Differential Regulation of Endoplasmic Reticulum Structure through VAP-Nir Protein Interaction*

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The endoplasmic reticulum (ER)1 is an extensive network of membranes comprised of an array of interconnecting tubules and cisternae that emerges from the nuclear envelope (NE) and extends peripherally throughout the cell cytoplasm (1). It contains several structurally distinct domains, including the NE, the rough and smooth ER (rER and sER), and the regions that contact other organelles, such as the Golgi apparatus, the late endosomes, the lysosomes, mitochondria, peroxisomes, and the plasma membrane (2). The ER functions in diverse metabolic processes including lipid synthesis, carbohydrate metabolism, and the detoxification of drugs. It is responsible for the synthesis, translocation, glycosylation, folding, assembly, and processing of secretory and membrane proteins, and it functions in intracellular calcium storage and sequestering (3, 4).

While the function of the ER in membrane trafficking, lipid biosynthesis, and calcium signaling and, calcium signaling have been extensively studied, the mechanism by which the ER maintains its characteristic structure in vivo remains largely unknown. Studies from yeast and mammalian cells have shown that the size and/or structure of the ER is extremely sensitive to certain cellular stress conditions, such as the unfolded protein response (UPR) or to the overexpression of a subset of ER-resident membrane proteins (5, 6). Overexpression of 3-hydroxyethyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase, microsomal aldehyde dehydrogenase (msALDH), cytochrome P-450, and malooled cytochrome P-450, causes the proliferation of ER membranes and stacking of the ER cisternae into organized structures known as crystallloid ER, sinusoidal ER, or karmellae (7–12). The formation of these structures is easily visible and can be used as a quantitative method for studying ER membrane biogenesis (5). Nevertheless, the underlying mechanism of their formation is not completely understood. It has been proposed that proteins that regulate lipid metabolism, such as HMG-CoA reductase, induce the formation of these structures because of the extensive requirement of membrane biogenesis. However, subsequent studies suggested that the oligomerization state of the ER integral membrane proteins is crucial for their production (7, 13, 14).

The organization of the ER is also sensitive to drugs that induce microtubule depolymerization, such as colchicine or nocodazole. These drugs cause retraction of the ER tubules from the cell periphery and consequently, the formation of ER membrane aggregates around the nucleus. It has long been known that the ER uses the microtubules as a framework for extending and maintaining its reticular organization in animal cells (1, 15). The interaction of the ER with microtubules is mediated by motor proteins, which interact directly with microtubules and concomitantly with the ER membranes via protein-protein interactions. Their interaction with the ER and their motor activity are required for the sliding of ER tubules along stationary microtubules and consequently, motility of the ER network. In contrast, the position of the ER within the cells and its motility by microtubule movement or tip-attachment mechanisms (3) require static association of the ER membranes with microtubules, and this is thought to be mediated by proteins such as p63 or cytoplasmic linker proteins (CLIPs). p63 is a type II integral ER membrane protein that binds directly to microtubules via its cytoplasmic domain and thereby links the
ER membranes to the microtubule network (16, 17). CLIPs are soluble non-motor microtubule-binding proteins that link microtubules to intracellular organelles by binding a putative membrane receptor. Among the CLIPs, CLIP-170 links endosomes to microtubules and might be involved in the regulation of ER extension (18).

VAP-B is also a type II integral membrane protein of ~31 kDa that has been previously localized to the ER and the pre-Golgi intermediates (19, 20). It belongs to a highly conserved family of proteins, which are implicated in the regulation of neurotransmitter release, ER-Golgi and intra-Golgi transport, Glu4 (glucose transporter 4) trafficking, stabilization of presynaptic microtubules, and the expression of phospholipid biosynthetic genes (19, 21–25). These diverse functions have been demonstrated in different species and cell types, and are mediated by different members of this family. Nevertheless, the overall structures of VAP proteins are similar and consist of a large N-terminal region facing the cytoplasm and a hydrophobic C terminus that functions as a transmembrane domain (TMD) (20). The cytoplasmic region contains a conserved N-terminal domain of 100 amino acids, which shares high sequence similarity with the nematode major sperm protein (MSP). This 100 amino acid domain contains a highly conserved sequence of 16 amino acids. The central part of the cytoplasmic region contains a coiled-coil domain of ~40 amino acids, which is a common motif in many t-SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptor) proteins (26).

