A new role for reticulon-4B/NOGO-B in the intestinal epithelial barrier function and inflammatory bowel disease

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Submitted 14 August 2014; accepted in final form 17 April 2015

Reticulon 4B (RTN-4B), a.k.a. NOGO-B, is a structural protein of the endoplasmic reticulum, in intestinal barrier function and IBD. We used immunohistochemistry, confocal microscopy, real-time PCR, and Western blotting to study tissue distribution and expression levels of RTN-4B/NOGO-B in control and IBD samples from mouse and humans. We also targeted RTN-4B/NOGO-B using siRNAs in cultured human intestinal epithelial cell (IECs). Epithelial barrier permeability was assessed by transepithelial electrical resistance (TEER) measurement. RTN-4B/NOGO-B is expressed in the intestine mainly by IECs. Confocal microscopy revealed a colocalization of RTN-4B, E-cadherin, and polymerized actin fibers in tissue and cultured IECs. RTN-4B mRNA and protein expression were lower in the colon of IL-10−/− mice compared with wild-type mice. Colocalization of RTN-4B/E-cadherin/actin was reduced in the colon of IL-10−/− mice. Analysis of endoscopic biopsies from IBD patients showed a significant reduction of RTN-4B/NOGO-B expression in inflamed mucosa compared with control. Treatment of IECs with H2O2 reduced TEER values and triggered phosphorylation of RTN-4B in serine 107 residues as well as downregulation of RTN-4B expression. Acute RTN-4B/NOGO-B knockdown by siRNAs resulted in a decreased TEER values and reduction of E-cadherin and α-catenin expression and in the amount of F-actin-rich filaments in IECs. Epithelial RTN-4B/NOGO-B was downregulated in human and experimental IBD. RTN-4B participates in the intestinal epithelial barrier function, most likely via its involvement in E-cadherin, α-catenin expression, and actin cytoskeleton organization at sites of cell-to-cell contacts.

Retinalon-4B/NOGO-B: intestinal epithelial cells; intestinal barrier; inflammatory bowel disease; Crohn’s disease; E-cadherin

Intestinal barrier dysfunction is a key phenomenon in the pathogenesis of inflammatory bowel diseases (IBD); Crohn’s disease (CD) and ulcerative colitis (UC). Even though it might be an epiphenomenon secondary to subepithelial inflammation, increased intestinal permeability has been proposed as a primary pathogenic event in IBD, being observed even at remission stages as well as in first-degree relatives (5, 12, 14). Proinflammatory cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), reactive oxygen species, and other mediators could be responsible agents, since they are known to cause intestinal barrier dysfunction by inducing alterations and/or disassembly of intercellular junctions (17, 20, 19).

Intestinal epithelial cells (IECs) constitute the main barrier element between the gastrointestinal mucosal immune system and the microbial and alimentary antigens on the mucosal surface. In addition to their digestive and absorptive functions, IECs exert pivotal roles both in the generation of tolerance toward alimentary antigens and commensal microbiota, and in the activation and orchestration of innate and adaptive immune responses (3, 8). Intestinal epithelial barrier and apical-basal polarity is built up and maintained by specialized plasma membrane structures that contain adhesive and scaffolding proteins known as the apical junction complexes (AJs) (27). These complexes, mainly composed by tight (TJs) and adherent junctions (AJs), seal the paracellular space between adjacent IECs and are tightly anchored into cytoskeletal structures such as actin cytoskeleton, intermediate filaments, and microtubules (13). Multiple molecules including peripheral membrane proteins such as zonula occludens (ZO), occludins, claudins, and the junctional adhesion molecule constitute TJs. On the other hand, AJs mainly include E-cadherin, members of the nectin family of proteins, catenins, and vinculin (13, 4, 11). Several studies have demonstrated changes in junctional molecules at different stages during the course of IBD (7, 13). Among others, E-cadherin has emerged as an important marker of intestinal epithelial integrity, permeability, and colorectal carcinoma (9). For example, the expression of E-cadherin is reduced, and its intracellular location is altered in the IECs of patients with IBD-related colorectal carcinoma (2).

