CADASIL mutations enhance spontaneous multimerization of NOTCH3

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Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is the most common monogenic cause of stroke and vascular dementia. Disease-causing mutations invariably affect cysteine residues within epidermal growth factor-like repeat domains in the extracellular domain of the NOTCH3 receptor (N3ECD). The biochemical and histopathological hallmark of CADASIL is the accumulation of N3ECD at the cell surface of vascular smooth muscle cells which degenerate over the course of the disease. The molecular mechanisms leading to N3ECD accumulation remain unknown. Here we show that both wild-type and CADASIL-mutated N3ECD spontaneously form oligomers and higher order multimers in vitro and that multimerization is mediated by disulfide bonds. Using single-molecule analysis techniques (‘scanning for intensely fluorescent targets’), we demonstrate that CADASIL-associated mutations significantly enhance multimerization compared with wild-type. Taken together, our results for the first time provide experimental evidence for N3 self-association and strongly argue for a neomorphic effect of CADASIL mutations in disease pathogenesis.

INTRODUCTION

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is an inherited small vessel disease leading to recurrent ischemic stroke and vascular dementia (1–3). CADASIL is caused by mutations in the NOTCH3 gene (4). NOTCH3 encodes a large type-I transmembrane receptor that, in adulthood, is predominantly expressed in vascular smooth muscle cells (VSMC) (4,5).

NOTCH3 (N3) belongs to the Notch receptor superfamily, which regulates cell fate during embryonic development (6). Notch receptors relay signals from the cell surface to the nucleus via regulated intramembrane proteolysis. They undergo several proteolytical cleavages: the first cleavage (termed S1) is executed by a furin-like convertase in the trans-golgi network yielding a heterodimeric receptor which is subsequently transported to the cell surface. Binding of ligands presented by neighboring cells induces an extracellular cleavage (S2) near the plasma membrane that results in shedding of the extracellular domain. S2 cleavage is followed by a third, gamma-secretase-dependent cleavage (S3) and liberation of the intracellular domain, which translocates to the nucleus to regulate gene expression by interaction with transcription factors such as RBP-Jk. It has previously been shown that NOTCH1 (N1) can form homodimers (7) in vitro. However, ectodomain shedding and S3-cleavage seems to be primarily executed with monomeric N1 receptors (8) and the physiological relevance of N1 dimerization is unknown. The ability of the N3 receptor to self-associate has not been characterized so far.

CADASIL-related mutations are highly stereotyped and invariably located in the extracellular domain of N3 (N3ECD) within epidermal growth factor-like (EGF-like) repeat domains (9–11). These domains show a modular structure with six highly conserved cysteine residues, which stabilize the domain by the formation of disulfide bonds (12). The vast majority of CADASIL mutations hitherto described results in a gain or loss of a cysteine within an EGF-like repeat domain thus altering the number of cysteine residues and leaving one unpaired (9–11). It has thus been hypothesized that

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CADASIL-mutated N3 may interact with adjacent mutant receptors or other proteins by aberrant disulfide bond formation. Indeed, a key feature of CADASIL is selective accumulation of N3\(\text{ECD}\) within small arteries in close vicinity to VSMC (8). These cells degenerate over the course of the disease through an unknown mechanism.

Using transfected cells, we and others have shown that some CADASIL-mutant receptors exhibit normal receptor trafficking, ligand binding and ligand-induced signaling (13–15). Moreover, rescue experiments have shown that the developmental phenotype seen in N3-deficient mice can be corrected by crossing these mice to CADASIL transgenic mice (16). These findings strongly argue against a gain- or loss-of-function mechanism in CADASIL pathogenesis. Instead, CADASIL mutations may act through a neomorphic or toxic gain-of-function mechanism.

Given the critical role of protein aggregation in other conditions including vascular disorders such as cerebral amyloid angiopathy (17), we explored both the ability of N3\(\text{ECD}\) to self-associate and the impact of CADASIL mutations on multimerization of N3\(\text{ECD}\). We demonstrate that wild-type, truncated extracellular N3 is able to spontaneously form oligomers and higher order multimers in vitro. We further show that the generation of multimers is significantly enhanced by CADASIL mutations.