The VAP proteins interact with several intracellular proteins and have the ability to interact with each other (27–29). Originally, the Aplysia ApVAP33 was isolated as a VAMP/synaptobrevin-interacting protein using the yeast two-hybrid screen (21). Subsequent studies demonstrated the interaction of the mammalian VAP-A with additional SNAREs, including syntaxin 1A, rBet1, rSec22, αSNAP, and NSF (29). VAP-A also interacts with the tight junction protein occludin (22), with microtubules (20, 24), and with OSBP (oxysterol-binding protein) (28). In this study, we isolated VAP-B as an interacting protein with Nir2, using a pull-down experiment and mass spectrometry analysis. Similar to VAP-B, Nir2 also belongs to a highly conserved family of proteins, the Nir/rdgB, which are implicated in the regulation of membrane trafficking, phospholipid metabolism, and signaling (30, 31). Here we show that the three Nir proteins: Nir1, Nir2, and Nir3, interact with VAP-B via their FFAT motif, and that Nir-VAP interactions differentially affect the organization of the ER.

**EXPERIMENTAL PROCEDURES**

**Recombinant DNA and Antibodies**—The cDNA of human VAP-B was isolated by PCR using a first-strand cDNA, which was synthesized from total RNA of HeLa cells (SuperScript II first strand synthesis system, Invitrogen), as a template and the following sense and antisense oligonucleotide primers: 5'-CCGGATCCGGAAGTTGGGACA-3' and 5'-CCGGATATCTACAAGGCAATCTTCCCAAT-3', respectively. The amplified PCR product was subcloned into the pCAN-myc1 and pGEK-X1 expression vectors. The FFAT mutant of Nir2 was generated by replacing amino acids 349–353 (EFPDA) with ALLAG using three sequential PCR steps with the following sense and antisense primers: 5'-GAGATCTCTCGGACCAACGG-3' and 5'-GGCCACGGTAGGATCATCTAACGAGG-3', respectively. The PCR product was subcloned into the pCAc-Myc vector (Sigma). Nir1 and Nir2 cDNAs were subcloned into pRK5 mammalian expression vector downstream of the CMV promoter. Hemagglutinin (HA)-tag was fused to the C-terminal of Nir1, Nir2, and Nir3 coding sequences, essentially as described previously (30). Restriction enzyme analysis and DNA sequencing verified the DNA constructs. YFP-VSV-G construct was kindly provided by Koret Hirschberg (Tel-Aviv University, Israel). Antibody against Nir2 was raised in rabbits as described previously (32). Polyclonal antibody against VAP-B was raised in rabbits immunized with a recombinant GST-VAP-B fusion protein. The antisera was first run through a GST-bound agarose column, to remove the anti-GST antibodies. The flow-through was then affinity-purified on a GST-VAP-B-bound agarose column. Monoclonal antibody against β-tubulin was purchased from Sigma. Monoclonal and polyclonal antibodies against HA and Myc were purchased from Santa Cruz Biotechnology, Inc. Antibody against PDI was purchased from ABR (Affinity BioReagents). Alexa-488 donkey anti-mouse and anti-rabbit IgG were purchased from Molecular Probes. Cy3-conjugated goat anti-rabbit or goat anti-mouse IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA).

**Cell Culture, Transfections, and Indirect Immunofluorescence**—HEK293 and HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 mg/ml). The cells were transfected by the calcium phosphate method as described previously (33). HeLa cells grown on glass coverslips were transfected as indicated, fixed in 4% paraformaldehyde in PBS for 20 min at room temperature, and immunostained essentially as described previously (34). GST fusion proteins were expressed in bacteria and purified by standard procedures (Amersham Biosciences). For protein identification by mass spectrometry, 10% or 5% SDS, as indicated. The gradients were centrifuged at 100,000 g for 18 h at 4 °C, fractionated from the top to the bottom into 20 fractions of variable density, and analyzed by silver staining. The gradients were centrifuged at 100,000 g for 18 h at 4 °C, fractionated from the top to the bottom into 20 fractions of variable density, and analyzed by silver staining.
encoding Nir2-HA, VAP-B-Myc, or Nir2-HA and VAP-B-Myc. Following 5 h, the cells were shifted to 40 °C to accumulate YFP-VSV-G in the ER. The cells were then incubated at 32 °C for different time periods in the presence of cycloheximide (100 μg/ml) to permit the transport of VSV-G from the ER along the secretory pathway. The cells were then washed, scraped, and embedded in Karmovsky’s fixative (3% paraformaldehyde, 2% glutaraldehyde, 5 mM CaCl₂ in 0.1 M cacodylate buffer, pH 7.4 containing 0.1 M sucrose). The cells were then incubated at 32 °C for different time periods in the presence of cycloheximide (100 μg/ml) to permit the transport of VSV-G from the ER along the secretory pathway. The cells were then washed, scraped, and embedded in Karmovsky’s fixative (3% paraformaldehyde, 2% glutaraldehyde, 5 mM CaCl₂ in 0.1 M cacodylate buffer, pH 7.4 containing 0.1 M sucrose). The cells were then incubated at 32 °C for different time periods in the presence of cycloheximide (100 μg/ml) to permit the transport of VSV-G from the ER along the secretory pathway.