Reticulon 4B (RTN-4B), a.k.a. NOGO-B, is a structural protein of the endoplasmic reticulum (ER) that participates in the maintenance of ER tubular shape and functions. Differential expression (up- or downregulation) of RTN-4B/NOGO-B has been associated with diverse conditions such as vascular remodeling (1, 22), pulmonary hypertension (24), and cirrhosis (30). Recent data suggest that RTN-4B/NOGO-B may participate in the control of cell-to-cell adhesion processes. NOGO-A/B null mice displayed a strong asthma phenotype when properly challenged, which was associated with highly perme-
able pulmonary epithelium (28). Furthermore, null mice showed a downregulation of palate lung and nasal clone (PLUNC), a molecule involved in the control of pulmonary epithelial permeability (28). Di Lorenzo et al. (6) demonstrated a role of endothelial RTN-4B/NOGO-B in the transmigration of leukocytes during the inflammatory process, via the modulation of ICAM-1-dependent signaling and VE-cadherin phosphorylation. The presence and potential functions of RTN-4B/NOGO-B within the intestinal tissue has not been investigated to date. Therefore, we hypothesized that RTN-4B/NOGO-B might be involved in the control of intestinal epithelial barrier function, and its expression levels might be altered within the mucosa of IBD patients.

Our findings identify RTN-4B/NOGO-B as a new AIC-associated molecule in the surface intestinal epithelium. RTN-4B mRNA and protein levels are lower in the colon of interleukin-10−/− vs. wild-type mice. Analysis of endoscopic biopsies from IBD patients revealed a significant reduction of RTN-4B/NOGO-B expression in inflamed mucosa. Oxidative disruption of epithelial barrier caused phosphorylation of RTN-4B/NOGO-B in Ser 107 residues accompanied by changes in its abundance. Acute downregulation of RTN-4B/NOGO-B by siRNAs in IECs increased permeability and reduced the amount of E-cadherin-, α-catenin-, and F-actin-rich filaments.

MATERIALS AND METHODS

Primers. The following primers (5′→3′) were employed: mTNF-α AC GGCA TGGAGTCTCAAAGAC (F), GTGGGTGAGGAGCACG TAGT (R); miNOS CAGCTGGGCTGTACAAACCTT (F), CATTGG GAAGTGAAGCGTTTCG (R); mZO-1 GGGCCATCTCAACTCTGTA (F), AGAAGGGCTGACGGGTAAAT (R); mE-cadherin CAAAGTGACGCTGAAGTCCA (F), TACACGCTGGGAAACAT-

![Fig. 1. Identification of reticulon-4B (RTN-4B)/NOGO-B in intestinal tissue. A: RTN-4B/β-actin mRNA levels and representative Western blot for RTN-4B and β-actin (loading control) in mouse brain (n = 3), ileum (n = 3), and colon (n = 3). Statistical differences were analyzed by ANOVA, *P < 0.05 compared with brain. B: RTN-4B immunohistochemistry in small bowel (ileum) from mouse (C, middle and right) and human (D). E and F show RTN-4B immunostaining in mouse and human colon, respectively. Negative control is showed in C, left. Scale bars are embedded in each image.](http://ajpgi.physiology.org/).
GAG (R); mβ-actin CGGTTCGGATGCCCTGAGGCTCTT (F), CGTC 去除重复字符 ACTTCATGATGGAATTGA (R); hE-cadherin ACCCCACAGCCCCGCCCTTAT (F), CCGCCCTCTTCGAGTCCCC (R); hVillin CTAACAGCAACCTCAGTTCT (F), CCTACAATCAGGGTAGGGTA (R); hReticulon-4B TCCTCGGGCTCAGTGGTTGTTG (F), TGCCCTGAATGGGTGGCCTT (R).

Antibodies. The following antibodies were used: RTN-4B/NOGO-B (Imgenex, San Diego, CA), E-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA), α-smooth muscle actin (α-SMA; Sigma, St. Louis, MO), F/480 (AbD Serotec, Oxford, UK), β-actin (Sigma), zonula occludens-1 (ZO-1; BD Transduction Laboratories), inducible nitric oxide synthase (iNOS; Pierce, Rockford, IL), GAPDH (Santa Cruz Biotechnology), phospho Ser 107 RTN-4B (University of Dundee/Dr. P. Cohen laboratory, Dundee, Scotland), α-catenin (BD Transduction Laboratories), GADD153/CHOP-10 (Santa Cruz Biotechnology), occludin (BD Transduction Laboratories), K-DEL (Santa Cruz Biotechnology).