RESULTS

Homophilic interaction of wild-type and CADASIL-mutated N3

To assess possible homophilic interactions of wild-type and CADASIL-mutated N3, we first performed reciprocal co-immunoprecipitation experiments using the full-length N3 receptor N-terminally tagged with either V5- or myc-epitopes. Constructs were transiently co-transfected into HEK293 cells. As shown in Figure 1A, both wild-type and R133C-mutant N3 bind to their respective counterparts and precipitation is reciprocally possible. Of note, there were no apparent differences in the amount of precipitated protein between wild-type and CADASIL-mutated receptors. We next repeated the co-immunoprecipitation experiments using C-terminally tagged extracellular (N3\(\text{ECD}\)) receptor fragments since this region of N3 accumulates in CADASIL-affected arteries. Again, both wild-type and R133C mutant N3 bind to their respective counterparts and precipitations are reciprocally possible (Fig. 1B). Similar results were observed with fragments carrying two other CADASIL mutations (C183R and C455R; Fig. 1C). Non-specific interaction of the fragments due to overexpression was excluded by control precipitations from mock measurements were quantified, representing various disease-related proteins including alpha-synuclein (18–20) and readily detects multimers larger than 10 particles. A confocal setup allows to study fluorescent dye labeled proteins (in our case indirectly labeled via fluorescent dye coupled antibodies) in a single molecule analysis, where the fluorescence intensity of every particle can be characterized. In order to improve sensitivity for rare protein species, the focus is moved through the sample (scanning). Quantification is based on fluorescence intensity distribution, which is plotted in 2D histograms. Thereby, signal from antibodies bound to multimers (dual color events) can be easily distinguished from monomers and antibody conglomerates (Fig. 3B). Lysates of HEK293 cells transiently transfected with V5-tagged N3\(\text{ECD}\) were cleared (16000 g, 20 min) and labeled with Alexa Fluor 488 (‘green’ fluorescence)- and Alexa Fluor 647 (‘red’ fluorescence)-conjugated anti-V5 antibody (Fig. 3A), respectively. For SIFT analysis, dual-color events with bright fluorescence above a threshold determined from mock measurements were quantified, representing higher order multimers of N3\(\text{ECD}\) (Fig. 3B). SDS–PAGE and SIFT analyses were performed both immediately after cell lysis and following incubation of cell lysates at 37°C for 24 h. Specificity of SIFT signal was confirmed by control experiments using fluorophor-conjugated anti-alpha-synuclein.

Spontaneous self-association and multimerization of wild-type and mutant N3\(\text{ECD}\)

We next analyzed whether de novo N3\(\text{ECD}\) multimerization can be observed in cell lysates. Non-reducing SDS–PAGE of fresh lysates from transiently transfected HEK293 cells revealed, in addition to monomers (Fig. 2A), high molecular weight bands most likely representing dimers and N3 oligomers. Incubation of these lysates for 24 h at 37°C resulted in a complete shift towards higher molecular weight bands within the stacking gel suggesting spontaneous self-association of N3\(\text{ECD}\). Incubation of cell lysates with N-ethylmaleimide (NEM, a thiol reactive alkylation compound and irreversible inhibitor of disulfide bridge formation) prior to incubation prevented the generation of high molecular weight bands to a great extent indicating that multimerization of N3\(\text{ECD}\) is dependent on disulfide bond formation. Higher order multimers were partly resistant to β-mercaptoethanol (Fig. 2A, lower panel). Incubation with 1.3 mM cupric phenanthroline (a disulfide-forming catalyst) for 10 min at 37°C followed by immediate SDS–PAGE resulted in enhanced accumulation of higher order multimers (Fig. 2B). In multiple experiments, formation of higher order multimers after incubation for 24 h appeared to be enhanced for mutant receptor fragments, as judged by the immunoreactive material retained in the stacking gel.

