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The small leucine-rich proteoglycan BGN accumulates in CADASIL and binds to NOTCH3

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Abstract

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is an inherited form of cerebral small vessel disease caused by mutations in conserved residues of NOTCH3. Affected arteries of CADASIL feature fibrosis and accumulation of NOTCH3. A variety of collagen subtypes (types I, III, IV, and VI) have been identified in fibrotic CADASIL vessels. Biglycan (BGN) and decorin (DCN), are Class I members of the small leucine-rich proteoglycan (SLRP) family that regulate collagen fibril size. Because DCN has been shown to deposit in arteries in cerebral small vessel disease, we tested whether BGN accumulates in arteries of CADASIL brains. BGN was strongly expressed in both small penetrating and leptomeningeal arteries of CADASIL brain. BGN protein was localized to all three layers of arteries (intima, media, and adventitia). Substantially more immunoreactivity was observed in CADASIL brains compared to controls. Immunoblotting of brain lysates showed a 4-fold increase in CADASIL brains (compared to controls). Messenger RNA encoding BGN was also increased in CADASIL and was localized by in situ hybridization to all three vascular layers in CADASIL. Human cerebrovascular smooth muscle cells exposed to purified NOTCH3 ectodomain upregulated BGN, DCN, and COL4A1 through mechanisms that are sensitive to rapamycin, a potent mTOR inhibitor. In addition, BGN protein interacted directly with NOTCH3 protein in cell culture and in direct protein interaction assays. In conclusion, BGN is a CADASIL-enriched protein that potentially accumulates in vessels by mTOR-mediated transcriptional activation and/or post-translational accumulation via protein interactions with NOTCH3 and collagen.

Keywords

CADASIL; small leucine rich proteoglycans; biglycan; collagen; Notch; arteries; protein interactions

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Compliance with Ethics Requirements

Xiaojie Zhang, Soo Jung Lee, Marian F.Young, and Michael M. Wang declare that they have no conflict of interest.
This article does not contain any studies with living human or animal subjects.

Introduction

Cerebral small vessel disease is a major cause of stroke and an important factor in the pathogenesis of dementia [1]. Genetic causes of cerebral small vessel disease provide opportunities to discover molecules which may participate in cerebral arteriopathy. The best-recognized inherited cause of small vessel disease is cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), a condition caused by mutations in conserved residues of *NOTCH3* [2].

CADASIL arteries are markedly thickened [3, 4], and there is significant loss of smooth muscle cells that are replaced by amorphous material that includes excessive NOTCH3 ectodomain [5]. These abnormally thickened vessels are dominated by hypocellular extracellular matrix, which includes type I, III, and VI collagens [6], major final components of tissue fibrosis, as well as type IV collagen [6]. At the ultrastructural level, small deposits of material known as granular osmiophilic material (GOM) accumulate around smooth muscle cells [7, 8]; these are likely consequences of accumulation and aggregation of extracellular proteins [9]. All of these changes, including accumulation of abnormal proteins in multiple layers of arteries, are present in both leptomeningeal and penetrating small arteries of the gray and white matter [9]. Molecules that contribute to arterial thickening in CADASIL are still emerging and, based on positive periodic acid-Schiff staining, may include glycoproteins [10].

The small leucine-rich proteoglycans (SLRPs) compose a class of multifunction glycoproteins secreted into the extracellular matrix in many tissues. There are 17 members in the SLRP familiy that are characterized by extensive post-translational glycosylation of a relatively small protein core backbone that is composed of repeats rich in leucine. Biglycan (BGN) and decorin (DCN) are the best characterized of the SLRP family members. Their functions include modification of extracellular matrix structure and modulation of a number of signaling systems, which have been well-documented in a wide range of tissues [11]. BGN and DCN bind collagen proteins [12–14] and are components of extracellular fibers which may in turn further modify fiber-forming complexes of the extracellular matrix. The function of BGN has been extensively examined in musculo-skeletal tissue including bone [15], cartilage [16], tendon [17], muscle [18] [19] and teeth [20, 19]. The molecular foundation for this regulation includes both structural regulation and fine-tuning of critical growth factors such as BMP-2, TGF-beta and Wnt3a [21–23]. BGN binds to Toll 2 and Toll 4 receptors to modulate the immune system [24]. Some of BGN’s functions have only been revealed by disease challenge and include the induction of myocardial infarction [25], oviarectomy [26] and bone fracture [27].