Mice and human samples. C57Bl6 and IL-10−/− (B6.129P2-Ii10tm1Cgn/J) mice were purchased from Jackson (Bar Harbor, MA).

Fig. 2. Identification of RTN-4B/NOGO-B-expressing cells. Confocal micrographs of mouse colon double labeled for RTN-4B/E-cadherin (A), RTN-4B/F-actin (B), RTN-4B/α-smooth muscle actin (α-SMA; C), and RTN-4B/F480 (D). Merge+DAPI images are also shown.
Animals were housed and bred according to the Guide for the care and use of Laboratory Animals (NIH publication no. 85-23, 1985). The ethical committee on animal experiments of our institution approved all experiments. Mice were euthanized by an overdose of CO2 at the age of 18–20 wk, and the entire colon was removed. Surgical (n = 9) and endoscopic (n = 6) samples from patients with CD showing ileocolonic involvement were obtained, respectively, at the Hospital General Universitario Gregorio Marañón and the Hospital de Ciudad Real. Ethics committees for clinical investigation from both centers approved the study.

**Histological scoring.** The small bowel and colon cross sections (5 μm) from mice were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E) and scored in a blinded manner. Histological scoring (HS) was based on a semiquantitative scoring system that graded the following features: extent of destruction of mucosal architecture (0, normal; 1, 2, and 3, mild, moderate, and extensive damage, respectively); presence and degree of cellular infiltration (0, normal; 1, 2, and 3, mild, moderate, and transmural infiltration, respectively); extent of muscle thickening (0, normal; 1, 2, and 3, mild, moderate, and extensive thickening, respectively); presence of crypt abscesses (0, absent or 1, present); and presence of goblet cell depletion (0, absent or 1, present). The scores for each feature were summed with a maximum possible score of 11. A HS ≤3 was considered to be normal, HS between 3 and 7 was considered to correspond to moderate disease, and HS >7 corresponded to severe IBD phenotype.

**Immunohistochemistry.** Tissue cross sections (5 μm) were deparaffinized and rehydrated, boiled in sodium citrate, and blocked in 5% goat serum. The sections were incubated overnight at 4°C with 1:100 polyclonal rabbit anti-RTN-4B/NOGO-B antibody, followed by incubation with reagents from the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to manufacturer’s instructions. Staining was developed with DAB (3,3’-diaminobenzidine) with Karachi’s hematoxylin as counterstaining. Negative controls were obtained by avoiding the primary antibody as well as by incubation with inactivated anti-RTN-4B/NOGO-B antibody generated as described below.

**Immunofluorescence staining and confocal microscopy.** Tissue specimens were embedded in Tissue-Tek (Dako, Glostrup, Denmark) at the end of each experiment. Cells and tissues were fixed with prechilled 95% ethanol for 20 min at -20°C and incubated with the corresponding primary antibodies overnight at 4°C, washed in PBS, and incubated with secondary antibodies or dyes plus DAPI for 1 h at room temperature. Thereafter, samples were washed and mounted with fluorescence mounting medium (Dako). A confocal scanning inverted AOBS/SP2-microscope (Leica Microsystems, Wetzlar, Germany) was used for image acquisition.

**Cell culture.** Caco-2, T84, HT-29, HCT-116, SW-480, and SW-620 were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were grown and propagated according to ATCC recommendations. Cells were used between passages 10 and 20. **RTN-4B/NOGO-B blocking peptide.** One milligram of a peptide with the sequence DSPPRPQPAFKYQFVRE was synthesized and HPLC purified by Biomedal (Seville, Spain) and used as blocking peptide. For blockage, peptides were incubated with RTN antibody in a 5:1 ratio for 1 h at room temperature in the antibody dilution solution.

