Cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a vascular dementia arising from abnormal arteriolar vascular smooth muscle cells. CADASIL results from mutations in Notch3 that alter the number of cysteine residues in the extracellular epidermal growth factor–like repeats, important for ligand binding. It is not known whether CADASIL mutations lead to loss or gain of Notch3 receptor function. To examine the functional consequences of CADASIL mutations, we engineered 4 CADASIL-like mutations into rat Notch3 and have shown that the presence of an unpaired cysteine does not impair cell-surface expression or ligand binding.

Cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is an inherited vascular dementia caused by mutations in NOTCH3. Pathologically, CADASIL is characterized by replacement of arteriolar vascular smooth muscle cells (VSMCs) by a granular material that closely associates with Notch3 extracellular domain (N3ECD) immunoreactivity.1,2 Consistent with the observed defects in CADASIL, arteriolar VSMCs express Notch3,2,3 a member of a family of transmembrane receptors crucial for cell fate determinations.4 Notch receptors consist of an intracellular domain (ICD) required for signal transduction and an extracellular domain (ECD) containing 29 to 36 epidermal growth factor–like repeats (EGFRs), each with 6 conserved cysteines.4 EGFR-11 and -12 are required for DSL (Delta, Serrate, LAG2) ligand binding,5 and CADASIL mutations introduce or delete a single cysteine1,6 within certain EGFRs. However, it is unknown whether CADASIL mutations lead to a loss or gain in Notch3 receptor function.

Mammalian Notch proteins are proteolytically processed by a furin-like convertase to produce a heterodimeric receptor.7 After DSL-ligand binding, additional proteolytic cleavage steps release the ICD, which translocates to the nucleus and interacts directly with the CSL (CBF/Su(H)/LAG1) transcription factor.8 In some systems, the Notch3 ICD (N3ICD) is a weak CSL activator, whereas in other systems it is comparable to the strong activity intrinsic to Notch1 ICD (N1ICD).9,10 Although N3ICD antagonizes N1ICD-induced CSL activation,11 ligand-induced activation of full-length Notch3 has not been reported. To study the effects of CADASIL mutations, CADASIL-like mutant Notch3 proteins were expressed in 293T human embryonic kidney (HEK) cells and assessed for cell surface expression and DSL ligand interaction.

**Materials and Methods**

**Cloning, Sequencing, and Mutagenesis of Rat Notch3 cDNA Clones**

A subcloned PCR fragment corresponding to mouse Notch3 nucleotides 288 to 1037 was used to screen E13 and adult rat brain cDNA libraries by standard procedures.12 Clones pHIP105 (nucleotides 1 to 754) and pRB702 (nucleotides 755 to 7319) were ligated at an Ndel site, generating a clone encoding the entire rat Notch3 (rNotch3) sequence (GenBank AF164486). This construct (pN3.1) was C-terminally epitope-tagged with a tandem repeat of hemagglutinin (HA) using PCR and subcloned into the expression vector pEF1α-BOS13 (pBOSN3HA). CADASIL-like mutations (R171C, H184C, C544Y, and R560C)1 were introduced using site-directed mutagenesis (QuikChange, Stratagene).

**Transfection, Biotinylation, and Immunoblotting**

HEK293T cells were propagated as recommended by the American Type Culture Collection. Cells were transfected with 4 μg of DNA using lipofection (Cytofectene, BioRad). Cell surface proteins were labeled with 0.5 mg/mL Sulfo-NHS-Biotin (Pierce) and biotinylated proteins were purified on streptavidin (SA) beads (Pierce) as previously described.14 Proteins were resolved by SDS-PAGE and identified by immunoblotting with either 1:1000 anti-HA antibody (12CA5) or 1:10 000 anti-erk1 antibody (C-16, Santa Cruz), followed by ECL or ECL-Plus (Amersham-Pharmacia).

**Ligand Binding Assay**

Transfected cells were incubated for 1 hour at RT with Delta1Fc (D1Fc) conditioned medium (CM) preclustered with Fc antibodies conjugated with Texas Red (TR) as previously described.15 D1Fc binding was monitored by the TR fluorescence, detected using a Leica DM RXA microscope and cooled CCD camera.