**RESULTS**

**Isolation of VAP-B as a Nir2-interacting Protein**—To gain a better insight into Nir2 cellular functions, we looked for Nir2-interacting proteins using coimmunoprecipitation assays. Control and Nir2-HA-transfected HeLa cells were metabolically labeled with [35S]methionine, lysed, and immunoprecipitated with anti-HA antibody. Several proteins were precipitated with the Nir2-HA is marked by an arrow. B, HeLa cells that express either the wild-type Nir2-HA, the indicated Nir2-HA-truncated mutants, or control non-transfected cells were metabolically labeled, lysed, and immunoprecipitated as described above. The 31 kDa protein was not detected in the control or the immunocomplex of a mutant lacking the first 440 amino acids of Nir2. C, GST or GST-Nir2-(205–424) fusion protein immobilized on glutathione-agarose beads were incubated with [35S]methionine-labeled HeLa cell lysate. The samples were extensively washed, resolved by SDS-PAGE, and subjected to autoradiography. D, HEK293 cells were transiently transfected with an expression vector encoding VAP-B-Myc. The cell lysate was incubated with GST or GST-Nir2-(205–424) bound to glutathione-agarose beads. Following washing, the samples were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-Myc antibody.

**Fig. 1. Isolation and identification of a 31 kDa protein that interacts with Nir2.** A, HeLa cells expressing wild-type Nir2-HA and control non-transfected cells were metabolically labeled with [35S]methionine, lysed, and subjected to immunoprecipitation with anti-HA antibody. The samples were separated by 10% SDS-PAGE, and then subjected to autoradiography. The 31 kDa protein that immunoprecipitated with Nir2-HA is marked by an arrow. B, HeLa cells that express either the wild-type Nir2-HA, the indicated Nir2-HA-truncated mutants, or control non-transfected cells were metabolically labeled, lysed, and immunoprecipitated as described above. The 31 kDa protein was not detected in the control or the immunocomplex of a mutant lacking the first 440 amino acids of Nir2. C, GST or GST-Nir2-(205–424) fusion protein immobilized on glutathione-agarose beads were incubated with [35S]methionine-labeled HeLa cell lysate. The samples were extensively washed, resolved by SDS-PAGE, and subjected to autoradiography. D, HEK293 cells were transiently transfected with an expression vector encoding VAP-B-Myc. The cell lysate was incubated with GST or GST-Nir2-(205–424) bound to glutathione-agarose beads. Following washing, the samples were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-Myc antibody.
**Nirs Interact with VAP-B via Their Conserved FFAT Motif**—Recently, the yeast homolog of VAP-B, Scs2p was shown to interact with Opi1p, through a conserved EFFDA X E motif designated FFAT (35). This motif is also found in the Nir2 protein at amino acids 349–355 and is highly conserved among the other Nir/rdgB family members. The presence of this motif within the region that mediates the interaction with the 31 kDa protein (amino acids 341 to 439; Fig. 1B) further supports our results and strongly suggests that the 31 kDa protein is indeed the VAP-B protein. To determine whether the interaction of Nir2 with VAP-B is mediated through its FFAT motif, the conserved EFFDA sequence was replaced by ALLAG. This mutagenesis completely abolished the interaction of Nir2 with VAP-B (Fig. 2A), indicating that the interaction of Nir2 with VAP-B is mediated by its FFAT motif. To demonstrate the interaction between endogenous VAP-B and Nir2 proteins, we raised a polyclonal antibody against VAP-B as described under “Experimental Procedures,” and used in immunoprecipitation or immunoblotting of lysate prepared from HEK293 cells expressing either the VAP-B-Myc or the Plk1-Myc protein, as indicated. Endogenous VAP-B was immunoprecipitated from HeLa cells (right panel) using either preimmune (PI) or VAP-B immune serum (I). The samples were resolved by SDS-PAGE, and immunoblotted with the indicated antibodies. C, immunoprecipitation of endogenous VAP-B and Nir2 proteins. HeLa cell lysate was subjected to immunoprecipitation following immunoblotting by the indicated antibodies. Preimmune (PI) and immune (I) serum.