Accumulation of DCN protein and mRNA was recently demonstrated in small penetrating arteries of CADASIL [28]. Strong expression of both collagen and DCN in brain arteries in CADASIL prompted us to hypothesize that BGN may also accumulate within thickened blood vessels in this disease. Here, we characterize the expression of BGN in CADASIL and identify candidate mechanisms that may drive the accumulation of this cerebrovascular protein.

Methods

Brain histology

Formalin fixed frontal lobes sections were acquired from the Brain Bank of the National Institute for Developmental and Childhood Disorders at the University of Maryland and the Alzheimers Disease Research Core at the University of Michigan and have been previously described [6, 29]. In addition, two CADASIL brains from patients with ischemic stroke and dementia with the NOTCH3 mutations R141C and R153C were studied [28]; both of these patients were Caucasian males in their 60s who died of complications of the disease. All brains in the CADASIL group showed severe small vessel disease, with significant white matter hyperintensities on premortem MRI imaging. The ages of CADASIL and controls were on average 66 years (n=8, range 46–83) and 63 years (n=6, range 47–82). Five micron sections from frontal cortex were analyzed using chromagenic immunohistochemical staining, counterstained with hematoxylin. We verified brain tissue antigen integrity using mouse monoclonal antibody BRIC231 (anti-H; Santa Cruz). The mouse monoclonal antibody 4E1-1G7 (Abnova) against human BGN was used for immunohistochemistry at 1:1000 dilution.

In situ hybridization

Localization of BGN mRNA in tissue sections was performed using a system developed by Advanced Cell Diagnostics. The protocol incorporated hybridization of nucleic acid probes, multiple non-enzymatic amplification steps, and probe detection using an alkaline phosphatase-conjugated terminal probed. Only three CADASIL brain blocks that resulted in signal with ubiquitous positive control probes were used in this study.

Protein quantification

Brain protein homogenates were prepared from frozen frontal cortex and analyzed by immunoblotting using 4E1-1G7 at 1:1000 dilution. Labeled secondary antibodies were detected using a Licor Odyssey infrared scanner. Expression levels were normalized to tubulin content.

RNA quantification

RNA from frozen brain tissue or cell cultures was purified using an RNeasy kit, reverse transcribed, and the cDNA was quantified by real time PCR, using HPRT, as a control; the primer sequences were: Human BGN sense: 5'-TCCGACCTGGGTCTGAAGT- 3' and antisense: 5'-GCCTTCTCATGGATCTTGGA-3'. Human HPRT: Sense: 5'-TGGCGTCGTGATTAGTGATG-3' and antisense: 5'-AATCCAGCAGGTCAGCAAAG-3'. RNA quality was determined by agarose gel electrophoresis, and samples included here demonstrated expected ribosomal RNA banding patterns. Samples which did not show clear banding were excluded from analysis.

Cell culture and immunoprecipitation

HEK 293 cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum. Transfections and immunoprecipitations were performed using

Lipofectamine 2000 (Invitrogen) as described before [30]. In brief, cell lysates were prepared after 48 hours after transfection, antibodies were added to lysates, and protein G agarose was used to pull down immune complexes which were then analyzed by immunoblotting.

Human cerebral smooth muscle cells were purchased from ScienCell Research Laboratories and grown in smooth muscle cell medium as recommended by the manufacturer. Cells were serum starved before challenge with purified recombinant NOTCH3-Fc fusion protein (400ng/mL). This protein was purified by protein A chromatography to homogeneity from stable 293 cell lines that secreted the protein into conditioned media as described [31]. Challenge with NOTCH3-Fc or Fc control protein (R&D Systems) was performed for 24 hours prior to RNA analysis. Cells challenged with NOTCH-Fc were simultaneously treated with rapamycin dissolved in DMSO.

Solid phase binding assays

Recombinant proteins human BGN and Notch-3-Fc (first 11 EGF repeats) were purchased from R&D Systems. Purified unlabeled binding target proteins were adsorbed to plates in PBS at 5 ug/ml. Solid phase assays were performed as described [30, 32]. We labeled purified proteins with Alexa 700 succinimide and then removed free label by gel filtration chromatography. Protein-coated plates were blocked using 0.5 % BSA in Tris-buffered saline (50 mM Tris, 150 mM NaCl) with 2 mM $CaCl_2$ for 1 h at room temperature. Labeled proteins were added to protein-coated ELISA plates in Tris-buffered saline with 2 mM $CaCl_2$ at 4 °C for 16 hours. Plates were washed three times with TBS with 2 mM calcium and quantified using a LiCor flatbed infrared scanner.

