids were the kind gift from J. Peranen, University of Helsinki. The pEGFP-Rab8-22N and pEGFP-Rab8-67L were constructed by cloning these Rab8 mutants into the EcoRI-BamHI sites of pEGFP-C1. pEGFP-Rab8wt was constructed by cloning Rab8wt into the EcoRI-HindIII sites of pEGFP-C1A (modified pEGFP-C1). The Myc-tagged Rab8 constructs were generated by cloning Myc-tagged Rab8wt, Rab8-22N, and Rab8-67L into the EcoRI-HindIII sites of pEGFP-N1.

PCR Site-directed Mutagenesis—Site-directed mutagenesis to generate E50K optineurin was performed using the commercially available QuickChange site-directed mutagenesis kit (Stratagene), in accordance with the manufacturer’s instructions. To introduce the E50K mutation into wild-type Optn, the following oligonucleotides were used: 5’ Opt E50K F, 5’GAA GGA ACT CCT GGT TAA GAA CCA CCA GCT GAAAS’ and 5’T TT CAG CGT GTG GTT CTT AAC CAG GAG TTC CTT3’.

The DNA sequence of the mutant optineurin was confirmed by sequence analysis. The mutant optineurin expression vectors were constructed by cloning the mutated optineurin coding region into the HA-pcDNA3, pMT21, c-Myc/GFP-pcDNA3, and FLAG-pcDNA3 (Invitrogen) as described above.

Northern Blot—Total RNA from various organs and different eye structures were isolated using Trizol reagent (Invitrogen) according to manufacturer’s instructions. 15 mg of total RNA was separated on 1% DNA labeling kit (Roche Applied Science) and32P-labeled dCTP. RNA fragments cloned 3’ were isolated using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. 15 mg of total RNA was separated on 1% DNA labeling kit (Roche Applied Science) and32P-labeled dCTP. RNA blots were hybridized in a mixture of 5× SSC, 50% formamide, 5× Denhardt’s solution, 1% SDS, 100 μg/ml salmon sperm DNA at 65 °C. The filters were washed at 65 °C in 2× SSC, 2% SDS, 0.2× SSC, 0.2% SDS before exposure to x-ray film.

In Situ Hybridization—Full-length Optn cDNA was cut with XhoI or HindIII and used as template to synthesize digoxigenin-labeled antisense and sense probes, with digoxigenin-labeled UTP and T7 or Sp6 polymerase, according to the manufacturer’s recommendations (Roche Applied Science). The Optn cDNA was labeled with a random primer DNA labeling kit (Roche Applied Science) and 32P-labeled dCTP. RNA blots were hybridized in a mixture of 5× SSC, 50% formamide, 5× Denhardt’s solution, 1% SDS, 100 μg/ml salmon sperm DNA at 65 °C. The filters were washed at 65 °C in 2× SSC, 2% SDS, 0.2× SSC, 0.2% SDS before exposure to x-ray film.

Immunohistochemistry—Eyes of adult mice were fixed in 4% PFA overnight at 4 °C, cryoprotected in 30% sucrose, and embedded in Tissue-Tek (Sakura). 7-μm-thick sections were cut in a cryostat and mounted on polylysine-coated slides for immunohistochemistry. Non-specific background was blocked by incubating the sections for 30 min in PBS, 0.1% Tween, 5% normal goat serum subsequently incubated with polyclonal anti-optineurin 1:200 for 3 h at room temperature, washed in PBS, 0.1% Tween, incubated for 45 min with biotinylated goat anti-rabbit, and reacted with the avidin-biotinylated peroxidase complex (Vector Laboratories). The reaction product was visualized by incubation for 5 min in diaminobenzidine (Vector Laboratories).

Cell Culture and Transfection—COS7 and NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% CO2 atmosphere. Neuro 2A cells were grown in the same medium supplemented with essential amino acids.

Transfections were performed using the PolyFect transfection reagent (Qiagen) following the manufacturer's instructions. 60-mm dishes were transfected with 2.5 μg of DNA. 12-Well plates transfected with 0.75 μg of DNA were used for immunofluorescence assays.