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**Fig. 3. Identification of RTN-4B/NOGO-B in cultured intestinal epithelial cells (IECs).** A: representative Western blot for RTN-4B in 6 different cultured IEC cell lines. B and C: immunofluorescence staining for RTN-4B in confluent HT-29 and T-84 cells. D: confocal images showing RTN-4B, E-cadherin, and merge + DAPI staining in polarized Caco-2 cells. E: confocal images showing RTN-4B, F-actin, and merge + DAPI staining in HT-29 cells. Magnification ×63. Scale bar = 10 μm.
Protein isolation and Western blotting. Proteins were isolated as described earlier (21). Equal amounts of proteins were denatured and subjected to SDS-PAGE in 10% polyacrylamide gels. Proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Molsheim, France); transfer was checked by Ponceau red S staining. Membranes were incubated with the corresponding primary antibody, followed by incubation with appropriate horseradish peroxidase-conjugated secondary antibodies. Signal was detected by enhanced chemiluminescence, and digital images were acquired with Alliance and quantified by use of Image J software.

Preparation of total RNA and real-time PCR. Total RNA from frozen tissue samples was extracted with glass homogenizers by using 1 ml Tri-pure isolation reagent (Invitrogen, Darmstadt, Germany) according to the manufacturer’s protocol. Contamination with genomic DNA samples was avoided by treatment with DNase. Reverse transcription was carried out with 500 ng of total RNA by using a Superscript II kit (Bio-Rad Laboratories, Hercules, CA). Specific sets of primers were obtained by using the NCBI/Primer-BLAST designing tool. Animal (Bio-Rad Laboratories, Hercules, CA). Specific sets of primers were used. Human data were compared by Wilcoxon test. P < 0.05 was considered statistically significant.

RESULTS

RTN-4B/NOGO-B expression and distribution within the intestinal tissue and IECs. To check for the presence of RTN-4B/NOGO-B within the intestinal tissue, we performed real-time PCR and Western blotting from samples of mouse brain (used as positive control), ileum (n = 3), and colon (n = 3). RTN-4B/NOGO-B mRNA was expressed similarly in colon and ileum, although at lower levels than brain (P = 0.0017) (Fig. 1A). The protein expression of RTN-4B/NOGO-B was also detected by Western blotting in mouse brain tissue, ileum, and colon (Fig. 1B), and by immunohistochemistry in mouse (Fig. 1, C and E) and human (Fig. 1, D and F) ileum and colon. RTN-4B/NOGO-B was widely distributed through the intestinal wall, appearing at sites of cell-to-cell junctions of surface epithelium (Fig. 1, D–F, right). To characterize the cell type(s) expressing RTN-4B, double labeling and confocal microscopy
analysis were performed. RTN-4B was expressed by colonic epithelial cells as demonstrated by colocalization with the epithelial marker E-cadherin (Fig. 2A). We observed colocalization between RTN-4B and polymerized actin (F-actin) as evidenced by phalloidin staining in colonic tissue (Fig. 2B) and cultured HT-29 IECs (Fig. 3E). We also found colocalization between RTN-4B/NOGO-B- and α-SMA-positive cells (Fig. 2C). RTN-4B was also expressed by macrophages as shown by colocalization with F4/80 (Fig. 2D). No staining was observed in the negative controls (Fig. 1C).

We also explored the presence of RTN-4B/NOGO-B in six different cultured IECs lines from human origin. All the IECs expressed both isoforms of RTN-4B/NOGO-B (B1 and B2), as assessed by Western blotting (Fig. 3A). Immunofluorescence revealed a pericellular distribution of RTN-4B/NOGO-B in a confluent monolayer of HT-29 and T-84 cells (Fig. 3, B and C). To confirm the presence of RTN-4B/NOGO-B at the ER, a confluent monolayer of Caco-2 cells were double labeled for RTN-4B/NOGO-B-B and the ER marker K-DEL (Fig. 4). Confocal microscopy analysis showed a strong RTN-4B and K-DEL colocalization. Moreover, treatment of Caco-2 monolayer with the following cocktail of cytokines [IFN-γ (20 ng/ml), TNF-α (25 ng/ml), and IL-1β (20 ng/ml)] did not modify RTN-4B/K-DEL costaining (Fig. 4). Consistent with the findings in the intestinal tissue, RTN-4B/NOGO-B was located at the sites of cell-to-cell contacts as demonstrated by the colocalization between RTN-4B/NOGO-B and E-cadherin in polarized Caco-2 cells (Fig. 3D).