**The Interaction of VAP-B with Nir2 Affects the ER Structure**—The VAP-B protein was previously localized to the ER and to pre-Golgi intermediates by biochemical and immunocytochemical methods (19). We have previously shown that Nir2 mainly localizes in the Golgi apparatus, but it is also found in the ER in interphase cells (36). To determine the localization of VAP-B in HeLa cells, we used the anti-VAP-B antibody in

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**Fig. 2.** The three Nir proteins interact with VAP-B. A, Nir2 interacts with VAP-B via its FFAT motif. HEK293 cells were either transfected with expression vectors encoding Nir2-HA or VAP-B-Myc or cotransfected with VAP-B-Myc and wild-type Nir2-HA, or its FFAT mutant (FM). The cells were lysed and subjected to immunoprecipitations followed by immunoblotting with the indicated antibodies. The expression level of the proteins was determined by Western blotting of cell lysate (10% of total) using the indicated antibodies. B, specificity of the anti-VAP-B antibody. Antibody against VAP-B was raised in rabbits as described under “Experimental Procedures,” and used in immunoprecipitation or immunoblotting of lysate prepared from HEK293 cells expressing either the VAP-B-Myc or the Plk1-Myc protein, as indicated. Endogenous VAP-B was immunoprecipitated from HeLa cells (right panel) using either preimmune (PI) or VAP-B immune serum (I). The samples were resolved by SDS-PAGE, and immunoblotted with the indicated antibodies. C, immunoprecipitation of endogenous VAP-B and Nir2 proteins. HeLa cell lysate was subjected to immunoprecipitation following immunoblotting by the indicated antibodies. Preimmune (PI) and immune (I) serum. D, structure of Nir and VAP-B proteins. The three Nir proteins contain a conserved C-terminal region of ~300 amino acids, a DDHD domain, six hydrophobic stretches that are marked by vertical lines, and an acidic region that consists of the FFAT motif, EFFDAXE. Nir2 and Nir3 contain an N-terminal PI transfer domain. VAP-B consists of a large cytoplasmic N-terminal region, a TMD, and a short luminal C-terminal tail of 4 amino acids. The cytoplasmic region consists of an N-terminal domain of 100 amino acids that shares high sequence homology with MSP (white) and within which is a very conserved domain (VCD) of 16 amino acids, and a coiled-coil domain (CCD). E, three Nir proteins; Nir1, Nir2, and Nir3 interact with VAP-B. HEK293 cells were either transfected with an expression vector encoding VAP-B-Myc or cotransfected with VAP-B-Myc and Nir1-HA, Nir2-HA, or Nir3-HA. The three Nir proteins were immunoprecipitated by anti-HA antibody, and their association with VAP-B was determined by immunoblotting with anti-Myc antibody, while VAP-B was immunoprecipitated with anti-Myc antibody and its association with Nirs was determined by immunoblotting with anti-HA antibody. The expression level of the proteins was determined by Western blotting of cell lysate (10% of total) using the specified antibodies.
indirect immunofluorescence and confocal microscopy analyses. The VAP-B protein exhibits a typical ER reticular localization. Similar localization was observed with the transfected VAP-B-Myc protein, and double immunostaining with antibody against protein-disulfide isomerase (PDI), an ER marker, revealed their strong colocalization (Fig. 3A), suggesting that endogenous and transfected VAP-B proteins are localized in the ER.

To gain a better understanding of Nir-VAP-B interactions, we coexpressed them together in HeLa cells and examined their subcellular localization using indirect immunofluorescence and confocal microscopy analyses. Overexpression of wild-type Nir2 with VAP-B caused to production of large heterogeneous granular structures that were dispersed around the nucleus throughout the cytosol, in which Nir2 and VAP-B were strongly colocalized. These structures were not detected in cells that express Nir2 alone (data not shown), or coexpressed the FFAT mutant of Nir2 and VAP-B (Fig. 3B). These results suggest that the interaction of Nir2 with VAP-B, and not simply their overexpression, induces the formation of these unusual structures. To determine the origin of these structures, we used several organelle-specific markers and analyzed their localization by confocal microscopy. As shown in Fig. 3B, the ER-resident protein PDI was strongly localized to these structures, whereas neither the Golgi markers, nor the endosomal or lysosomal markers were detected in these structures (data not shown). These results suggest that these structures were formed from the ER, and that Nir2-VAP-B interaction rearranges the normal structure of the ER. Furthermore, coexpression of Nir2 and VAP-B had no detectable effect on the cis, medial, or trans Golgi morphology, on COPI vesicles, on early or late endosomes, or on lysosomes, as determined by localization of their respective markers; p58, NAGT-I, sialyltransferase, β-COP, EEA1, rab7, and LAMP1 (data not shown). Thus, coexpression of Nir2 and VAP-B specifically modifies the ER structure.

To better characterize the effect of the Nir2-VAP-B interaction on ER structure, we used transmission electron microscopy (EM) analysis. The EM images shown in Fig. 4, demonstrate the remarkable reorganization of the ER, which is
characterized by various structures consisting multiple membrane arrays that were organized in diverse forms. Some of them emerged from the NE, while others appeared in peripheral locations, consistent with our confocal microscopy analysis. Sometimes they were visualized as circular packed cisternae, or linear stacks of cisternae located far from the cell nucleus. Membrane wheels, or closed or partially opened loops were also observed (data not shown). Similar structures have been previously obtained by overexpression of several integral ER membrane proteins, including HMG-CoA (9), msALDH (7), and cytochrome b₅ (37), among others.