In order to be stimulated, the cells were incubated with 25 mM H2O2 for the defined period of time after which cells were washed with PBS and fixed. When serum-starved, cells were grown for 24 h in medium containing 0.5% of serum. Immunofluorescence—Cells were grown on coverslips to 50% confluence and transfected as described above; 36 h after transfection, the cells were rinsed in PBS and fixed in 4% paraformaldehyde for 15 min. Those cells transfected with the GFP fusion proteins (optineurin or E50K optineurin) were mounted and observed under the fluorescence microscope. For those cells transfected with HA-optineurin or for the endogenous proteins, cells were incubated with the blocking solution PBS, 0.1% Tween, 5% normal goat serum for 30 min at room temperature after fixation. The antibody incubation was performed for 3 h at room temperature using the following dilutions: αHA 1:400, α-optineurin 1:200, α-cytochrome c 1:200, and anti-58K 1:50. Staining was completed by incubation with fluorescein isothiocyanate-conjugated anti-rabbit antibodies (Dako) or with TRITC-conjugated anti-mouse antibodies (Dako). Cells were washed in PBS, 0.1% Tween, and nuclei were counterstained with Hoechst (1:5000) for 10 min at room temperature and mounted with Vectastain (Vector Laboratories). The anti-rabbit Cy3-conjugated and anti-mouse Cy2-conjugated antibodies (Jackson ImmunoResearch) were used for confocal analysis. Each immunofluorescence experiment described was repeated at least five times.

MitoTracker—Cells were grown on coverslips, and 36 h after transfection the cells were washed with PBS and incubated with MitoTracker Red and MitoFluor mitochondrion-selective probes at 350 nm, diluted in Dulbecco’s modified Eagle’s medium for 45 min. After incubation the cells were fixed in 3.7% formaldehyde. Finally, nuclei were counterstained with Hoechst (1:5000) for 10 min and mounted with Vectastain (Vector Laboratories).

Immunoblot—Proteins were boiled for 5 min in reducing sample buffer, separated in 8% SDS-PAGE, and electroblotted on nitrocellulose
membranes (Schleicher & Schuell). The membranes were rinsed in PBS, 0.1% Tween and blocked in PBS, 3% bovine serum albumin, 0.1% Tween for 30 min at 37 °C. Incubation with the antibodies was performed using anti-c-Myc monoclonal supernatant (1:5 dilution), anti-HA monoclonal antibody (final concentration 0.8 μg/ml; Babco), or polyclonal anti-optineurin in PBS, 3% bovine serum albumin, 0.1% Tween. Antibody binding was detected with a secondary anti-mouse or anti-rabbit IgG coupled with horseradish peroxidase (Amersham Biosciences) followed by visualization with the enhanced chemiluminescence kit (Pierce).

Nuclear Extracts—1 × 10⁶ cells were washed in 10 ml of PBS and then scraped and collected in 1 ml of PBS and centrifuged at 13,000 × g for 15 min. The pellet was resuspended in 400 μl of cold buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor mixture (Roche Applied Science)) and left on ice for 15 min. After the addition of 25 μl of Nonidet P-40, cells were vortexed for 30 s and centrifuged for 30 s at 4 °C. The pellet was resuspended in 50 μl of buffer C (20 mM Hepes, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride), incubated with agitation for 15 min at 4 °C, and centrifuged for 5 min at 4 °C. The supernatant was then loaded on an SDS-polyacrylamide gel as described above.

Apoptotic Assay—Cells were grown on a 12-well plate to 50% confluence and transfected with GFP-optineurin or GFP-E80K as described above. 36 h later cells were stimulated with 25 mM H₂O₂ for 1 h and fixed, and nuclei were counterstained with Hoechst. Ten different fields for each well were analyzed by fluorescence microscopy to count apoptotic or nonapoptotic transfected cells versus the not stimulated control. This experiment was performed five times.

Real Time PCR—Total RNA from cells stimulated with H₂O₂ at different time points was extracted using Trizol reagent (Invitrogen). Three micrograms of RNA were retrotranscribed by SuperScript II first strand kit (Invitrogen). A quantitative PCR was performed using the following primers: 5′TTC AAA GAG AAA TCA GAA AAG CCA3′ and 5′CTC CTC CCA GGC TCT GGG A3′ and 2× SYBR Green MasterMix (Applied Biosystem) according to the manufacturer’s recommendations at Tm 60 °C for 40 cycles. An Applied Biosystem ABI PRISM 7000 sequence detector system was used.