CADASIL mutations enhance the formation of higher order multimers of N3\(\text{ECD}\)

Due to the known limitations of PAGE analysis and western blotting in resolving and quantifying high molecular weight protein complexes, we sought to reliably quantify higher order multimers of N3\(\text{ECD}\) with ‘scanning for intensely fluorescent targets’ (SIFT) technology. SIFT technique has successfully been used to characterize de novo aggregation of various disease-related proteins including alpha-synuclein (18–20) and readily detects multimers larger than 10 particles. A confocal setup allows to study fluorescent dye labeled proteins (in our case indirectly labeled via fluorescent dye coupled antibodies) in a single molecule analysis, where the fluorescence intensity of every particle can be characterized. In order to improve sensitivity for rare protein species, the focus is moved through the sample (scanning). Quantification is based on fluorescence intensity distribution, which is plotted in 2D histograms. Thereby, signal from antibodies bound to multimers (dual color events) can be easily distinguished from monomers and antibody conglomerates (Fig. 3B). Lysates of HEK293 cells transiently transfected with V5-tagged N3\(\text{ECD}\) were cleared (16000 g, 20 min) and labeled with Alexa Fluor 488 (‘green’ fluorescence)- and Alexa Fluor 647 (‘red’ fluorescence)-conjugated anti-V5 antibody (Fig. 3A), respectively. For SIFT analysis, dual-color events with bright fluorescence above a threshold determined from mock measurements were quantified, representing higher order multimers of N3\(\text{ECD}\) (Fig. 3B). SDS–PAGE and SIFT analyses were performed both immediately after cell lysis and following incubation of cell lysates at 37°C for 24 h. Specificity of SIFT signal was confirmed by control experiments using fluorophor-conjugated anti-alpha-synuclein.
antibodies (Fig. 3C, lower panels), where no aggregate signal was detectable by SIFT. Consistent with SDS–PAGE analyses (Fig. 2), there were few multimers formed by N3ECD at baseline. Also, there was an increase in multimer signal following incubation for 24 h for both wild-type and CADASIL-mutated N3ECD as observed with SDS–PAGE analyses. Quantification of dual-color signal events at 24 h revealed significantly increased SIFT signals for all three CADASIL-mutated constructs (R133C, C183R and C455R) when compared with wild-type N3ECD (Fig. 3D) indicating enhanced multimer formation of CADASIL-mutated constructs.

DISCUSSION

Aggregation and accumulation of abnormally folded proteins has been recognized as a key pathological event in various neurological disorders (21). Given the accumulation of N3ECD in systemic arteries of CADASIL patients, we analyzed homo- and heterophilic interactions of wild-type and CADASIL-mutated N3. We demonstrate a propensity of the N3 receptor to spontaneously form oligomers and higher order multimers in vitro. Using SIFT technology, we further show that CADASIL mutations significantly enhance receptor multimerization in higher order multimeric complexes.

The ability of wild-type N3 to form homophilic complexes agrees with data for N1 (7,8). While interactions between Notch receptors and their ligands are crucial for proper signaling (22), the physiological role of Notch receptor oligomerization remains elusive. Studies with N1 suggest that oligomerization is not necessary for receptor cleavage or signaling (8).

The highly stereotyped nature of N3 mutations in CADASIL suggests a role of aberrant disulfide bridge formation of EGF-like repeat domains in CADASIL pathogenesis. It has been shown previously that small CADASIL-mutated receptor fragments containing EGF repeats 1–5 form homodimers on SDS–PAGE under non-reducing conditions (23). Our current...
findings extend these data in several ways: first, we demonstrate spontaneous self-association and aggregation of N3 ECD, the actual molecular component of CADASIL deposits. Second, we show that this process is enhanced by CADASIL mutations and—in contrast to data from EGF repeat 1–5 constructs—occurs with wild-type receptor fragments as well. This suggests that protein sequences outside EGF repeats 1–5 are relevant for wild-type receptor multimerization. Third, by blocking or facilitating disulfide bridge formation, we show that multimerization of N3, at least in part, depends on disulfide bridges. Conceivably, the unpaired cysteine residue might render CADASIL-mutated N3ECD more susceptible to multimerization in higher order complexes. However, the partial resistance of N3 multimers to reducing agents indicates that other mechanisms such as conformational changes of N3 might also play a role.

