The Nicastrin-like Protein Nicalin Regulates Assembly and Stability of the Nicalin-Nodal Modulator (NOMO) Membrane Protein Complex*\(^5\)

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The assembly of the \(\gamma\)-secretase complex, an Alzheimer disease-related protease required for \(\beta\)-amyloid generation, is tightly regulated and predominantly limited by the stoichiometric availability of its components. We have identified a novel endoplasmic reticulum-located protein complex that is regulated in a similar fashion. It contains the recently identified Nodal signaling antagonists Nicalin (a distant homolog of the \(\gamma\)-secretase component Nicastrin) and NOMO (Nodal modulator). Using an RNA interference approach, we found that Nicalin and NOMO became unstable in the absence of the respective binding partner, suggesting that complex formation has a stabilizing effect. Overexpression of Nicalin resulted in an increase in NOMO, whereas endogenous Nicalin was reduced below the detection limit. Both effects were shown to occur at a post-transcriptional level. Thus, NOMO is most likely produced in excess amounts and either stabilized by Nicalin or rapidly degraded. In contrast, Nicalin levels are limited independently of NOMO. We, therefore, propose that Nicalin controls the assembly and stability of the Nicalin-NOMO complex.

Membrane protein complexes are involved in a wide variety of cellular functions, including energy generation through the respiratory chain at the inner mitochondrial membrane, protein translocation across the mitochondrial and endoplasmic reticulum (ER)\(^2\) membranes, and ion transport as well as signal transduction across various membranes. An extensively studied example is \(\gamma\)-secretase, a membrane protein complex with an unusual proteolytic activity (1). It mediates the intramembrane cleavage of various type I proteins, including the \(\beta\)-amyloid precursor protein (2). The \(\gamma\)-secretase complex consists of the four membrane proteins Npsenilin, Nicastrin, APH-1 (anterior pharynx defective-1), and PEN-2 (presenilin enhancer-2), with Presenilin representing the catalytically active component (3, 4). Nicastrin was identified as a Presenilin-interacting protein (5) and subsequently shown to be involved in the assembly of the \(\gamma\)-secretase complex (6–8) as well as in the selection of substrates via its aminopeptidase-like domain (9). We have recently identified Nicalin, a Nicastrin-like protein that also contains an aminopeptidase-like domain (10). Similar to Nicastrin, point mutations at critical positions within this domain exclude a proteolytic activity. Nicalin was found to be part of a high-molecular-weight membrane protein complex unrelated to \(\gamma\)-secretase (10, 11). No interactions between Nicalin and \(\gamma\)-secretase components could be observed, and \(\textit{in vivo}\) binding studies identified a novel \(~130\)-kDa membrane protein, which we termed NOMO (Nodal modulator), as a major Nicalin-binding partner. A functional analysis of Nicalin and NOMO in the zebrafish model system using gain-of-function and loss-of-function approaches resulted in phenotypes similar to those described for Nodal signaling mutants (10). Nodals are secreted ligands of the transforming growth factor-\(\beta\) superfamily required very early in vertebrate embryogenesis for the development of the three germ layers and the formation of the major body axes (12). Ectopic expression of Nicalin and NOMO resulted in partial cyclopia, a phenotype caused by a reduction in Nodal signaling (13). Moreover, a knockdown of NOMO led to defects typical for enhanced Nodal activity. Finally, a block in Nodal signaling induced by the established Nodal inhibitor Lefty could be reversed by the down-regulation of NOMO, demonstrating a genetic interaction between these two proteins. We, therefore, concluded that Nicalin and NOMO act as antagonists of the Nodal signaling pathway (10).

We have now analyzed Nicalin and NOMO biochemically and show that they are part of an endoplasmic reticulum-located membrane protein complex with a size of \(200–220\) kDa. Assembly of the complex results in the stabilization of Nicalin and NOMO and represents a major mechanism in controlling their expression levels. Whereas Nicalin is sufficient for NOMO stabilization, its own expression is limited independently of NOMO. We, therefore, propose that Nicalin is the rate-limiting factor for the Nicalin-NOMO complex.