**VAP-B Undergoes Oligomerization in Mammalian Cells**—The mechanism underlying the formation of stacked ER membrane arrays is not completely understood. However, several lines of evidence suggest that oligomerization of the membrane protein is critical to this process. Accordingly, interaction between the cytoplasmic domains of proteins on apposing ER membranes causes stacking of the ER cisternae (7, 13, 14, 38). Other studies have suggested that oligomerization of the ER membrane protein is insufficient for the induction of alterations in membrane assembly; rather, proper folding of the TMD is the critical induction factor (10, 37). We therefore assessed the oligomerization state of VAP-B in mammalian cells. VAP-B-Mye was expressed in HEK293 cells, and its oligomerization state was analyzed following treatment with DSP, a membrane-permeable reducible cross-linker. The results (Fig. 5A) indicated that VAP-B undergoes oligomerization in mammalian cells. A clear band of ~63 kDa, probably representing a dimer, was detected, whereas a band with the expected migration for a tetramer, at ~120 kDa, was less abundant. Furthermore, deletion of the first 63 amino acids of VAP-B had no effect on its oligomerization, as demonstrated by the same experimental approach (data not shown). These results suggest that VAP-B undergoes self-oligomerization in mammalian cells, and that the TMD is probably critical for its oligomerization. We further confirmed this result by sucrose density gradient analysis (Fig. 5B). In this set of experiments, we expressed either VAP-B or Nir2 alone, or coexpressed VAP-B with wild-type Nir2 or its FFAT mutant in HEK293 cells. The cells were lysed as described under “Experimental Procedures” and layered onto a continuous sucrose density gradient in the presence or absence of 1% SDS. Following sedimentation, fractions were collected and analyzed by Western blotting. Protein markers, including thryoglobulin (669 kDa), catalase (232 kDa), and bovine serum albumin (67 kDa), were separated on a parallel sucrose gradient, fractionated under the same conditions, and detected by SDS-PAGE and Coomassie Blue staining. A logarithmic plot of the molecular mass of the marker proteins as a function of gradient fraction indicated that VAP-B sediments as expected for a dimer, consistent with the data shown in Fig. 5A. The peak intensity of the VAP-B protein under native conditions was found in fraction 5, which has an estimated molecular mass of ~63 kDa, while its peak under denaturing conditions (1% SDS) was shifted to fraction 3. Nir2 was detected in fractions 6 through 12, with a peak in fraction 10 corresponding to ~153 kDa. Coexpression of VAP-B and the FFAT mutant of Nir2 had no effect on the sedimentation profile of either VAP-B or Nir2, consistent with these proteins’ inability to interact with one another. However, coexpression of wild-type Nir2 and VAP-B induced a dramatic shift in VAP-B sedimentation. The peaks of VAP-B and Nir2 intensities appeared in fraction 12, corresponding to ~220 kDa, and continued through to fraction 16 (~440 kDa). Collectively, these results suggest that by itself, VAP-B is a dimer, and that its interaction with Nir2 creates complexes of various sizes that may represent different oligomerization forms. Accordingly, we suggest (Fig. 5C) that VAP-B undergoes dimerization in mammalian cells, probably mediated by the GXXXY motif present in its TMD (20, 27). The binding of Nir2 to VAP-B induces a conformational change in the VAP-B cytoplasmic domain and facilitates its trans-oligomerization. This trans-oligomerization is mediated by a head-to-head interaction of VAP-B cytoplasmic domains on apposing ER membranes, which zips up the apposing membrane, yielding stacked ER cisternae. Alternatively, a head-to-head interaction of Nir2 molecules could bridge VAP-B proteins on apposing ER membranes and induce their stacking. The sedimentation profile of Nir2 alone (Fig. 5B), which demonstrates the formation of larger Nir2 structures, may support this possibility. Both of these possibilities rely on head-to-head interactions, involving either VAP-B or Nir2 molecules. However, it could be that the binding of Nir2 to VAP-B modifies the folding of VAP-B TMD, which would then transmit a signal for the production of stacked membrane arrays (Fig. 5C, option 3).