Statistical analysis

Results are displayed with standard deviations. All quantitative PCR studies were done with groups of three. T-tests were applied with statistically significant differences considered for $p<0.05$.

Results

BGN protein in CADASIL

We examined the distribution of BGN in genetically-confirmed CADASIL brains (Figure 1). Immunohistochemical analysis of BGN showed abundant vascular staining predominantly in arteries affected by diseaseIn small penetrating arteries of the white matter, which typically show marked hyaline thickening, BGN was deposited densely through the entire thickness of the vessel. Capillaries in CADASIL were also stained, albeit more lightly than arteries (not shown). BGN was found predominantly in all layers of leptomeningeal arteries, with increasing density moving away from the lumen.

Normal appearing penetrating (Figure 1C) and leptomeninigeal arteries of controls showed light adventitial staining without smooth muscle or endothelial expression, and capillary staining was variable. Immunoblot analysis of proteins from brains demonstrated significant increases in BGN in CADASIL compared to control brain (Figure 1D).

BGN transcripts in CADASIL

In situ hybridization revealed predominantly vascular expression of *BGN* in CADASIL (Figure 2A). RNA was expressed in cells located in the same vascular layers in which we identified BGN protein: the adventitia, media, and intima of leptomeningeal arteries. BGN mRNA was also detected in cells of penetrating arteries of the white matter (Figure 2B). Light capillary expression of *BGN* matched protein staining. In controls, BGN mRNA was reduced (Figure 2C). Quantitative RT-PCR revealed a substantial increase in BGN mRNA in CADASIL brain compared to controls (Figure 2D).

Regulation of BGN mRNA by excessive NOTCH3 ectodomain

NOTCH3 is dramatically deposited in CADASIL vessels [5], suggesting a pathogenic role of NOTCH3 excess in pathogenesis of small vessel disease. We investigated the effects of excessive NOTCH3 on BGN and related factors in human cerebrovascular smooth muscle cells (HC-SMC) grown in culture (Figure 3). Incubation of HC-SMC with purified NOTCH3-Fc fusion protein significantly increased BGN mRNA relative to levels in cells treated with Fc protein.

Rapamycin, a potent and specific inhibitor of mTOR signaling, has been used clinically to block stent stenosis by intimal hyperplasia, which is associated with BGN elevation [33, 34]. We therefore investigated whether this drug would affect NOTCH3 ectodomain-driven BGN gene activation (Figure 3, bars on the right). The elevation of BGN transcripts in response to NOTCH3-Fc was blocked by rapamycin. Messenger RNA encoding additional components of pathological vessels, DCN [28] and COL4A1 [6], were also upregulated by incubation with NOTCH3 ectodomain. Stimulation of both DCN and COL4A1 message was also blocked by rapamycin.

Interactions between BGN and NOTCH3 proteins

In addition to regulation at the transcriptional level, CADASIL-enriched proteins may accumulate in vessels by virtue of protein-protein interactions that trap molecules with the degenerating arterial matrix. A large increase in NOTCH3 protein has been reported in CADASIL arteries [5]; thus, binding to NOTCH3 may result in anchoring of proteins in diseased arteries [35]. To test whether BGN binds to NOTCH3, we performed co-immunoprecipitation assays in cells expressing both proteins. Protein immunopurified with BGN antibodies contained NOTCH3 protein. Conversely, NOTCH3 immunoprecipitates also contained BGN protein (Figure 4A–B).

To confirm molecular interactions between BGN and NOTCH3, we tested the ability of labeled BGN to bind to purified NOTCH3 in vitro. We observed dose dependent binding of BGN to NOTCH3 but not to Fc (control protein) (Figure 4C). Similar results were obtained when labeled NOTCH3 was applied to BGN coated wells (Figure 4D).

Discussion

Thickening of degenerating arteries of the brain is a striking feature of CADASIL. In extracerebral vessels, proteoglycans have been implicated in atherosclerosis [36, 37].