Fig. 5. Characterization of IL-10−/− mice and mRNA expression profiles in the colon. A: macroscopic (left) and hematoxylin and eosin images (middle and right) of wild-type and IL-10−/− mouse colons. B: colon length (left) and weight-to-length ratio (right). C: TNF-α/β-actin (left) and iNOS/β-actin (right) mRNA levels. D: ZO-1/β-actin (left) and E-cadherin/β-actin (right) mRNA levels. E: RTN-4B/β-actin mRNA levels. mRNA data correspond to wild-type (n = 12) and IL-10−/− (n = 19) mice. Statistical differences were analyzed by Mann-Whitney U-test *P < 0.05 compared with wild-type.
RTN-4B expression levels in experimental and human IBD.

We next explored the RTN-4B/NOGO-B expression levels in wild-type mice ($n = 12$) and IL-10$^{-/-}$ mice ($n = 19$), a murine model of IBD. Body weight was similar in both groups. IL-10$^{-/-}$ mice showed evident changes in the gross anatomy and inflammation in the colon (Fig. 5A) and a moderate IBD phenotype according to HS compared with wild-type mice (wt: $1.9 \pm 0.3$ vs. IL-10$^{-/-}$: $6.2 \pm 1.4$, $P < 0.0001$). Macroscopic and microscopic observation of H&E staining showed a remarkable increase in size (Fig. 5A, middle and left). Colon length (measured as the distance from anus to cecum) was also larger in IL-10$^{-/-}$ mice ($8.15 \pm 0.21$ cm vs. $9.2 \pm 0.21$ cm, $P = 0.0064$, Fig. 5B, left). The colon weight-to-length ratio was also higher in IL-10$^{-/-}$ mice ($0.032 \pm 0.001$ vs. $0.069 \pm 0.003$, $P < 0.0001$, Fig. 5B, right). Whereas the expression of iNOS and TNF-$\alpha$ mRNA was very low or undetectable in the colon of wild-type mice, the expression of TNF-$\alpha$ mRNA was increased up to 6-fold higher ($P < 0.0001$) and the expression of iNOS up to 45-fold ($P < 0.0001$) in IL-10$^{-/-}$ mice (Fig. 5C). Moreover, the mRNA expression of ZO-1 and E-cadherin was downregulated in IL-10$^{-/-}$ mice, which showed 0.55-fold lower levels ($P < 0.001$) and 0.48-fold lower levels ($P < 0.001$), respectively, than wild-type mice (Fig. 5D). IL-10$^{-/-}$ mice expressed 0.51-fold lower levels of RTN-4B/NOGO-B mRNA ($P < 0.001$) than wild-type (Fig. 5E).

To confirm the mRNA data, Western blotting for RTN-4B/NOGO-B was performed in the colon from wild-type and IL-10$^{-/-}$ mice. As shown in Fig. 6, A and B, RTN-4B expression levels were significantly lower in the colon of IL-10$^{-/-}$ mice compared with wild-type mice. Immunohistochemistry and immunofluorescence staining showed that the reduction of RTN-4B/NOGO-B in the colon was more evident at the surface epithelium.
of IL-10\(^{-/-}\) mice compared with wild-type mice (Fig. 6, C and D). Furthermore, the loss of RTN-4B/NOGO-B immunostaining followed a patchy pattern and did not affect the entire surface epithelial monolayer in the same way (Fig. 6C). In addition, triple labeling of RTN-4B/NOGO-B, E-cadherin, and F-actin revealed a decrease in the colocalization between these three molecules in the surface epithelium of the colon of IL-10\(^{-/-}\) compared with wild-type mice (Fig. 6D).