**Coexpression of VAP-B and Nir2 Attenuates Protein Export from the ER**—Next, we assessed whether these structures have any effect on protein export from the ER to the Golgi apparatus and subsequently to the plasma membrane. For this purpose, HeLa cells were cotransfected with either VAP-B and a temperature sensitive mutant of vesicular stomatitis virus-glycoprotein (ts045 VSV-G) fused to YFP, or with Nir2, VAP-B, and YFP-VSV-G. At 40 °C the VSV-G is synthesized and retained in the ER because of its misfolding (39). However, upon shifting...
the temperature to 32 °C, the VSV-G protein folds and is transported to the Golgi apparatus and then to the cell surface within 20–30 min and 60–90 min, respectively. The results shown in Fig. 6, clearly demonstrate the accumulation of VSV-G in the granular structures at 40 °C (time 0) in cells coexpressing Nir2 and VAP-B, consistent with the localization of PDI in these structures and their ER origin. However, 30 min after shifting the temperature to 32 °C, VSV-G was mainly localized in the Golgi apparatus in the control non-transfected, Nir2-transfected (data not shown), or the VAP-B-transfected cells, and at 90 min was mainly at the plasma membrane. In contrast, in cells coexpressing Nir2 and VAP-B, VSV-G was retained in the granular structures even 1 h following shifting the temperature to 32 °C, suggesting that these structures inhibit the export of VSV-G from the ER. The accumulation of VSV-G in these structures was not caused by a continuous synthesis of VSV-G protein, because the experiment was performed in the presence of cycloheximide.

Ectopic Expression of Nir3 and VAP-B Induces Microtubule Bundling along the ER Membranes—Since Nir1 and Nir3 also interact with VAP-B, we assumed that their coexpression with VAP-B would affect the ER structure in a similar manner to that of Nir2. We therefore coexpressed them with VAP-B and examined their localization by immunofluorescence and confocal microscopy analyses. Coexpression of Nir1 with VAP-B had no apparent effect on either Nir1 or VAP-B localizations (Fig. 7A). In contrast, coexpression of Nir3 and VAP-B strikingly changed the typical localization of VAP-B. It was visualized as thick bundles surrounding the nucleus that were extended peripherally throughout the cytosol, to which Nir3 was colocalized (Fig. 7B). To determine whether coexpression of Nir3 and VAP-B-Myc has any effect on the ER structure, HeLa cells that coexpress them were double immunostained with anti-PDI and anti-Myc antibodies and their localization was analyzed by confocal microscopy. The results shown in Fig. 7C demonstrate that PDI staining was strikingly different in cells that coexpress VAP-B and Nir3 as compared with the control non-transfected HeLa cells (Fig. 3A). In the cotransfected cells, PDI immunostaining appeared in tubular-like structures that were colocalized with VAP-B. Thus, in contrast to Nir1 and Nir2, coexpression of Nir3 with VAP-B modified the ER structure into a tubular pattern. A similar pattern has been previously obtained when the integral ER-membrane protein p63 was overexpressed in COS cells (16). Its overexpression caused to rearrangement of the ER and concomitantly bundling of microtubules along the altered ER membranes. This similarity led us to examine the morphology of the microtubules in cells that coexpress Nir3 and VAP-B compared with their morphology in cells that either express Nir3 or VAP-B alone. As shown in Fig. 7D, coexpression of Nir3 and VAP-B dramatically modified the organization of the microtubules; they were unusually thick and not organized, the microtubule organization center was not visible, and, more importantly, they aligned with VAP-B or Nir3 immunostaining. These results suggest that the VAP-B-Nir3 interaction links the ER membranes to the microtubule network, and thereby modifies both the ER and microtubule organization. It is noteworthy that coexpression of Nir3 and VAP-B caused to partial dispersal of the Golgi (data not shown), consistent with the role of microtubules in positioning and maintenance of the Golgi apparatus (1).

The mouse and the Drosophila VAP-A proteins have been previously shown to directly interact with microtubules, yet, through an unidentified motif (20, 24). Since the Drosophila VAP-A (DVAP-33A) was proposed to stabilize microtubules, we
examined the effect of nocodazole treatment on the microtubule architecture in cells that overexpress VAP-B or coexpress VAP-B and Nir3. As shown in Fig. 7E, treatment with nocodazole for 3 h caused to complete depolymerization of microtubules in the VAP-B overexpressing or in the non-transfected control cells. However, in cells that coexpress VAP-B and Nir3, some microtubules were still detected following such long treatment with nocodazole, suggesting that Nir3-VAP-B interaction enhances the microtubule stability. These results are consistent with the proposed function of DVAP-33A in stabilizing microtubules (24).

**DISCUSSION**

Nir1, Nir2, and Nir3 belong to a highly conserved family of proteins, the Nir/rdgB, which have been implicated in regulation of phospholipid trafficking, metabolism, and signaling. Nir2 and Nir3 contain an N-terminal phosphatidylinositol (PI)-transfer domain, followed by a short acidic region, six hydrophobic stretches, and a long highly conserved C-terminal domain (Fig. 2D). The N-terminal PI-transfer domain is not present in Nir1 or the zebrafish pl-RdgB (31, 40), but all the other family members, including *Drosophila* retinal degeneration B (rdgB), have a functional PI-transfer domain that has the ability to transfer PI and phosphatidylcholine (PC) between membrane bilayers in vitro (41).