Studies identifying a number of types of collagen [38, 6, 35] in CADASIL vessels led us to hypothesize that proteoglycans known to bind collagen may localize to diseased arteries. Here, we demonstrate abundant accumulation of the collagen-binding proteoglycan BGN in CADASIL arteries.

Our studies suggest two mechanisms by which BGN could accumulate in CADASIL vessels. First, transcripts encoding BGN are increased in CADASIL; furthermore, in situ hybridization demonstrates an increase in vascular BGN mRNA specifically in intimal, medial and adventitial cells within arteries. Thus, arterial BGN transcriptional upregulation likely contributes to protein accumulation in vivo. Our studies suggest that in CADASIL, accumulation of NOTCH3 ectodomain in arterial smooth muscle cells may be sufficient to trigger increased BGN expression. Furthermore, experiments in cell culture implicate involvement of the mTOR pathway in activation of BGN mRNA. Stimulation of mTOR initiated by NOTCH3 ectodomain also resulted in upregulation of additional components of thickened vessels in CADASIL, DCN and COL4A1. Thus, BGN, DCN, and COL4A1 appear to be coordinately regulated in cerebral smooth muscle cells via a common signaling pathway. To our knowledge, this is the first signaling pathway identified that regulates transcripts enriched in CADASIL vessels. The mTOR pathway has been previously implicated in the development of intimal hyperplasia [39], which has resulted in deployment of rapamycin-eluting stents [40]; this is the first example of interactions between mTOR, Notch proteins, and BGN, and these studies suggest that pharmacological blockade of mTOR could modify intimal hyperplasia through BGN regulation. Of note, intimal hyperplasia is a core feature of CADASIL pathology [3].

Secondly, BGN accumulation in CADASIL may result from post-translational mechanisms. Specifically, BGN could become anchored to the vessel wall via protein binding partners that are up-regulated in CADASIL. These partners include collagen [6] and NOTCH3, which we show here for the first time is a BGN molecular partner. Attachment of BGN to extracellular components of CADASIL vessels may impair clearance of the protein. Of note, several additional proteins that build up in CADASIL vessels have been shown to physically interact with NOTCH3 protein (eg. vWF [41], collagen [42], TIMP3 [35], and NOTCH3 itself [31]). The binding of BGN to both NOTCH3 and collagen suggests the possibility that complex multimolecular complexes may form in CADASIL. The complexes, which likely feature multiple domains of interactions, could amplify protein accumulation and vascular thickening.

As a hydrophilic proteoglycan, BGN alter properties of the extracellular matrix by tissue- and gender-specific modulation of collagen structure [16, 43, 44] which may, in the brain, contribute to arterial thickening. On the other hand, the possibility of a homeostatic role of BGN is supported by Heegaard et al [44] who described a potential role of BGN in stabilizing vascular smooth muscle in mice and preventing aneurysm formation. In addition, in vitro, BGN binds TGFbeta [45] and blocks signaling [46], which is implicated in human small vessel disease [47]. The role of BGN in inhibiting TGFbeta mediated fibrosis in vivo is not as clear as for other SLRPs [46]. Assignment of a functional role of BGN awaits overexpression and knockout studies of BGN in the context of accelerated CADASIL animal models.

In conclusion, we have identified for the first time the accumulation the proteoglycan BGN in arteries of CADASIL patients. Accumulation of BGN may result from a combination of mTOR-dependent transcriptional upregulation and/or binding to proteins that contribute to molecular networks that drive vascular wall thickening.