**Experimental Procedures**

**Antibodies**—The rabbit polyclonal anti-Nicalin antibody Ncl-2221 has been described previously (10) and was used 1:1,500 for immunoblotting. The rabbit polyclonal anti-
NOMO antibody NOMO-900 was generated against amino acids 526–799 of human NOMO2 fused to maltose-binding protein (MBP-NOMO-900). The obtained antisera (Immuno-globe, Himmelstadt) was affinity-purified by high pressure liquid chromatography using N-hydroxysuccinimide-activated HiTrap columns (Amersham Biosciences) coupled with MBP-NOMO-900 according to the manufacturer’s instructions and used 1:1000 for immunoblotting. The following other antibodies were used: monoclonal anti-Myc (9E10; American Type Culture Collection (ATCC), Manassa, VA), polyclonal anti-Nicastrin (N1660, Sigma), and polyclonal anti-calnexin (Stressgen).

Cell Culture—Human embryonic kidney 293T (HEK293T) and T-REx-293 (293TR) cells (Invitrogen), human cervix HeLa cells, and SH-SY5Y neuroblastoma cells were cultured in Dulbecco’s modified Eagle’s medium with Glutamax (Invitrogen), human liver HepG2 cells and human breast MCF-7 cells were cultured in Eagle’s minimum essential medium (ATCC) supplemented with 2 mM L-glutamine; human epidermoid A431 cells, and human pancreatic PANC-1 cells (obtained from M. Dauer and A. Eigler) were cultured in advanced RPMI 1640 medium (Invitrogen) supplemented with 2 mM L-glutamine. All media were supplemented with 10% fetal calf serum (Invitrogen) and penicillin/streptomycin. Transfections were carried out using Lipofectamine 2000 (Invitrogen), and stable cell clones were selected using either zeocin (200 μg/ml) or blasticidin (10 μg/ml). The expression of Nicalin or NOMO in T-REx-293 cells was induced with 1 μg/ml tetracycline. Cycloheximide treatment of HEK293T cells was performed at a final concentration of 100 μg/ml.

Glycosylation Analysis and Polyacrylamide Gel Electrophoresis—Membrane protein lysates were prepared by hypo-tonically lysing cells in 50 mM sodium citrate, pH 6.4, 1 mM EDTA, 1× protease inhibitor mix (Sigma), pelleting cell membranes from the post-nuclear supernatant fraction for 30 min at 16,000 × g and solubilizing them in 50 mM Tris, pH 7.6, 150 mM NaCl, 2 mM EDTA containing 1% Nonidet P-40 and 1× protease inhibitor mix. After pelleting the insoluble components for 10 min at 16,000 × g, the protein concentrations were determined using the Bio-Rad protein assay. Deglycosylation experiments were carried out on membrane protein lysates (~25 μg) containing 0.1% SDS using 1 unit of N-glycosidase F or 10 μl milliliters of endoglycosidase H (Roche Applied Science) for 16 h at 37 °C. SDS and blue native-PAGE as well as immunoblotting were performed as described previously (8). Quantification of the immunoblots signals was carried out using the FluorChem system (AlphaInnotech).

Subcellular Fractionation—Cells from four 10-cm dishes were harvested in homogenization buffer (10 mM HEPES, pH 7.4, 1 mM EDTA, 0.25 M saccharose, 1× protease inhibitor mix (Sigma)) and lysed by 10 strokes in a Dounce homogenizer followed by passage 10 times through a 23-gauge needle. After preparation of a post-nuclear supernatant, cell membranes were pelleted at 100,000 × g for 1 h, resuspended in homogenization buffer, loaded onto a 2.5–30% discontinuous Optiprep (Sigma) gradient, and run for 2.5 h at 200,000 × g. 1-ml fractions were collected, and 25-μl aliquots loaded onto an SDS gel.

Immunofluorescence Microscopy—Cells were grown on poly-l-lysine-coated coverslips, fixed 20 min with phosphate-buffered saline/3.7% paraformaldehyde, permeabilized 20 min with phosphate-buffered saline/0.2% Triton X-100, and blocked 1 h with phosphate-buffered saline/1% bovine serum albumin. Antibody incubations were performed for 1 h at room temperature in phosphate-buffered saline using a humid chamber. For detection, Alexa 488- and Alexa 594-coupled secondary antibodies (Molecular Probes) were used, and after mounting the samples in Mowiol (Hoechst), they were analyzed with a Zeiss Axioscop fluorescent microscope.