RESULTS

Optineurin Is Mainly Expressed in the Ganglion Cells of the Mouse Retina—The mouse homologue of OPTN is located on chromosome 2 in a region syntenic to human chromosome 10 and is composed of 15 exons. We cloned the full-length mouse Optn from total eye RNA using primers designed on a sequence derived from transcript predictions of the genomic sequence. A bioinformatic analysis on the genomic sequence of the Optn gene, using Celera (Santa Cruz Biotechnology) and expressed sequence tag data bases plus the cDNA sequence we amplified from eye, revealed alternative splice variants in the mouse not described in the human gene. The form we cloned from whole mouse eye, revealed alternative splice variants in the mouse not expressed in the adult (see below) or that it has a low ubiquitous expression level. An immunohistochemistry analysis in adult retina revealed optineurin expression in the retinal ganglion layer (Fig. 1D). Optineurin is specifically expressed in the retina. A, Northern blot analysis on RNA extracted from different eye structures of an adult mouse. c, cornea; l, lens; r, retina; ch, choroids. The expression of β-actin (bottom panel) was used as control. B, in situ hybridization on developing mouse embryos at E10.5 revealed the expression of Optn in the developing eye (white arrow). C, in situ hybridization with the sense control probe at the same embryonic stage (E10.5). D, immunohistochemistry on adult mouse retina reveals the expression of optineurin in the RGC layer.

Optineurin Increases Cell Survival—We next determined the response of cells overexpressing optineurin over an apoptotic stimulus. NIH3T3 cells transiently transfected with optineurin were stressed with 25 mM H₂O₂ for 20 min; the cells were then harvested and fixed, and we performed immunofluorescence to observe optineurin localization. Most unexpectedly, we observed that optineurin translocates to the nucleus after the apoptotic stimuli (Fig. 2D). The nuclear translocation of optineurin was also observed when cells were serum-starved for 24 h (Fig. 2F). This result was consistently observed in each experiment performed.

To determine that the nuclear translocation is not because of the overexpression of the protein, we decided to look at the behavior of the endogenous protein. To this end, we used the polyclonal antibodies against the optineurin protein we produced (see “Material and Meth-
This antibody reveals both the transfected and the endogenous protein by immunoprecipitation, Western blot (data not shown), and immunofluorescence (see Fig. 2). NIH3T3 cells were stimulated with 25 mM H₂O₂ for 20 min and subjected to immunofluorescence. We observed that the endogenous protein translocates to the nucleus as we had observed with the transfected protein (Fig. 2J). To further confirm this result, we examined the subcellular localization of Optn after H₂O₂ stimulation in the cytosolic and nuclear fractions using a biochemical approach. NIH3T3 cells were stimulated with 25 mM H₂O₂ and samples taken at several time points. The cytosolic and nuclear fractions were separated by centrifugation, resolved by SDS-PAGE, and analyzed in Western blots with the anti-optineurin antibody. In control cells we observed a high concentration of optineurin in the cytosolic fraction. As expected, we revealed optineurin in the nuclear fraction of cells that received the H₂O₂ treatment. These results confirm our immunofluorescence data and demonstrate that optineurin translocates to the nucleus upon an apoptotic stimulus.