Adding to previous studies in cell culture or transgenic mice that showed no evidence for a gain- or loss-of-function mechanism (13,15,16) in CADASIL pathogenesis, our results strongly argue for a neomorphic (e.g. toxic) effect of CADASIL-mutated N3ECD. Our finding of quantitative differences in multimerization between wild-type and mutant receptors represents a link between CADASIL and other protein aggregation disorders such as Alzheimer’s (AD) or Parkinson’s disease (PD): for instance, wild-type alpha-synuclein exhibits spontaneous aggregate formation that is strongly enhanced by mutations associated with autosomal dominant PD (24,25). Similar to these conditions, strategies that inhibit or reverse protein aggregation might offer therapeutic perspectives in CADASIL.

Figure 2. Spontaneous generation of N3 multimers dependent on disulfide bond formation. (A) HEK293 cells were transiently transfected with N3ECD constructs (C-terminal V5-tag, (C) and analyzed by western blot under reducing (+$\beta$-mercaptoethanol, +$\beta$-ME) and non-reducing conditions (−$\beta$-ME). Note the shift toward higher molecular weight bands within the stacking gel (**) after incubation (24 h, 37°C, middle panel) and the lower abundance of these bands after addition of NEM (right panel) prior to incubation. Higher molecular weight complexes are partly resistant to $\beta$-ME (lower panel). (B) A 10 min incubation with cupric phenanthroline (a disulfide-forming catalyst) enhances high molecular weight bands. Note that high molecular weight complexes (**) after incubation concentrate in the loading wells and do not entirely enter the gel. (# putative monomeric receptor fragments, note the low apparent molecular weight between 100 and 150 kDa most likely due to native conformation; * putative dimers; ** putative oligomers; *** putative multimers).

Some technical aspects of our study need to be addressed. First, overexpression of proteins may interfere with the cell’s protein synthesis apparatus thus causing artificial interactions, especially of large proteins. However, control precipitations with APP, another type-I transmembrane protein cleaved by gamma secretase, confirm the specificity of N3 protein–protein interactions. Second, the use of non-purified cell lysates for SIFT and SDS–PAGE analyses limits conclusions as interaction of N3 fragments might depend on other proteins or cellular compounds. Ideally, these experiments would be performed with recombinant receptors. However, purification of N3ECD (comprising 210 kDa) was not feasible using various approaches including baculovirus expression (data not shown). Nevertheless, the enhanced aggregation properties of CADASIL-mutated receptors in incubated lysates in vitro clearly argue for a specific functional effect of these mutations.

As in many disorders associated with the accumulation of abnormally folded proteins, the exact relationship between aggregate formation and cellular dysfunction and eventually death remains to be clarified (26–28). While in other conditions, such as AD, small oligomers have been identified as being disease relevant, our results suggest that, in CADASIL, higher order complexes of N3 might be the major culprit in disease pathogenesis. Functional studies using mono-, oligo- or multimeric N3 in cell culture or overexpression of full-length or truncated N3 in transgenic mice will be crucial to evaluate a possible functional (e.g. toxic) effect on VSMC in vitro and in vivo. Since the SIFT technology proved to be a reliable tool in detecting higher order
aggregates, it might allow screening for anti-aggregatory substances in a high-throughput approach similar to previous studies on other protein aggregation disorders (19,20,29). Moreover, SIFT technology might aid analyzing the effects of rare non-canonical N3 mutations not involving cysteine residues which have been sporadically linked to CADASIL.

In summary, our study for the first time demonstrates spontaneous self-association of N3 that is significantly enhanced by CADASIL mutations. This finding eventually provides experimental evidence for a neomorphic mechanism of N3 mutations that has been frequently postulated. Better understanding of N3 aggregation and its relation to VSMC degeneration in CADASIL may help developing novel therapeutic strategies.