RNA Interference—For the construction of plasmids expressing Nicalin- and NOMO-specific short hairpin RNAs, the following oligonucleotides were used: N2a, 5'-AGTCGGAGTCTAGATCTTACTTCGACGAC-3'; NOMO-2s, 5'-ATCACTTCAAGAGTTGATGACTTGACCAGTTTCTTCGCA-3'; Ncl-1531s, 5'-GAAAAAGCAATGTAGATGATCGTGACTCTCCCTGAGCTCATCAG-3'; Ncl-1531as, 5'-GCAAAGTGAATGCGTAGTTAGCAGAGTAGCATTCCATCGTTTCTCGA-3' (pur- chased from Thermo Electron, Ulm). After denaturation, oligonucleotides were annealed in 60 μM Tris–HCl, pH 7.5, 500 mM NaCl, 60 mM MgCl2, and 10 μM dithiothreitol for 15 min at 37 °C and subcloned into the BSENU6 vector (14) using Pmel and Pstl restriction sites. Knockdown efficiency was evaluated by co-transfection of HEK293T cells with the shRNA-expressing BSENU6 vectors and the psiCHECK2 plasmid (Promega) either expressing a luciferase-Nicalin or luciferase-NOMO fusion mRNA and measuring luciferase activity. Stable cell clones were generated by co-transfection of the short hairpin RNA-encoding BSENU6 vector with pcDNA4/TO/Myc-His and selection with zeocin (Invitrogen). For the generation of the RNA interference (RNAi)-resistant Nicalin rescue construct, silent mutations were introduced into the Nicalin cDNA by PCR using the partially overlapping primers NclRNAi-mutF (5'-AGCAGGTATGAACTGCTTATAGAGTCAACGC-3') and NclRNAi-mutR (5'-CTATAGGCGTACTCAGAGAATAGCGTACTATTTCGGAG-3). Full-length Nicalin containing the mutations was subcloned into the pcDNA6 vector (Invitro-gen) which provides a blasticidin resistance. A NOMO rescue construct was generated in an analogous manner using the primer NOMO-mutF (5'-AGTCGGAGTCTAGATCTTACTTCGACGAC-3') and NOMO-mutR (5'-CTCAGAACTATTTCGAGCAGCCTTCTC-3'). Real-time PCR—Total RNA from HEK293T cells and stably transfected cell lines were isolated using the RNaseq Kit (Qiagen), and 4 μg were used for the synthesis of cDNA using oligo(dT) primers and the SuperScript first-strand synthesis system (Invitrogen). 1/50 of the cDNA was used for a SYBR Green (Bio-Rad) real-time PCR reaction (40 cycles with 15 s at 95 °C and 60 s at 65 °C) on a Bio-Rad IQ5 machine using the following primers: for total Nicalin, 5'-ACCACCTTGGACGCCTGTACGGAGAAGAGGAGAGT-3'; for total NOMO, 5'-AGCGGCTGAGCCAGGCTTCTCTAGT-3'; and 5'-ACATCCGGCAAGAATCAC-3'. Reactions were per-
formed in triplets, and the obtained C<sub>T</sub> values normalized to β-actin expression by the comparative C<sub>T</sub> method.

RESULTS

Nicalin and NOMO Are Components of a 200–220-kDa Membrane Protein Complex—The human genome contains three NOMO genes, NOMO1, NOMO2, and NOMO3, which are located as tandem repeats on chromosome 16. All three are expressed according to cDNA data base entries, and their coding regions have identical lengths with amino acid identities between 99.5 and 99.8% (supplemental Fig. 1). Thus, these genes almost certainly encode functionally identical proteins, and we, therefore, refer to them collectively as NOMO. The NOMO2 transcript can undergo alternative splicing, which results in the generation of an mRNA lacking a ~330-bp exon and encoding a NOMO variant with an extended carboxyl terminus (NOMO-L). In a Northern blot analysis, we had detected only a single NOMO mRNA species of ~4.5 kb (10), which most likely corresponds to the long NOMO transcripts. Thus, NOMO-L, which is encoded by the shorter transcript, might represent a rare NOMO isoform.

To detect endogenous NOMO protein, we generated a polyclonal antibody (NOMO-900) directed against a fragment of the large NOMO amino-terminal domain. NOMO-900 and the Nicalin antibody Ncl-2221 (10) were used to analyze Nicalin and Nicalin protein levels in various human cell lines. Whereas Ncl-2221 recognizes a ~60-kDa protein under denaturing conditions, NOMO-900 labels a band of ~130 kDa (Fig. 1A), which is in good agreement with the calculated molecular mass of NOMO after cleavage of its putative signal peptide (131.5 kDa).