As mentioned, optineurin is known to interact with Rab8, a small GTPase involved in membrane trafficking that promotes radical changes in cell shape (18). To test whether optineurin needs to be associated to Rab8 to change its localization, we generated a truncated version of optineurin that lacks the first 206 amino acids, the region that interacts with the small GTPase Rab8 (55–208 amino acids) (13). Interestingly, we observed that this truncated version localizes to a different cellular compartment and is found diffuse in the cytoplasm as well as in the nucleus, as assayed by confocal microscopy (Fig. 3, A and B). The localization of the amino-terminal deletion mutant does not change upon H₂O₂ stimulation (data not shown). These data suggested that the nuclear translocation and the Golgi localization could be dependent on the association of optineurin with Rab8 or on Rab8 activity. To deter-
mine whether the activity of Rab8 is necessary, we utilized a dominant negative (Rab8-22N) and a constitutive active Rab8-67L protein (kind gift of J. Peranieen) to understand the mechanism behind the translocation of optineurin to the nucleus. When NIH3T3 cells were transfected with Rab8-22N and stimulated with H$_2$O$_2$, the endogenous optineurin failed to translocate to the nucleus (Fig. 3, I–N). Similarly, the overexpression of Rab8-67L forced the endogenous optineurin into the nucleus even in the absence of a stress stimulus. We also observed some optineurin diffuse in the cytoplasm after stimulation (Fig. 3, C and D). These results were also confirmed by confocal microscopy (data not shown). The endogenous optineurin behaved as in control cells in the presence of overexpressed wild-type Rab8, remaining perinuclear when nontreated and translocating to the nucleus upon H$_2$O$_2$ stimulation (Fig. 3, C–H). These results indicate that the nuclear translocation of optineurin is dependent on Rab8 GTPase activity.

To gain further insight into the biological mechanism of optineurin function, we studied the behavior of the mutant form found in patients suffering NTG glaucoma. We performed site-directed mutagenesis on the wild-type optineurin to recreate the E50K mutation. NIH3T3 cells were transfected with a tagged version of E50K, and we determined its subcellular localization in control and H$_2$O$_2$-treated cells. The mutated form of optineurin, E50K, shows a mainly diffuse staining in the cytoplasm, with some discrete punctate specks, when overexpressed (Fig. 4, A and B). In these studies we observed that polyclonal antibody used to reveal the endogenous protein does not recognize the E50K form of optineurin. When cells transfected with the E50K form were stimulated with H$_2$O$_2$, there was no nuclear localization of the protein as observed with the wild type (Fig. 4, C and D). When we performed a double transfection experiment in NIH3T3 cells with both the wild-type and E50K optineurin, we observed that the wild-type protein no longer associates with the Golgi but translocates to the nucleus (Fig. 4, E–G) as when stimulated with H$_2$O$_2$ or serum-starved. This same behavior was observed with the endogenous optineurin, when NIH3T3 cells overexpressing the mutant form E50K of the endogenous protein translocates to the nucleus (Fig. 4, H–J, white arrow). These results indicate that the disease causing E50K optineurin mislocalizes and loses its ability to respond to H$_2$O$_2$ stimulation as its wild-type counterpart. Furthermore, these results also suggest that cells respond to the overexpression of the E50K optineurin by translocating the overexpressed and the endogenous wild-type optineurin to the nucleus, in an analogous manner as observed upon H$_2$O$_2$ stimulation. It is important to note that after transfection the expression levels of the wild-type and E50K optineurin are comparable, as shown in Fig. 4K.

**Wild-type Optn Protects from H$_2$O$_2$-induced Apoptosis**—The translocation of optineurin to the nucleus upon H$_2$O$_2$ addition suggests that
the protein might be involved in response to oxidative stress. NIH3T3 cells were transiently transfected with wild-type or E50K optineurin (optn). The total amount of cells transfected or not and with or without H$_2$O$_2$ addition were counted in 10 different fields and averaged (A, top panel). The percentage of either optineurin or E50K-transfected cells was determined revealing an increase in optineurin-expressing cells (almost 90%, compared with the 28% before stimuli) after H$_2$O$_2$ addition. The cells expressing E50K remained the same before and after stimuli (~20%) (A, bottom panel). Error bars represent the standard deviation of five different experiments. The transcription of Optn is increased upon H$_2$O$_2$ addition. Real time PCR analysis revealed a 2-fold increase in Optn mRNA upon H$_2$O$_2$ addition. The numbers on the x axis (10', 20', and 30'; where ' represents minute) represent time of incubation with H$_2$O$_2$; the C stands for control cells (not stimulated). Error bars represent the standard deviation of three experiments.

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24 h after transfection, the cells were split and G418 was added to the medium; 10 days after selection, we counted the amount of colonies formed with cells expressing the wild-type or E50K. We observed a 50% reduction in colony formation in those plates transfected with E50K Optn when compared with the empty vector control and wild-type optineurin (this experiment was repeated three times; data not shown). The data suggest that the mutated optineurin is interfering with a cell survival pathway.