MATERIALS AND METHODS

Constructs/cloning

Human N3 constructs have been described previously (15). N-terminal tags (V5/myc) were inserted in the N3 full-length vector construct by an oligonucleotide containing the tag flanked by NotI cleavage sites, which were used for cloning. S1-truncated extracellular N3ECD constructs (amino acid
Cell culture, transfection and immunoblotting

HEK293 cells (DSMZ, Germany) were propagated in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen). For transient transfection, HEK293 cells were grown in 35 mm plates and transfected with 1.5 µg cDNA using 9 µg polyethyleneimine (Polysciences Europe, Eppelheim, Germany) as described previously (30). Twenty-four hours after transfection, cells were washed with PBS, harvested and lysed with TNT-lysis buffer (50 mM Tris–HCl, pH 8; 200 mM NaCl; 0.5% NP-40; protease inhibitor Complete EDTA-free, Roche Applied Science, Mannheim, Germany). After 20 min on ice, cell lysates were centrifuged at 16000 × g for 20 min and supernatants were analysed for total protein concentrations (BCA protein assay, Pierce, Bonn, Germany). Lysates were adjusted to 1 mg/ml total protein with lysis buffer. Protein gel electrophoresis and semi-dry immunoblotting were carried out using standard protocols and equipment (Biorad, Munich, Germany). V5-epitopes were detected using a 1:10000 dilution of monoclonal mouse anti-V5 antibody (Invitrogen), myc-epitopes using a 1:4000 dilution of monoclonal mouse anti-myc (9E10, a gift of E. Kremmer, Helmholtz Zentrum, Munich, Germany). Detection was performed using HRP-labeled anti-mouse secondary antibody (1:8000 dilution, Dako, Hamburg, Germany), followed by high-sensitivity enhanced chemiluminescence detection (Immobilon Western HRP Substrate, Millipore, Schwalbach, Germany; Kodak Biomax MR films, Stuttgart, Germany). For incubation experiments, NEM (Sigma-Aldrich, Hamburg, Germany) was added to a final concentration of 20 mM, cupric phenanthroline to 1.3 mM, 1,10-phenanthroline and 0.25 mM CuSO₄ (both Sigma-Aldrich).

Immunoprecipitation

Cell lysates were immunoprecipitated using anti-V5 or anti-myc antibody coupled agarose beads (Sigma-Aldrich, 25 µl of slurry) using standard procedures. After incubation for 3 h, beads were washed three times with PBS and boiled in Laemmli buffer (375 mM Tris–HCl, pH 6.8; 30% (v/v) glycerol; 6% (w/v) SDS, 5% (v/v) β-Mercaptoethanol) for 5 min at 95°C. Proteins were then resolved and detected by SDS–PAGE and western blotting as described above.

Confocal single particle analysis of N3ECD in cell lysates

Cleared cell lysates from HEK293 cells (prepared as described above) were adjusted to a protein concentration of 1 mg/ml. Incubation was performed in TNT-lysis buffer in 0.5 ml reaction tubes at 37°C. For analysis at time points 0 h and 24 h, aliquots were diluted 1:10 into an antibody mix of anti-V5 antibodies labeled with Alexa Fluor 488 and Alexa Fluor 647, respectively (Invitrogen) at a final concentration of four fluorescent particles per focal volume each (Fig. 3). Fluorescence intensity distribution analysis (FIDA) and SIFT were performed on an Insight Reader (Evotec-Technologies, Düsseldorf, Germany) with excitation laser wave lengths at 488 nm (excitation power 200 µW) and 633 nm (300 µW). Measurement time was 10 s with five repeats per well. Scanning parameters were set to 50 Hz beam scanner, 100 µm scan path length and 2000 µm positioning table movement. Data of FIDA measurements were analyzed with FCSPP evaluation software version 2.0 (Evotec-Technologies). SIFT data were recorded in two-dimensional intensity distribution histograms Hiₜ;Ig with intensity values Ir, Ig from 0 to 255 photons/bin and bin width 40 µs (18) and analyzed with a 2D-SIFT software module (Evotec-Technologies) as described previously (20,31). Analysis of receptor multimerization using NEM and cupric phenanthroline to evaluate disulfide bridge formation was not feasible by SIFT because of interference with antibody integrity and quenching of fluorescence, respectively (data not shown).

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Conflict of Interest statement. None declared.

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