We next analyzed surgical specimens from patients with CD who underwent ileocolonic resections, as well as endoscopic biopsies collected from patients with CD. We observed a discontinuous (patchy) staining pattern for RTN-4B/NOGO-B mostly at the surface epithelium (Fig. 7A) by immunohistochemistry. Figure 7A shows staining of areas in which the signal from RTN-4B/NOGO-B was very low or undetectable in contrast to adjacent areas in which the staining was preserved within the same cross section derived from a diseased segment. In addition, we analyzed the expression of RTN-4B/NOGO-B by Western blotting in macroscopically unaffected (control) and inflamed colonic endoscopic biopsies harvested from the same patient diagnosed of CD (n = 6) (Fig. 7, B and C). RTN-4B/NOGO-B abundance was significantly lower in the diseased compared with healthy segments (Fig. 7C). RTN-4B expression levels were considered as 1 in healthy segments, and the relative amount was calculated for each diseased segment (Fig. 7C). Expression levels of GAPDH and iNOS were evaluated by Western blot (Fig. 7B). As demonstrated in Fig. 7B, GAPDH, iNOS, and RTN-4B/NOGO-B expression patterns differ markedly between healthy and diseased samples, indicating the specificity of RTN-4B/NOGO-B down-regulation.

Effects of oxidative stress on epithelial barrier function and RTN-4B/NOGO-B expression levels. We next questioned whether molecules that disrupt epithelial barrier function might affect RTN-4B/NOGO-B expression and/or localization. T84

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Fig. 7. RTN-4B localization and expression levels in human Crohn’s disease samples. A: representative images of RTN-4B immunohistochemistry in colonic surgical samples from healthy (left) and diseased mucosa (right). Arrows point to cells lacking RTN-4B signal. B: representative Western blots for RTN-4B, GAPDH, and iNOS from 6 human endoscopic biopsies. H, healthy; D, diseased. C: densitometric analysis of RTN-4B expression levels. Statistical differences were analyzed by Wilcoxon test, *P < 0.05 compared with healthy sample.
cells were grown for 10–12 days in Transwells until they formed stable monolayers and achieved TEER values between 1,000 and 1,200 Ω·cm². Thereafter, cells were washed and kept overnight in a serum-free media and further challenged with H2O2 (10 mM) for 4 h. TEER values were monitored before stimulation (baseline values considered as 100%) and every 20 min after H2O2 addition (Fig. 8A). TEER values were reduced by ~15% at 120 min after stimulation and continued to decrease to a maximal reduction of around 60% after 4 h. Incubation of control cells with PBS did not modify TEER values (Fig. 8A). At the end of the experiment, Transwells were fixed and immunostained for ZO-1, RTN-4B/NOGO-B, and DAPI. Figure 7B shows an apical localization of ZO-1 and a lateral distribution of RTN-4B/NOGO-B in untreated cells. Upon stimulation with H2O2 for 4 h, both molecules were redistributed within the cytosol and the intensity of the signal was lower than in untreated cells (Fig. 8B).

In a parallel experiment, cells were stimulated with H2O2 (10 mM) for 30, 60, 180, and 240 min. Total protein extracts were subjected to SDS-PAGE and blotted for RTN-4B/NOGO-B and β-actin (used as loading control). Western blots revealed changes in RTN-4B/NOGO-B electrophoretic mobility 30 min upon H2O2 stimulation (an upward band shift was detected). This upper band was not observed in PBS-treated cells or after 60, 180, and 240 min of H2O2 stimulation (Fig. 8C). Quantitative analysis revealed a decrease of total RTN-4B/NOGO-B expression levels (P < 0.05, Fig. 8D). To identify the upper band, we performed a Western blot for RTN-4B phosphorylated in Ser 107 (P-RTN-4B). As shown in Fig. 8E, we observed a 3.5-fold increase in P-RTN-4B levels 30 min upon H2O2 stimulation compared with PBS-treated cells. Moreover, immunofluorescence staining showed a reduction in RTN-4B/NOGO-B signal 30 min after H2O2 stimulation compared with untreated cells (Fig. 8F).

Effects of RTN-4B/NOGO-B depletion in epithelial barrier function. In a preliminary set of experiments, siRNAs (600 pmol) were nucleofected into Caco-2 cells. RTN-4B/NOGO-B mRNA levels were efficiently reduced 72 h after nucleofection with RTN-4B/NOGO-B siRNA, compared with control siRNAs (90 ± 3%, P < 0.001) (Fig. 9A). Western blotting revealed similar results (not shown). MTT assay showed no differences on cell growth and viability between RTN-4B/NOGO-B-depleted and control cells after 24, 48, and 72 h (data not shown). To test the effect of RTN-4B knockdown on cell permeability, nucleofection of siRNAs were performed and equal amount of cells were seeded in Transwells and the TEER values were measured after 2, 4, and 6 days postnucleofection. As indicated in Fig. 9B, RTN-4B/NOGO-B silenced cells had significantly
lower TEER values than control cells 2, 4, and 6 days upon nucleofection.