Optineurin Inhibits Cytochrome c Release from the Mitochondria—We decided to address whether optineurin could regulate the intrinsic apoptotic pathway by measuring cytochrome c release upon H$_2$O$_2$ stimulus. We performed immunofluorescence staining to reveal cytochrome c in NIH3T3 cells overexpressing wild-type or E50K optineurin treated with H$_2$O$_2$. The results indicated that optineurin delays cytochrome c release.
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from the mitochondria after 20 min of H₂O₂ treatment (compared with control, nontransfected cells) (Fig. 6, K and M). Cytochrome c staining appears more diffuse in nontransfected cells after 20 min of H₂O₂ stimulation than in cells overexpressing optineurin at the same time point (compare Fig. 6, K with M). On the contrary, expression of E50K accelerates cytochrome c release upon H₂O₂ treatment, and cytochrome c is already released after 10 min of H₂O₂ stimulation compared with cells expressing wild-type optineurin and the control nontransfected cells (Fig. 6, F, H, and J). Interestingly, some cytochrome c release is observed also in nontreated cells that overexpress E50K (Fig. 6E).

The observation that E50K overexpression accelerates cytochrome c release in stressed cells prompted us to determine whether the overexpression of either wild-type or E50K optineurin may have an impact on mitochondrial membrane potential. Cytochrome c release is accompanied by the loss of membrane potential. We measured membrane potential in NIH3T3 cells using MitoTracker (see “Materials and Methods”), and this dye is released from the mitochondria if the membrane potential is disrupted, losing the characteristic punctate staining of the mitochondria. We observed that increasing doses of transfected E50K-expressing plasmid resulted in the loss of MitoTracker staining compared with control or cells transfected with the wild-type optineurin and the control nontransfected cells (Fig. 6, F, H, and J). The overexpression of optineurin prevents cytochrome c release after 20 min of H₂O₂ stimulation compared with control cells (M and K) and to cells overexpressing E50K (O). This experiment was repeated several times (a minimum of four times) and showed consistent results.

**DISCUSSION**

This is the first study that explores the role of optineurin in cell survival. The expression of optineurin in the retinal ganglion cells and the fact that mutations in optineurin are responsible for a form of NTG strongly suggest that this protein could have a cell autonomous role in the development of glaucoma by interfering with the retinal ganglion cell survival pathway when mutated. We present evidence that optineurin has a protective role and stimulates cell survival.

The functional characterization of optineurin that we performed revealed that both the endogenous and transfected protein changes in its localization, translocating from the Golgi to the nucleus upon apoptotic or stress stimuli. This same phenomenon was also observed when cells were serum-starved. This is not the first report that states optineurin changes its subcellular localization. A chick homologue of optineurin, which is also associated with the Golgi, translocates to the marginal band during chicken erythrocyte differentiation.

We have uncovered the mechanism by which optineurin changes its subcellular localization, which is dependent on its interaction with Rab8. Rab8 is a small GTPase involved in membrane trafficking that promotes radical changes in cell shape. At least 60 different Rab family members are encoded in the human genome, each of which may participate in a different trafficking pathway. The nuclear translocation of optineurin is dependent on the GTPase activity of Rab8 and is inferred from the experiments with the active GTP-bound Rab8. The endogenous optineurin translocates to the nucleus in absence of a stress stimuli when a constitutively active Rab8 (Rab8-67L) is overexpressed. The overexpression of a GDP-bound Rab8 resulted in the lack of optineurin translocation upon H₂O₂ addition. An analogous translocation mechanism was described for the AAPL1 protein, which is released from endocytic structures upon GTP hydrolysis by Rab5.