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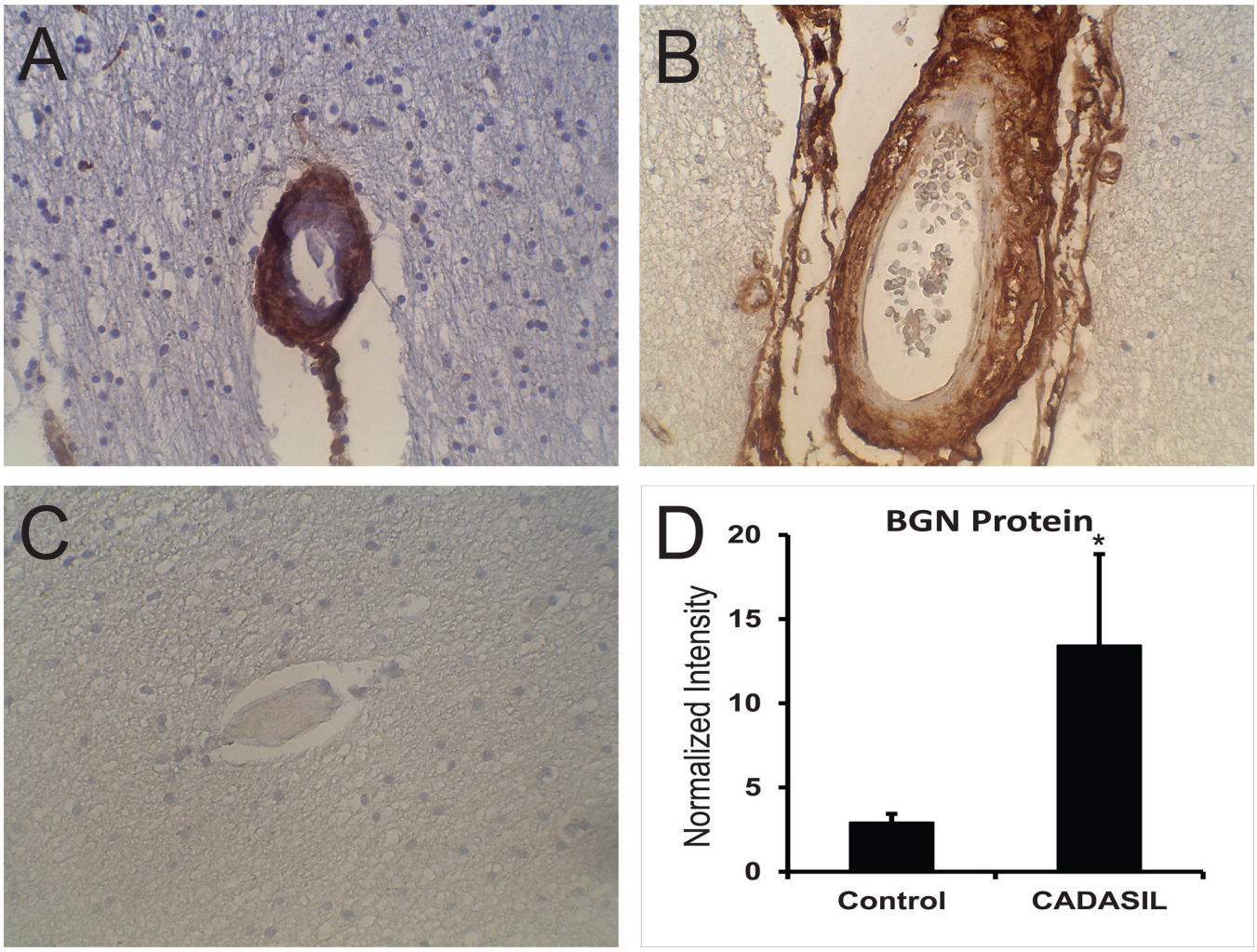

Figure 1.

BGN protein in CADASIL and control brain. Immunohistochemical analysis of genetically-defined CADASIL brains (A and B) and matched controls (C) was performed on frontal cortex sections with antibody 4E1-1G7. Penetrating arteries (A, C) and leptomeningeal (B) vessels are shown. Magnification is at 400x. (D) Brain lysates were analyzed by immunoblotting with 4E1-1G7 and the core BGN protein band was quantified for CADASIL and control frontal cortex, normalized to tubulin levels. Protein was not treated with glycosidases. Difference were significant ($p<0.05$) for CADASIL samples versus controls.