In this study, we show that the three Nir proteins interact with VAP-B through their conserved FFAT motif (Fig. 2), which is present within their acidic region. This motif consists of a conserved EFFDAXE sequence, which acts as an ER-targeting determinant by its direct interaction with VAP proteins (35). The FFAT motif has been identified in 17 distinct eukaryotic proteins, 14 of which are directly implicated in lipid binding or lipid sensing, including homologs of OSBP, homologs of Goodpasture’s antigen-binding protein (GBBP), the Nir/rdgB proteins, and Opi1p, a transcriptional regulator of phospholipid synthesis in yeast. The FFAT motif in Opi1p mediates the interaction of Opi1p with Ssc2p and thereby targets it to the ER. A similar targeting mechanism has been shown for the yeast homologs of OSBP, Osh1p, Osh2p, and Osh3p (35). In mammalian cells, OSBP also interacts with VAP-A protein (28), probably through its FFAT motif. However, in this particular case, the binding affinity of OSBP to VAP-A is largely dependent on its pleckstrin homology (PH) domain. A specific mutation in this domain, W174A, enhances its binding to VAP-A, and overexpression of this mutant in mammalian cells causes the production of ER inclusions, in which OSBP and VAP-A are colocalized (28). Very similar structures were obtained upon overexpression of Nir2 and VAP-B (Fig. 3B), suggesting that the interaction of FFAT-motif-containing proteins with VAPs can induce the formation of these unusual ER structures. However, our results indicate that these structures are formed in a specific manner, which is largely dependent on the VAP-interacting protein, as neither Nir1 nor Nir3 induced their formation (Fig. 7), despite their interaction with VAP-B protein (Fig. 2E). Electron microscopy analysis revealed that these structures consist of multiple membrane arrays that are organized in diverse forms. The structures were found either emerging from the NE or peripherally throughout the cytosol (Fig. 4). Similar structures have been previously obtained in different cell types by overexpressing HMG-CoA reductase, an integral membrane protein that catalyzes the rate-limiting step in cholesterol biosynthesis (42). Detailed analysis using different mutants or chimeric proteins of HMG-CoA reductase suggests that its catalytic activity is not required for stimulation of membrane proliferation; rather, its membrane domain appears to be both necessary and sufficient for the induction of these structures (9, 43). Subsequent studies using other transmembrane proteins led to the hypothesis that the proper folding of the transmembrane domain is critical for their formation, and that this folding is required for the transmission of a signal for membrane biogenesis and the production of stacked membrane arrays (5, 37). In contrast, other studies proposed that homodimeric interactions between cytoplasmic domains of ER-resident proteins consisting of TMDs is sufficient for generating these structures (14). Accordingly, head-to-head interaction between the cytoplasmic domains of the integral membrane protein on apposing ER membranes zips

![Fig. 6. Attenuation of YFP-VSV-G export from the ER of cells coexpressing the Nir2 and VAP-B proteins.](http://www.jbc.org/)

| Time  | YFP-VSV-G | VAP-B |
|-------|-----------|-------|
| 0 min |           |       |
| 15 min|           |       |
| 30 min|           |       |
| 60 min|           |       |
| 90 min|           |       |

Shown are confocal images of YFP-VSV-G (ts045) export from the ER of either VAP-B-expressing cells (upper panels) or VAP-B and Nir2-coexpressing cells (lower panels). The cells were fixed, immunostained with anti-Myc antibody, and analyzed by confocal microscope at the indicated time points following shifting the temperature from 40 to 32 °C. The localization of YFP-VSV-G in VAP-B-expressing cells is shown in the upper panels. The localization of YFP-VSV-G in the Nir2/VAP-B-coexpressing cells is shown in the middle panels, along with the localization of VAP-B in the same cells (lower panels). Bar, 10 μm.
the apposing membrane, thereby stacking the ER cisternae (7, 13, 14). This hypothesis prompted us to characterize the oligomerization state of VAP-B in mammalian cells (Fig. 5).

Our finding that VAP-B undergoes dimerization in mammalian cells (Fig. 5) is consistent with previous studies performed in yeast and in vitro (27–29). According to the yeast two-hybrid analysis (28), truncated VAP-A mutants lacking either amino acids 41–59, the TMD, or the last 84 amino acids (amino acids 1–160) interact with wild-type VAP-A but fail to interact with OSBP, suggesting that the C-terminal domain, including the TMD, is not required for VAP-A self-oligomerization. On the other hand, Weir et al. (29) showed that both the N- and

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**Fig. 7. Nir3-VAP-B interaction leads to rearrangement of the ER and the microtubule network.**