Is the interaction of optineurin with Rab8 necessary for Golgi localization of optineurin? When optineurin fails to interact with Rab8, as observed with the 1–206 amino-terminal truncated version, it is mislocalized and found diffuse in the cytoplasm and in the nucleus. Hattula and Peranen (13) demonstrate that optineurin (herein known as FIP-2) does not bind to the GDP-bound Rab8; however, neither we nor that report see optineurin mislocalized when co-expressed with Rab8-22N. These observations suggest that the localization of optineurin at the Golgi is not dependent on its interaction with Rab8 and that the most amino-terminal domain (1–55) might be interacting with some other protein that anchors optineurin at the Golgi. It is important to note that Hattula and Peranen (13) did not observe a mislocalization of overexpressed optineurin when co-transfected with Rab867L as observed in this report. An important point differs in the experimental layout of these two studies; we observe the translocation of the endogenous protein and use a different cell type, which could account for the differences observed. A recent report has demonstrated that optineurin binds to myosin VI and demonstrates that optineurin is the link between myosin VI and Rab8; when optineurin is knocked down, using RNA interference, myosin mislocalizes and no longer co-localizes with Rab8. Myosin VI is a multifunctional motor protein found in several endocytic compartments, including the Golgi and the secretory vesicles.

**FIGURE 6. Optineurin prevents cytochrome c release from the mitochondria.** Cytochrome c immunofluorescence on NIH3T3 cells overexpressing wild-type or E50K optineurin. A, F, and K, cytochrome c immunofluorescence on nontransfected cells in control cells and after 10 (10') and 20 min (20') of H₂O₂ stimulation. B, G, and L, GFP-optineurin (GFP-optn) localization in control and stimulated cells. C, H, and M, cytochrome c localization in cells overexpressing optineurin in control and stimulated cells. D, I, and N, the localization of GFP-E50K in control and stimulated cells. E, J, and O, the localization of cytochrome c in control and stimulated cells overexpressing E50K. After 10 min of H₂O₂ stimulation cytochrome c is released from cells overexpressing E50K (compare J, H, and F). The overexpression of optineurin prevents cytochrome c release after 20 min of H₂O₂ stimulation compared with control cells (M and K) and to cells overexpressing E50K (O). This experiment was repeated several times (a minimum of four times) and showed consistent results.
that the fraction of optineurin that is not bound to myosin is free to translocate to the nucleus upon an overexpression of the constitutively active Rab8. It is also possible than under certain conditions optineurin interacts with specific partners. It was clearly shown that optineurin is phosphorylated and that kinase inhibitors inhibit this phosphorylation (11). The phosphorylation state of optineurin might determine with which partner it interacts.

The cells that overexpress optineurin are more fit to survive under “stress” conditions, i.e. H$_2$O$_2$ stimulation, that control cells. It is known that mitochondria are central to many forms of cell death usually via the release of proapoptotic factors from the mitochondrial intermembrane space (24). Cytochrome c release from the mitochondria represents a critical event during apoptosis (25), and several reports have indicated that the “mitochondrial” pathway is active in diverse neuronal cell apoptosis, including retinal ganglion cells (26). We observed that the increase in survival in cells overexpressing optineurin when stimulated with H$_2$O$_2$ is in accordance with the inhibition of cytochrome c release. We still have no evidence of where optineurin may be interfering with this pathway, and this will be the subject of further studies. It is important to note that although this characterization was performed in NIH3T3 cells, we also observed that optineurin translocates to the nucleus upon an apoptotic stimulus in a neuronal cell line (Neuro2a). A second step will be to repeat these findings in primary retinal ganglion cells or using a transgenic mouse model that specifically expresses optineurin in the retinal ganglion cell layer.

The response to the stress of optineurin is not limited to a change of localization; the transcription of optineurin also increases (2-fold) 20 min after stress stimuli. The fact that optineurin translocates to the nucleus and the existence of a bZIP domain, a conserved transcription factor motif normally involved in DNA binding and protein dimerization, in its protein structure suggest that optineurin might directly or indirectly regulate its own transcription to further increase the cell sur-
vival signal. Interestingly, the recurrent E50K mutation found in glaucoma patients is located within the putative bZIP motif, which is conserved in the mouse, bovine, and macaque genomes (1). This region has not been described as interacting with any other protein to date.