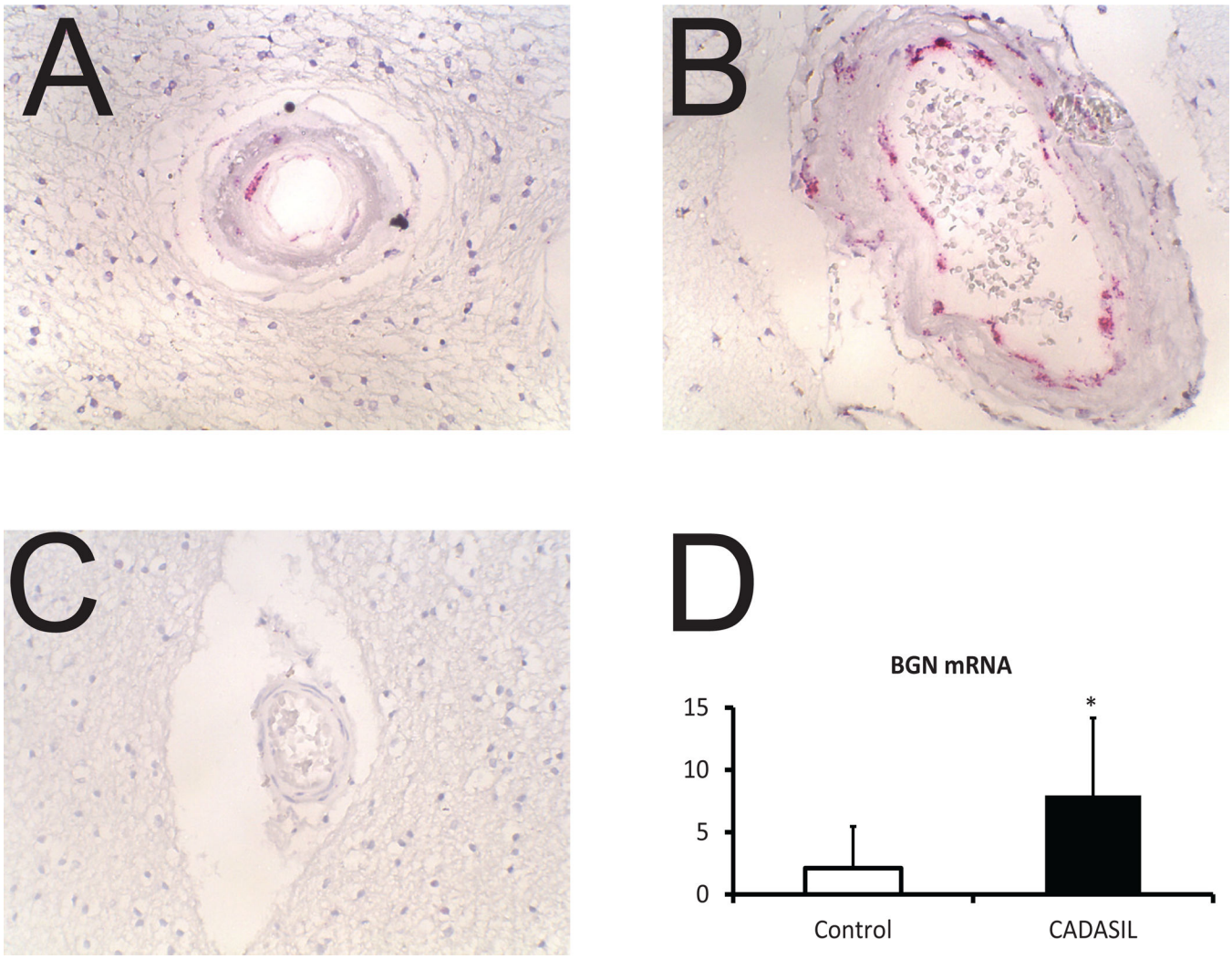

Figure 2.

Localization of BGN mRNA in CADASIL and control brain vessels. In situ hybridization was performed on frontal cortex of genetically-defined CADASIL brains (A and B) and controls (C). Small penetrating arteries (A and C) and leptomeningeal vessels (B) are shown. Magnification is at 400x. (D) Quantitative RT-PCR analysis of BGN mRNA from controls and CADASIL brains are shown; differences were significant with $p<0.05$.