A. Coexpression of Nir1 and VAP-B has no effect on VAP-B localization. Shown are confocal images of HeLa cells coexpressing Nir1 (red) and VAP-B (green), along with the merged image. Bar, 10 µm. B. Nir3 changes the localization of VAP-B protein. HeLa cells expressing Nir3-HA (left panel), or coexpressing VAP-B-Myc and Nir3-HA, were fixed and immunostained with antibodies against the corresponding proteins as indicated. Bar, 10 µm. C. Structure of the ER in Nir3/VAP-B-expressing cells. Shown are confocal images of HeLa cells coexpressing Nir3-HA and VAP-B-Myc double-immunostained with anti-Myc and PDI antibodies. Bar, 10 µm. D. Coexpression of Nir3 and VAP-B affects the organization of the microtubules. HeLa cells expressing VAP-B-Myc alone (upper panels) or coexpressing VAP-B-Myc and Nir3-HA (middle and lower panels) were double-immunostained with anti-β-tubulin and either anti-Myc or anti-HA. Colocalization appears in yellow. Bar, 10 µm. E. Effect of nocodazole treatment on depolymerization of the microtubules in VAP-B- (lower panels) and VAP-B/Nir3- (upper panels) expressing cells. The cells were incubated for 3 h in the presence of nocodazole (5 µM), fixed, and double-immunostained for β-tubulin and VAP-B-Myc. Transfected cells are labeled with an arrow, non-transfected control cells with an arrowhead. Bar, 10 µm.
C-terminal domains of VAP-A are required for its oligomerization, and Nishimura et al. (27) suggested that the TMD is critical for both hom- and hetero-oligomerization of VAP-A and VAP-B proteins in vitro. Indeed, the TMD of VAP-A/B contains a GXXGX motif, which has been identified in a number of TMDs and was proposed to induce strong self-assembly (44). Thus, it could be that several structural domains, including the TMD, mediate the oligomerization of VAP-A/B. We show here that VAP-B expression is insufficient to produce stacks of ER membrane arrays unless Nir2 is coexpressed (Fig. 3). We also show that VAP-B is found mainly as a dimer in mammalian cells, and that its interaction with Nir2 induces the formation of oligomers of various sizes (Fig. 5). These results suggest that the binding of Nir2 to VAP-B induces a conformational change in the VAP-B protein, which either facilitates the trans-oligomerization of VAP-B proteins on apposing ER membranes through interaction of their cytoplasmic domains, or induces specific folding of VAP-B TMD that transmits a signal for membrane assembly (Fig. 5C). Although the first possibility is consistent with the results of the yeast two-hybrid interaction assays suggesting that the cytoplasmic region of VAP-A is involved in VAP oligomerization, at present we cannot exclude the second possibility. We also cannot exclude the possibility that a head-to-head interaction of Nir2 molecules bridges VAP-B proteins on apposing ER membranes, allowing them to stack together (Fig. 5C). While this is a reasonable possibility in light of the sedimentation profile of Nir2 (Fig. 5B), our preliminary studies indicate that overexpression of a truncated VAP-B protein lacking the highly conserved 16 amino acids within the N-terminal region induces the formation of stacked ER membrane arrays (data not shown). These results suggest that these structures are formed by conformational changes in the VAP-B protein.

In contrast to Nir2, coexpression of Nir1 with VAP-B had no effect on ER structure (Fig. 7A). Since Nir1 lacks the PI-transfer domain, we assumed that this domain is required for induction of the unusual morphology of the ER seen in Nir2/VAP-B-expressing cells. However, deletion of most of the PI-transfer domain (amino acids 1–240), or even its flanking region (1–340), did not abolish the effect of Nir2/VAP-B interaction on the ER structure (data not shown). These results suggest that despite the high sequence similarity between the three Nir proteins, and their ability to interact with VAP-B via their FFAT motif (Fig. 2E), they distinctly affect the ER structure in the presence of VAP-B protein. Indeed, coexpression of Nir3 with VAP-B caused a striking rearrangement of the ER and concomitantly, bundling of microtubules along the altered ER membranes, to which Nir3/VAP-B-expressing cells are more resistant to nocodazole treatment than the non-transfected control or VAP-B-transfected cells (Fig. 7E), suggesting that this interaction stabilizes the microtubules, and is consistent with the proposed function of DVAP-33A.

Although, our results were obtained through overexpression of VAP-B and the different Nir proteins, they imply that VAP proteins play essential roles in the regulation of ER structure. Thus, VAPs can be considered ER-receptors for many FFAT-containing proteins, which are differentially expressed in various tissue and cell types, and might be differentially regulated under specific physiological conditions. These receptors may undergo different conformational changes upon binding of their cognate ligands, and consequently, distinctly affect the ER structure. Thus, our results suggest that the interaction of VAPs with FFAT-containing proteins is not only required for targeting of FFAT-containing proteins to the ER, but is also involved in the regulation of ER organization and positioning.

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