**Results and Discussion**

CADASIL mutations identified to date alter cysteine residues in EGF modules, a structure that is dependent on 3 disulfide bridges formed between 6 invariant cysteines. To study the consequences of CADASIL mutations on protein stability and function, we cloned a rNotch3 cDNA that encodes a protein 93% and 84% homologous to mouse and human NOTCH3, respectively, and contains all characteristic structural motifs found in Notch proteins. We introduced the following single CADASIL-like mutations in rNotch3 (Figure 1): R171C and H184C lie in EGF-4 where CADASIL mutations are seen, whereas C544Y and R560C alter EGF-13 and -14, which are near the Drosofila split loss-of-function mutant allele.16
The effects of these mutations on proteolytic processing and cell surface expression were assessed by transiently expressing wild-type (wt) and mutant Notch3 constructs in 293T cells. After biotinylation and immunoblotting with the 12CA5 antibody (Figure 2A), unprocessed full-length 280-kDa and processed 97-kDa polypeptides were detected in whole-cell lysates and SA precipitates from biotinylated transfected cells (Figure 2B). To ensure that detection of p280 Notch3 was not an artifact of inadvertent cell lysis, SA-precipitated proteins were probed with antibodies to the intracellular protein erk1. Although present in whole-cell lysates (Figure 2C), p44 erk1 was not detected among the biotinylated proteins (Figure 2D). Moreover, the ratio of p280 to p97 after SA precipitation was reversed relative to whole-cell lysates, indicating that the signal detected with SA precipitates is due to p280 and p97 cell surface expression. Normal processing and cell surface expression of these mutants suggest that the odd number of cysteines imposed by these mutations does not disrupt receptor maturation.

The constructs were expressed in 293T cells and tested for their ability to bind a soluble form of the rat Delta1 ligand, D1Fc. D1Fc consists of the extracellular portion of Delta1 fused to the Fc domain of human IgG and has been shown to bind and activate Notch1.15,17 D1Fc binding was detected with each Notch3 CADASIL mutant protein (Figures 3A through 3D) and appeared comparable to that detected for wt Notch3 (Figure 3E). No D1Fc binding was detected for cells transfected with the pBOS vector (Figure 3F) nor Notch3-expressing cells treated with clustered Fc CM (Figure 3G), indicating that the signal was specific for Delta1 and Notch3 sequences. Binding was also compared for D1Fc CM diluted 1:20, 1:30, and 1:50, and although binding affinities and on/off rates were not measured, no differences were detected.

Figure 1. Schematic representation of the processed rNotch3 protein with a C-terminal HA epitope tag. Like other mammalian Notch3 orthologues, the ECD of the receptor has 34 EGF-like repeats and LNR repeats. The p97 fragment contains the transmembrane domain, ankyrin repeats, and PEST sequences. The positions of the mutations examined in the present study are indicated.

Figure 2. Cell surface expression and processing of wt and mutant Notch3 in transfected 293T cells. A, Expression of HA-tagged wt (pBOSN3HA) and mutant Notch3 proteins in transfected 293T cells were detected by immunoblotting whole-cell lysates with the 12CA5 HA antibody followed by ECL. Both the p280 unprocessed receptor and the processed p97 form were detected. No protein was detected in cells transfected with empty vector (pBOS) or with a vector encoding GFP-tagged Notch1. B, Transiently transfected 293T cells were biotinylated then precipitated with SA beads overnight. Immunoblotting with 12CA5 and ECL-Plus detected both the processed p97 fragment and the full-length p280 protein among the precipitated proteins from cells expressing wt or mutant Notch3. No protein was detected in cells transfected with empty vector or a vector encoding GFP-tagged Notch1. Differences in the apparent abundance of proteins between A and B result from the increased sensitivity of ECL-Plus. C and D, To ensure that the detection of p280 and p97 in the SA precipitates represents cell surface expression, evidence of intracellular p44 erk1 biotinylation was determined using α-erk1 (C-16) and ECL. p44 erk1 was detected in whole-cell lysates from each of the transfected biotinylated cells (C) but was not detected in the SA-precipitated proteins (D).

Figure 3. D1Fc ligand binding by wt and mutant Notch3 detected by TR fluorescence. Transiently transfected 293T cells expressing the mutant Notch3 proteins (A through D), wt Notch3 (E and G), or empty vector (F) were incubated with a 1:5 dilution of D1Fc CM (A through F) or Fc CM (G) and examined by fluorescence microscopy. The CADASIL mutants (A through D) bind D1Fc similar to wt Notch3 (E). No binding is detected for cells transfected with control vector pBOS (F) or for those incubated with Fc CM (G).
with the different D1Fc CM concentrations (data not shown), suggesting that mutant and wt receptor–ligand interactions were qualitatively similar.

Our data suggest that CADASIL mutations act downstream of ligand binding, perhaps through disruption of ligand-induced shedding/clearance of the N3EC in CADASIL vessels has been reported, although accumulation was not detected with HEK cells expressing CADASIL mutant Notch3 forms in the absence of DSL ligands. Determination of whether ligand binding by these CADASIL mutants results in productive signaling is not straightforward, because ligand-induced Notch3 signaling has yet to be reported and both positive and negative activities have been reported for N3ICD. Nonetheless, experiments are in progress to determine whether abnormal accumulation or shedding of mutant ECD or disruption of Notch3 signaling underlies the pathogenesis of CADASIL.

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References

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