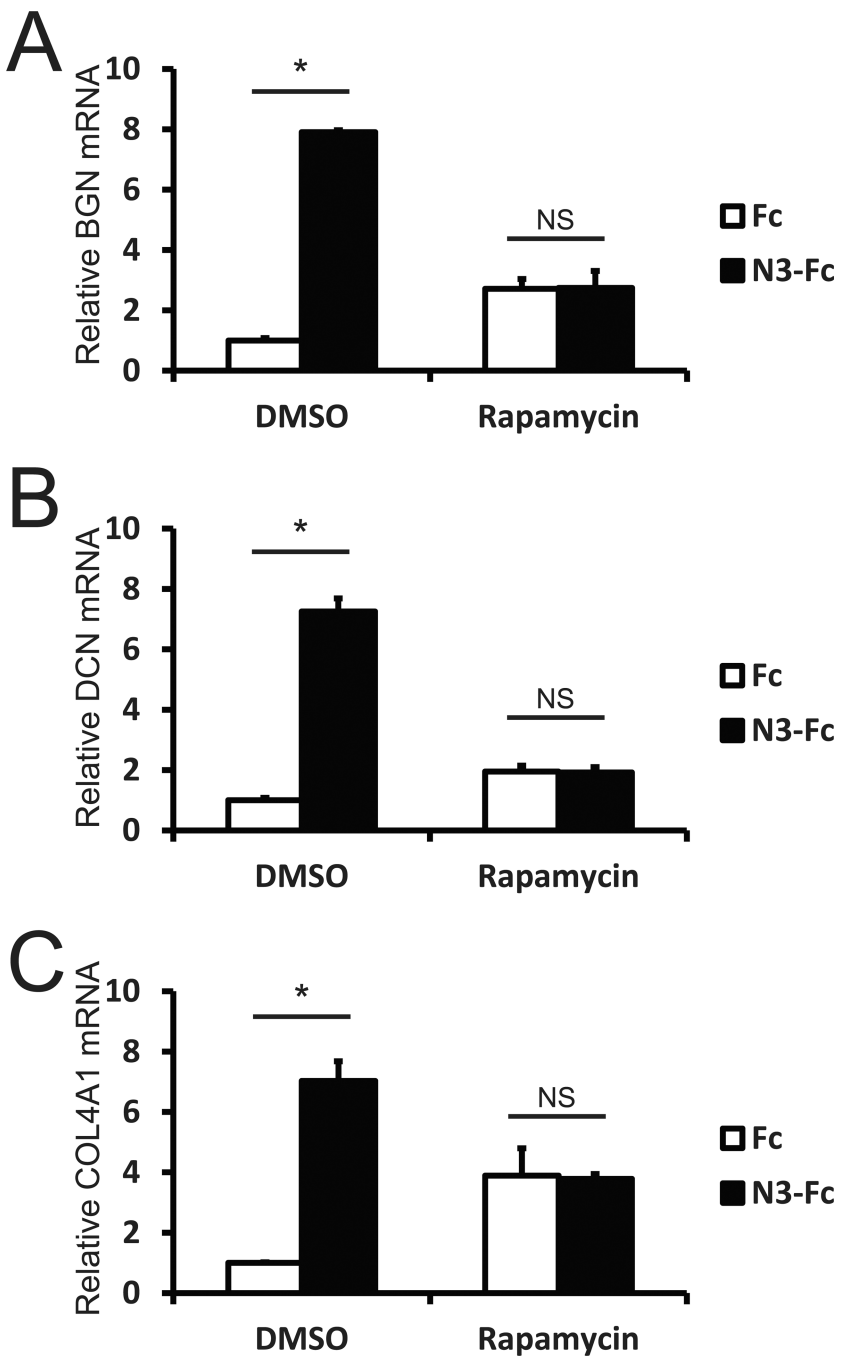

Figure 3.

Regulation of BGN mRNA levels in human cerebrovascular smooth muscle cells. Primary vascular smooth muscle cells from human brain were serum starved and then challenged with purified NOTCH3-Fc fusion protein or Fc control protein. One day after challenge with proteins, BGN (A), DCN (B), and COL4A1 (C) mRNA levels were assessed using quantitative RT-PCR. During the protein challenge, cells were exposed to DMSO vehicle or rapamycin to assess the role of PI3K or mTOR signaling on BGN transcript induction.

Differences between NOTCH3 and control treated cells were all significant with $p<0.05$. There were no significant differences between these groups after rapamycin treatment.