Both proteins were present in varying amounts in all of the cells examined, and their levels were found to be highly proportional. This result is consistent with our previously published Northern blot analysis, which had also shown ubiquitous expression and similar expression patterns in human tissues (10). We next performed blue native-PAGE to examine the native molecular weights of Nicalin and NOMO. Immunoblot analysis of membrane proteins from HEK293T cells with either the Ncl-2221 or the NOMO-900 antibody resulted in the specific labeling of a single band of 200–220 kDa (Fig. 1B). This not only demonstrated that Nicalin and NOMO exist in a high-molecular-weight form but, together with our co-precipitation data (10), strongly suggested that they are components of the same membrane protein complex. Because no bands of lower molecular weight were detected, Nicalin and NOMO monomers might exist only at low abundance.

Nicalin and NOMO Localize to the Endoplasmic Reticulum—Nicalin and NOMO are predicted to represent type I transmembrane proteins containing N-glycosylation sites within their large amino-terminal domains (10). To analyze their glycosylation status, which might provide an indication of their localization within the secretory pathway, we used sugar-cleaving enzymes. Treatment of HEK293T cell lysates with N-glycosidase F resulted in a small but reproducible reduction in Nicalin and NOMO molecular weights, demonstrating the presence of N-linked carbohydrates (Fig. 2A). Because N-glycosylation occurs co-translationally in the lumen of the ER, the amino termini of Nicalin and NOMO cannot reside in the cytoplasm, which is in agreement with the topology predictions. Treatment with endoglycosidase H resulted in similar molecular weight shifts (Fig. 2A), demonstrating that the N-linked carbohydrates are of the high-mannose type; i.e. they have not undergone Golgi-specific modifications. This suggested that Nicalin and NOMO represent proteins of the early secretory pathway.

We next examined the subcellular localization of Nicalin and NOMO in HEK293T cells by immunofluorescence microscopy. Endogenous levels of both proteins were too low to be detected, and therefore, cells overexpressing Nicalin and NOMO were used. Nicalin as well as NOMO immunoreactivities were found in a widespread tubular compartment that was identified as ER by staining for calnexin, an ER-resident protein (Fig. 2B). To analyze the localization of endogenous Nicalin and NOMO, we performed a fractionation of cellular membranes using density gradient centrifugation followed by immunoblot detection. ER membranes were enriched in the bottom four
gradient fractions as demonstrated by the presence of calnexin and immature Nicastrin (Fig. 2C). Nicalin and NOMO immunoactivities could only be detected in these four fractions. Taken together, these data demonstrate that the Nicalin-NOMO complex localizes to the ER membrane.

Nicalin and NOMO Expression Levels Are Mutually Regulated—The expression of γ-secretase components is tightly controlled by complex assembly (3). The high proportionality of Nicalin and NOMO expression levels in wild-type cells (see Fig. 1A) raised the question of whether they are regulated in a similar manner. To analyze Nicalin and NOMO expression levels in the absence of the respective complex partner, we used RNA interference (RNAi) in HEK293T cells. Clonal cell lines stably expressing Nicalin- or NOMO-specific short hairpin RNAs were generated, and efficient silencing was demonstrated by immunoblotting. Surprisingly, the knockdown of Nicalin caused a strong reduction of NOMO and vice versa, suggesting that Nicalin and NOMO are mutually dependent (Fig. 3A). This was supported by the observation that, in cell clones with a less efficient Nicalin knockdown, the reduction of NOMO was also incomplete (supplemental Fig. 2). The expression of other membrane proteins was not altered, demonstrating the specificity of the observed effects (Fig. 3A).
Regulation of the Nicalin-NOMO Complex

Next, we generated a cell line in which the Nicalin knockdown was rescued by expressing an RNAi-resistant Nicalin mRNA encoding Myc-tagged Nicalin, which has a slightly higher molecular weight, allowing its separation from endogenous Nicalin. Immunoblotting revealed that, in addition to Nicalin, NOMO expression was also restored in these cells (Fig. 3B). Moreover, analysis of clones with varying rescue efficiencies showed that the extent of NOMO restoration always closely followed that of Nicalin, corroborating the idea that their expression is tightly linked. To further show that this linkage is reciprocal, NOMO expression was rescued in NOMO knockdown cells in an analogous manner (Fig. 3C). Again, Nicalin and NOMO protein levels were restored in parallel, demonstrating a mutual dependence. To examine whether this increase in protein levels is accompanied by an enhanced interaction between Nicalin and NOMO, we performed immunoprecipitation of Nicalin-Myc from two Nicalin rescue cell clones with different expression levels. In the high expression clone (Fig. 3B, #2), higher amounts of NOMO were co-precipitated than in the low expressing clone (Fig. 3B, #1). This suggested that an increase in cellular Nicalin/NOMO levels results in enhanced complex formation.