Is optineurin a sensor of Golgi stress? The importance of the secretory pathway in apoptosis is becoming increasingly apparent (27). Sensing stress at the endoplasmic reticulum or at the Golgi might be the first signal for the cell to activate the survival mechanism and apoptosis if the cell is doomed. Important players in the apoptotic pathway are found in the Golgi such as caspase 2, caspase 12, and some death receptors such tumor necrosis factor receptor 1 and TRAIL R1, among others (28–30). The role of the endoplasmic reticulum in apoptosis has been better determined (for review see Ref. 31), and the role of the Golgi in this pathway is emerging. It is clear that these organelles are important to locally sense signals and ensure inter-organellle cross-talk. It is in this organelle cross-talk that optineurin might be playing a crucial role. Optineurin, through protein-protein interaction, links Huntingtin to Rab8 and promotes the relocalization of Huntingtin to vesicular structures. Huntingtin up-regulates the transcription of brain-derived neurotrophic factor, a pro-survival factor produced by cortical neurons that is necessary for survival of striatal neurons in the brain (32). Perhaps optineurin, by translocating to the nucleus, liberates Huntingtin from its links to the vesicular apparatus and is free to activate the transcription of brain-derived neurotrophic factor and to stimulate survival.

The mutant optineurin E50K is unable to translocate to the nucleus upon an apoptotic stimulus. The amino acidic change because of the mutation replaces a glutamic acid (negatively charged) with a lysine (positively charged) leading to the predicted conformational change. In fact, by using the tools available on line, we determined the predicted secondary structure of wild-type and E50K optineurin, and we observed that the mutation leads to a predicted conformational change in the protein that loses a β-sheet and gains a random coil. We have biochemical evidence that wild-type optineurin self-interacts and that the mutant fails to interact with the wild-type protein. Furthermore, we have preliminary evidence that E50K can no longer interact with Rab8. It is plausible to hypothesize that the conformational change might block the interaction with Rab8 explaining why the protein cannot respond to stress by translocating to the nucleus. However, our data indicate that the pathogenic mechanism of the mutant optineurin is not a mere “loss of function”; on the contrary, our results indicate that the mutant protein induces apoptosis or that the overexpression of E50K optineurin stresses the cell. The observation that the wild-type optineurin, both endogenous and exogenous, translocates to the nucleus when E50K is overexpressed indicates that the overexpression of the E50K stresses the cell. We cannot exclude that the mutant form might have a higher affinity for some anchor protein and displace the wild-type protein from its normal localization that moves by default to the nucleus. However, the fact that cells expressing the mutant are less fit to survive, as demonstrated in the colony assay, the release of cytochrome c and the loss of mitochondrial membrane potential in the absence of stimuli in cells overexpressing E50K favor a gain of function for this mutant.

OPTN mutations leading to NTG reported to date are autosomal dominant; however, it remains unclear whether the mechanism involves haploinsufficiency or a gain of function effect. Our results suggest a dual mechanism could be operating in the retinal ganglion cells of glaucoma patients that contain one copy of the wild-type and one mutated form. We know that although the wild-type protein self-interacts, there is no interaction between the mutant and the wild-type form. Therefore, the disease mechanism does not involve a structural hindrance of the wild-type protein. The RGC of NTG patients have half a dose of wild-type optineurin to respond to stress or cytotoxic signals, on the other hand the E50K might by itself be inducing stress to the cell. We cannot exclude that E50K only stresses the cells when overexpressed, and in a normal dose, as in patients, might not have a cytotoxic effect. Concomitantly, we know that the expression of optineurin is induced under stress or by cytokines (this paper and see Ref. 11); therefore, in a degenerating retina, where death is abundant, cytokines are produced resulting in the increase of expression of wild-type and mutant optineurin. The increase in expression of the mutant in this case might lead to the synergistic effect we see in overexpression jeopardizing the ability of the cell to survive. Experiments on cell lines derived from the patients could answer this enigma. In this scenario, optineurin might have a “survival signal” role and be necessary, in complete dosage, when the cells are stressed, as in an aging retina. This study highlights the point of the iceberg of a complex “stress sensor” mechanism functioning in the cell. Additional studies utilizing RGC cells and a more in depth dissection of the role of optineurin in the regulation of the intrinsic apoptotic pathway are necessary to fully unravel the role of this protein. However, in a disease with a complex etiology such as glaucoma, the understanding of some of the basic mechanisms operating in the cells during the disease pathogenesis is crucial to design future therapeutic strategies.

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