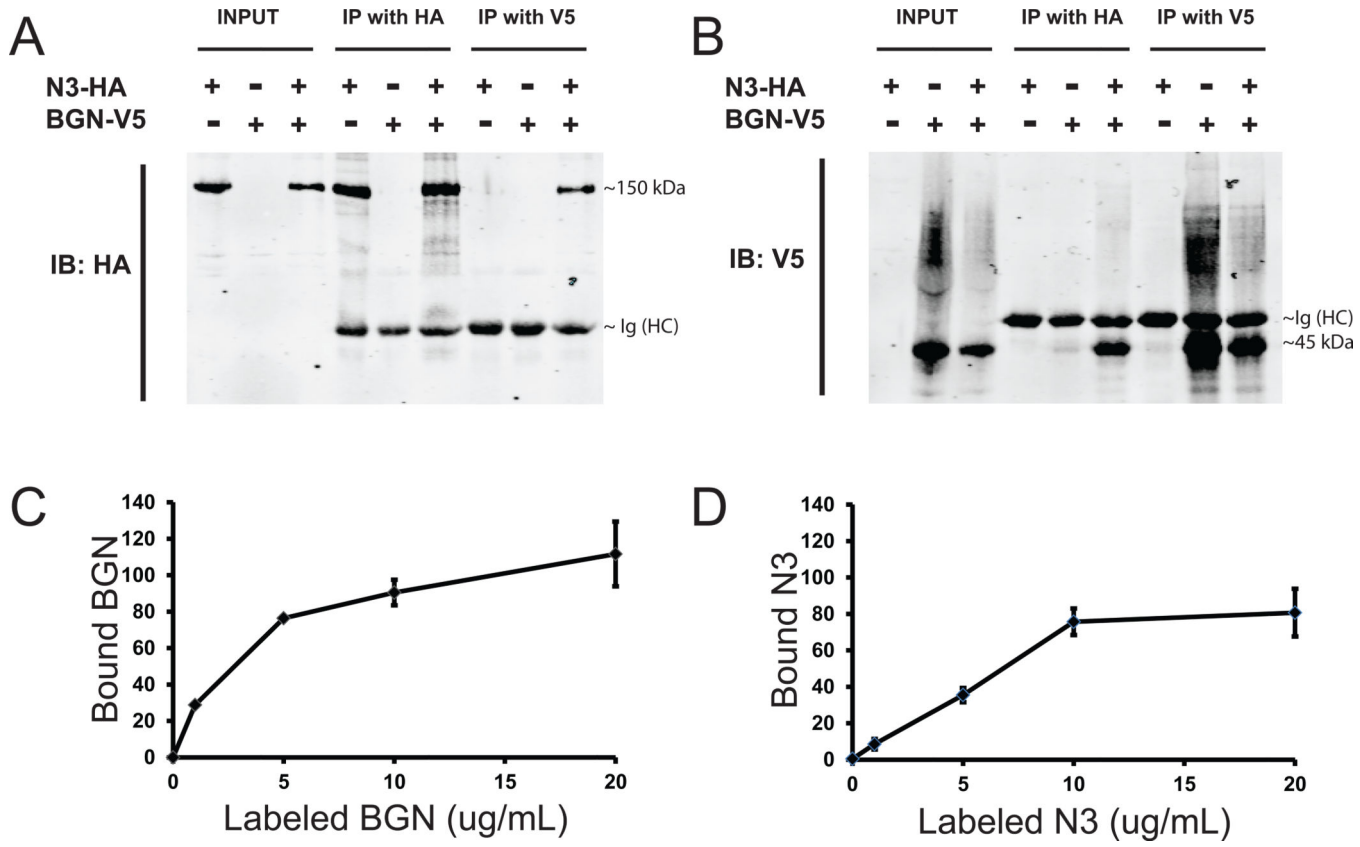

Figure 4.

Molecular interaction between BGN and NOTCH3 proteins. (A-B) 293 cells were transfected with genes encoding the indicated epitope-tagged proteins, as indicated in the legend above. Expression levels in cell lysates are shown on the left of each panel (INPUT). After treatment of lysates with indicated antibodies (IP with HA or IP with V5), proteins were pulled down using protein G-agarose were analyzed by immunoblotting (IB) with either HA (A) or V5 (B) monoclonal antibodies. NOTCH3 ectodomain tagged with HA (N3-HA) migrates at approximately 150kDa. The 50kDa band in lanes with IP proteins corresponds to the heavy chain of immunoglobulin used for the IP (Ig (HC)). The tagged BGN (BGN-V5) band migrates at approximately 45 kDa, with an upward smear that corresponds to glycosylated product. (C-D) Direct protein binding assays were performed by first adsorbing pure recombinant NOTCH3 (C) or BGN (D) to plastic dishes. Labeled BGN (in C) or NOTCH3 (in D), at increasing concentrations shown on the X-axis, was incubated with coated plates, which were washed and then quantitatively imaged to assess protein binding (on the Y-axis). Labeled Fc protein was used as a negative control and resulted in negligible binding to BGN- and NOTCH3-coated plates.