The use of a strong promoter to rescue Nicalin expression in knockdown cells led to clones with Nicalin levels severalfold higher than in wild-type cells (Fig. 3B, clones #2 and #9). In these clones, NOMO levels were similarly increased, suggesting that Nicalin might control NOMO expression. We sought to verify these results and generated cell lines stably overexpressing Myc-tagged Nicalin (293T/Ncl) or Myc-tagged NOMO (293T/NOMO). As in the cells with rescued Nicalin expression, an increase in NOMO levels was detected in 293T/Ncl cells (Fig. 4A). In contrast, 293T/NOMO cells showed no change in endogenous Nicalin (Fig. 4A, asterisk), suggesting that cellular Nicalin levels are limited independently of NOMO. This limitation was also apparent in 293T/Ncl cells, which show strongly reduced levels of endogenous Nicalin (Fig. 4A). A similar phenomenon has been described in detail for the γ-secretase component presenilin and termed “replacement” (15), raising the possibility that the expression of γ-secretase and the Nicalin-NOMO complex might be subject to similar regulatory mechanisms. No replacement of endogenous NOMO was observed in cells expressing a Myc-tagged version of the long isoform NOMO-L (Fig. 4A). Thus, only cellular expression levels of Nicalin (but not of NOMO) appear to be limited.

Nicalin and NOMO Expression Is Regulated on a Post-transcriptional Level—To examine whether the observed effects on Nicalin and NOMO expression are due to transcriptional or post-transcriptional events, Nicalin and NOMO mRNA levels were determined using real-time PCR. In Nicalin knockdown cells, Nicalin mRNA was reduced ~7-fold (Fig. 4B), in good agreement with the reduction on the protein level. NOMO mRNA in these cells was increased 1.5-fold, in contrast to the reduction in protein expression. Similarly, in NOMO knockdown cells, NOMO mRNA was reduced ~7-fold, whereas Nicalin mRNA even showed a 3.3-fold increase. Thus, the reduction of Nicalin or NOMO protein levels upon silencing of the respective binding partner occurs by post-transcriptional mechanisms. Similar results were obtained with cell lines over-expressing Nicalin, which contain strongly increased amounts of NOMO protein (see Figs. 3B and 4A). The corresponding NOMO mRNA levels were only slightly higher (in Ncl knockdown rescue cells) or even lower (in 293T/Ncl cells) than in wild-type cells (Fig. 4B), demonstrating that the up-regulation of NOMO by Nicalin is a post-transcriptional event. To examine the mechanism of Nicalin replacement, we investigated mRNA levels of endogenous Nicalin in 293T/Ncl cells by using 3′-untranslated region-specific primers. Only a ~2.4-fold reduction could be detected, which can only partially account for the decrease on the protein level (see Fig. 3A). We, therefore, conclude that mainly post-transcriptional mechanisms are responsible for the observed alterations in Nicalin and NOMO protein expression, with the possible exception of
the Nicalin replacement, which might be partially caused by reduced transcription.

**Nicalin Controls NOMO Expression via Protein Stabilization**—To analyze the relationship between Nicalin replacement and NOMO up-regulation, we examined their kinetics in a cell line with tetracycline-inducible Nicalin expression (293TR/Ncl). Nicalin and NOMO protein levels were analyzed at various time points after induction. Although strong Nicalin overproduction was observed already after 8 h, changes in endogenous Nicalin and NOMO levels could be detected only after 16–24 h and became more and more prominent at later time points (Fig. 5A). After 85 h, endogenous Nicalin levels had dropped below the detection limit, and NOMO expression had reached highest levels, demonstrating that both effects show a similar time course and proceed very slowly. The increase in NOMO expression might be the result of its stabilization by increased complex formation (see Fig. 3B). To verify this hypothesis, we analyzed the half-life of NOMO in cells overexpressing Myc-tagged NOMO, which show wild-type levels of Nicalin and thus do not contain increased amounts of complex (see Fig. 4A). Consequently, these cells have a large pool of free Myc-tagged NOMO that can be distinguished from endogenous NOMO by its slightly higher molecular weight. To rule out a displacement of endogenous NOMO from existing complexes, we used cells with tetracycline-regulated NOMO expression and induced them for a limited period of time (6 h). Subsequent treatment of these cells with the translation inhibitor cycloheximide for 26 h resulted in a significant reduction of Myc-tagged NOMO but not of endogenous NOMO (Fig. 5B), in agreement with the idea that NOMO is stabilized by complex formation. To examine the NOMO half-life under various conditions, we transiently expressed NOMO in cells with different Nicalin levels and quantified NOMO immunoblot signals in the presence or absence of cycloheximide (Fig. 5C). Whereas in wild-type cells NOMO was reduced to 55% after a 10-h treatment, 86% of the NOMO signal was still present in Nicalin-overexpressing cells, and only 14% remained in Nicalin knockdown cells. Thus, NOMO is most stable in cells with high Nicalin levels, and we conclude that Nicalin regulates the stability of NOMO most likely via complex formation.