In parallel, cells were plated in six-well plates and total proteins were obtained and separated by SDS-PAGE and subjected to Western blotting. Nucleofection of RTN-4B/NOGO-B siRNAs led to a reduction in E-cadherin and α-catenin protein levels after 4 and 6 days (Fig. 9C). Since RTN-4B/NOGO-B is an ER-associated protein, we investigated whether its downregulation by siRNA might induce ER stress. As indicated in Fig. 9C, the expression levels of GADD153/CHOP-10 (a well-established marker of ER stress) were not affected. Confocal microscopy analysis of IL-10 staining might be produced as a consequence of local accumulation and action of barrier dysfunction inducers (i.e., oxidative stress) and might contribute to increase intestinal permeability and bacterial translocation in these discrete areas of the

DISCUSSION

Intestinal barrier dysfunction is a hallmark of a variety of diseases, including IBD (8, 25). Elevated permeability is associated with maintained stimulation of mucosal immune cells by luminal antigens, and, consequently, with chronic inflammation. Thus identification of novel molecular players involved in the control of the normal epithelial barrier function is of great interest for the better understanding and management of IBD. The presence, expression pattern, and intracellular localization of RTN-4B/NOGO-B and its potential function(s) in intestinal tissue have not been established yet. Here we provide evidence that supports a new role for RTN-4B/NOGO-B in the control of epithelial barrier integrity and intestinal permeability, due to its relationship with E-cadherin, α-catenin, and actin cytoskeleton at the AJCs.

RTN-4B/NOGO-B is a structural protein of ER that participates in the maintenance of membrane curvature of ER and the number of ER tubules, owing to the presence of the reticulon domain (26, 31). ER forms an intricate intracellular network of membranes and vesicles closely associated with cellular structures and organelles like cytoskeleton and mitochondria, and it plays a key role in the control of protein translation and trafficking (10). The role of this organelle in IBD pathogenesis has been investigated in the context of ER stress due to the presence of misfolded proteins. However, the role of ER structural proteins such as RTN-4B/NOGO-B in IBD has not been investigated so far. Therefore, we initially evaluated the presence of RTN-4B/NOGO-B in the small bowel and colon of animals and humans. RTN-4B/NOGO-B was expressed by most of the cell types within the intestinal wall. Strikingly, RTN-4B was enriched in surface epithelium at sites of cell-to-cell contacts in the human and mouse large and small intestine, as well as in cultured IECs. RTN-4B colocalized with AJCs molecules like E-cadherin and F-actin. To our knowledge, this is the first study reporting such an association between these three molecules in IECs. To get insights into the participation of RTN-4B in IBD, we used mice lacking functional IL-10, an established model of IBD. The presence, expression pattern, and intracellular localization of RTN-4B/NOGO-B and its potential function(s)

![Western blots for RTN-4B, E-cadherin, and α-catenin, occludin, GADD153/CHOP-10, and β-actin in Caco-2 cells at 2, 4, and 6 days postnucleofection. C, control siRNA; RTN: reticulon-4B siRNA.](image)

Fig. 9. Effects of siRNA-mediated RTN-4B depletion. A: mRNA levels of RTN-4B and E-cadherin in Caco-2 72 h postnucleofection with control or RTN-4B siRNAs. *P < 0.05 vs. Control siRNA. B: TEER values of Caco-2 cells at 2, 4, and 6 days postnucleofection with control or RTN-4B siRNAs. Data are representative of 4 independent experiments and statistical differences were assessed by ANOVA. *P < 0.05 vs. Control siRNA. C: representative Western blots for RTN-4B, E-cadherin, α-catenin, occludin, GADD153/CHOP-10, and β-actin in Caco-2 cells at 2, 4, and 6 days postnucleofection. C, control siRNA; RTN: reticulon-4B siRNA.