**DISCUSSION**

Nicalin and NOMO were originally identified by our group as physically and functionally interacting membrane proteins that modulate Nodal signaling in zebrafish embryos (10). Based on the observation that Nicalin exists in a high-molecular-weight form under native conditions, we had proposed the existence of a Nicalin-NOMO complex. We now show that both proteins are indeed found in a complex with a molecular mass of 200–220 kDa. This size is close to the combined molecular masses of monomeric Nicalin (60 kDa) and NOMO (130 kDa) and, allowing for inaccuracies inherent to the method of Blue-Native-PAGE, is in agreement with a heterodimeric complex. This is supported by our inability to identify additional Nicalin-binding partners (10). However, we cannot rule out the existence of another small component such as PEN-2 in the γ-secretase complex (16). The considerably larger size of native Nicalin reported previously (500–550 kDa) (10) was most likely the result of Nicalin overexpression, which might lead to Nicalin aggregates or cause self-association, an assumption supported by co-immunoprecipitation data. Similar size inconsistencies have also been reported for the γ-secretase complex (8, 17–19). Examination of the subcellular distribution of Nicalin and NOMO by immunofluorescence microscopy and membrane fractionation demonstrated that they both localize to the ER. The identification of high-mannose-type N-linked carbohydrates within the amino-terminal domains of Nicalin and

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NOMO supported the localization data and, in addition, suggested that Nicalin and NOMO are type I proteins with a large luminal and a short cytoplasmic domain. These results ruled out the possibility of Nicalin or NOMO pools present in different cellular compartments and corroborated the idea of a close physical and functional association. The exclusive localization in the ER is in contrast to γ-secretase which, after its assembly within the ER, is transported to the plasma membrane (20). Thus, γ-secretase and the Nicalin-NOMO complex differ not only in their size and function but also in their subcellular distribution.

Upon RNAi-mediated knockdown of Nicalin in HEK293T cells, we observed a concomitant decrease of NOMO expression, an effect reminiscent of the reduction of Presenilin, APH-1, and PEN-2 expression in the absence of Nicastrin (8, 21). Similarly, Nicalin was reduced in NOMO knockdown cells, suggesting that both proteins, similar to γ-secretase components, are destabilized in the absence of complex formation. mRNA measurements confirmed that this destabilization indeed occurs on the protein level. Further analogies to γ-secretase were found when we examined the effects of Nicalin overexpression, which resulted in a strong reduction of endogenous Nicalin. A similar phenomenon (replacement) had first been described in detail for Presenilin (15) and was later also reported for APH-1 (22). It can be explained by the rapid degradation of excess protein molecules (23), which cannot be incorporated into γ-secretase complexes due to the limitation of the other components. In agreement with this hypothesis, endogenous Nicalin mRNA levels were only slightly reduced in Nicalin-overexpressing cells, demonstrating that the Nicalin replacement is at least partially mediated by post-transcriptional mechanisms.

Enhanced Nicalin expression also resulted in an increase in endogenous NOMO levels in wild-type cells as well as in Nicalin knockdown cells. Thus, Nicalin might stabilize endogenous NOMO leading to an increase in NOMO steady-state levels. NOMO half-life measurements in the absence or presence of excess Nicalin supported this idea. In contrast, NOMO overexpression resulted neither in replacement of endogenous NOMO nor in an increase in Nicalin levels. We, therefore, propose a model in which NOMO is synthesized in excess amounts and stabilized by complex formation with Nicalin. Nicalin itself is limited independently of NOMO and represents the rate-limiting factor of the Nicalin-NOMO complex. Further studies are required to elucidate the molecular mechanisms responsible for the limitation of Nicalin expression. They might also lead to a better understanding of the replacement phenomena described for γ-secretase components. Thus, the analysis of the Nicalin-NOMO complex will not only provide insight into the regulation of the Nodal signaling pathway but might also help to elucidate mechanisms controlling γ-secretase function.

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