![Graph showing fold change vs villin](image)

![Graph showing TEER (Ω·cm²) vs days post-nucleofection](image)

![Table showing siRNA (600 pmoles):](image)
diseased epithelium (Fig. 11). Animal findings were further confirmed in human CD samples. Within the same patient, inflamed mucosa contained less RTN-4B/NOGO-B than healthy segments. Altogether, these data support the idea that downregulation of RTN-4B/NOGO-B levels, in parallel to E-cadherin and F-actin decrease, might contribute to alterations in epithelial barrier function and perpetuation of the intestinal inflammatory response (Fig. 11). Mechanistically, lower levels of RTN-4B/NOGO-B in the surface epithelium might lead to a reduction in the amount of ER tubules. This decrease could modify certain ER functions, e.g., protein trafficking and/or the interaction with cellular components such as mitochondria or actin cytoskeleton. Our data are consistent with reports describing a RTN-4B/NOGO-B and actin cytoskeleton relationship in monocytes/macrophages (29).

The colocalization between RTN-4B/NOGO-B and AJC molecules led us to explore its participation in the control of epithelial permeability. Acute knockdown of RTN-4B/NOGO-B in IECs reduced TEER values and decreased the amount of E-cadherin, α-catenin, and F-actin. In contrast, members of the tight junctions such as ZO-1 and occludin remained unaffected. Under these conditions, absence of RTN-4B/NOGO-B seems
not to be related to the induction ER stress markers. Since E-cadherin mRNA levels were not affected by RTN-4B/NOGO-B depletion, we think that RTN-4B/NOGO-B down-regulation affects E-cadherin protein either during its translation at the ER and/or during its vesicular ER-Golgi trafficking and transport to its final destination at cell membrane. We cannot rule out the possibility that the silencing of RTN-4B/NOGO-B has a direct effect on F-actin polymerization by impairing the actions of actin-polymerizing proteins such as small GTPases like RhoA, or potentiating actin-depolymerizing proteins. These changes in the amount of F-actin could further modify the presence of E-cadherin and α-catenin at the cell surface.

The molecular mechanism(s) and the mediator(s) that regulate local levels of epithelial RTN-4B/NOGO-B in vivo are presently unknown. To get insights into this issue, we evaluated the effects of oxidative barrier disruption. Stimulation of cultured IECs with H₂O₂ increased epithelial permeability (measured as a reduction in TEER values), as reported earlier (20). These changes were preceded by alterations in RTN-4B electrophoretic mobility and phosphorylation degree as early as 30 min upon stimulation. Further incubation resulted in a significant reduction of its expression levels. Rousseau et al. (23) reported the only posttranslational modification known so far for RTN-4B (a phosphorylation in Ser 107 residues) in response to lipopolysaccharide in macrophages. Moreover, the function of this phosphorylation remains unknown; however, by itself, a single phosphorylation event cannot explain the reported changes in RTN-4B electrophoretic mobility. Therefore, future studies should focus in the identification and characterization of other(s) postranslational modification(s) besides phosphorylation that may be related to RTN-4B functions in intestinal epithelium in response to oxidative stress. In line with our findings, it has been shown that H₂O₂ also induced downregulation of the neuronal isoform of RTN in neurons by an unknown mechanism (15). These data link the downregulation of RTN-4B with situations of elevated permeability (Fig. 11). Future studies should elucidate the precise mechanism(s) underlying RTN-4B downregulation in H₂O₂-stimulated IECs and the relevance of RTN-4B/NOGO-B modifications in the control of total RTN-4B/NOGO-B and AJC molecules expression levels.

In conclusion, we have identified for the first time RTN-4B/NOGO-B as a new player in intestinal epithelial barrier function and IBD pathophysiology. Therefore, elucidation of new ways to upregulate intestinal epithelial RTN-4B levels and/or prevent its loss during the course of IBD may represent a novel approach to preserve intestinal epithelial barrier function and mucosal homeostasis through stabilization of AJC components such as E-cadherin, α-catenin, and actin cytoskeleton.

ACKNOWLEDGMENTS
The authors are grateful to Rafael Samaniego and Isabel Peligros for technical assistance and helpful comments.

GRANTS
This research has been supported by grants from Instituto de Salud Carlos III (FIS10/01912) to L. Menchén and from CIBEREHD to R. Bañeres. J. Vaquero is recipient of a Ramón y Cajal grant (2010-05838) from MINECO.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).
AUTHOR CONTRIBUTIONS

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