Conformational variability of the intracellular domain of *Drosophila* Notch and its interaction with Suppressor of Hairless

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The Notch receptor is the central element in an evolutionarily conserved signal transduction pathway that controls cell fates in metazoans. Receptor–ligand interactions trigger a cascade of proteolytic events that release the entire Notch intracellular domain (NICD) from the membrane, permitting its translocation into the nucleus and participation in a transcriptionally active complex. Using electron microscopy, we examined the structure of NICD and its interaction with the DNA-binding effector of Notch signaling, Suppressor of Hairless [Su(H)]. In conjunction with biochemical analyses, we found that *Drosophila* NICD is monomeric and exists in two primary conformational states, only one of which can bind Su(H). Furthermore, we show that changes in divalent cation concentrations lead to NICD self-association, which seems to be mediated by the polyglutamine-containing, opa-repeat region of NICD. Our study suggests that conformational modulation of NICD may define a mechanism of Notch pathway control.

biochemistry | electron microscopy

Notch signaling couples the cell fate acquisition of an individual cell with the cell fate choices made by its next-door neighbors. This cell signaling mechanism relies on interactions between the Notch receptor expressed on the surface of one cell with membrane-bound ligands expressed on the surface of adjacent cells. Receptor–ligand interactions trigger proteolytic events that eventually release the intracellular domain of Notch (NICD). The NICD then translocates to the nucleus, where it can assemble with the DNA-binding effector Suppressor of Hairless [Su(H)] and other nuclear proteins into a transcriptionally active complex that drives Notch-dependent gene expression (1–3). The accumulated molecular evidence indicates that the signal transduction mechanism of Notch does not involve an enzymatic amplification step but relies on a cascade of stoichiometric molecular interactions that lead to the transmission of the Notch signal into the nucleus. The signal itself is, therefore, the ≈1,000-aa long NICD, which participates directly in the initiation of downstream gene activity.

The molecular steps leading to the translocation of NICD into the nucleus and regulating the formation of the active complex remain unknown. However, it is clear that NICD physically interacts with many proteins both inside and outside the nucleus, and, thus, competitive protein binding could potentially influence the signaling capacity of NICD. For instance, under certain experimental conditions, Su(H) has been shown to associate with NICD in the cytoplasm (4). This interaction appears to involve Notch binding sites that overlap with those mediating the interaction between NICD and the cytoplasmic Notch signal modulator, Deltex (5, 6).

Despite the wealth of information that has accumulated over the past two decades regarding the mechanism by which signals are transmitted through the Notch cell surface receptor, very little is known about the stoichiometry of pathway–component interactions and the structural changes associated with these interactions. Additionally, because of the large size and domain

complexity of the Notch receptor, structural studies have thus far been confined to only fragments of the receptor. The crystal structures of NICD fragments relevant to the recruitment of transcriptional coactivators on DNA, as well as the ankyrin-repeat region of NICD, have been recently solved (7–10). However, information about the conformation of the entire NICD molecule or, indeed, its oligomeric state, is lacking. Given the nature of Notch signaling, such information is important if we are to understand the molecular mechanisms that govern the transmission of Notch signals from the cell surface to the nucleus. Therefore, using EM, we sought to examine the structure of the entire NICD and its interaction with the major Notch signal effector Su(H).

Here we report the results of our studies involving single-particle EM on purified NICD and Su(H), as well as the complex formed between these two molecules. In combination with analytical ultracentrifugation studies, we show that under physiological conditions, full-length NICD is a monomer and that it interacts with the transcription factor Su(H) in a 1:1 stoichiometry. We also show that both monovalent and divalent cations can affect the conformation of NICD and its ability to form a complex with Su(H). Finally, we provide evidence indicating that divalent cations can induce the formation of filamentous structures via the polyglutamine-rich, opa-repeat domain of NICD (11), demonstrating the potential of NICD to self-associate.

Results

***Drosophila* NICD Is an Asymmetric Monomer.** A polypeptide fragment corresponding to the *Drosophila* NICD (amino acids 1765–2703) (Fig. 1*a*) and the full-length Su(H) protein were independently expressed in Sf9 insect cells (see *Methods*). Both proteins were engineered with C-terminal Flag-tags and sequentially purified by anti-Flag M2 affinity chromatography and gel filtration chromatography. Elution profiles from the gel-filtration column revealed that each preparation contained a single component (Fig. 1). NICD eluted from the gel filtration column with an M_r of 250 kDa (Fig. 1*b*), whereas Su(H) eluted with an M_r of 75 kDa (Fig. 1*c*). To assess further the purity of the preparations, eluate fractions were resolved by SDS/PAGE. Coomassie staining confirmed the presence of a single predominant protein in each preparation, with NICD migrating at an M_r of ≈110 kDa and Su(H) migrating at an M_r of ≈70 kDa (Fig. 1*d*), consistent with the respective values of 98 and 67 kDa expected from their primary sequences.

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Abbreviations: NICD, Notch intracellular domain; Su(H), Suppressor of Hairless.

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Fig. 4. Influence of divalent cations on NICD structure. (a) Raw image of NICD incubated with 1 mM CaCl_2 at room temperature for 2 h, showing the marked increase in molecules adopting an extended conformation. (b) Raw image of NICD filaments formed in 10 mM CaCl_2 after 4 h. Thin NICD filaments are indicated with thin arrowheads, and a thick NICD filament is indicated with a thick arrowhead. (c) Raw image of negatively stained NICD particles in the presence of 200 mM MgCl_2 incubated for 24 h at 4°C. Both compact (white circles) and extended (white arrows) monomers are present. Thin filaments are indicated with black arrowheads. (Scale bars: 100 Å.)

formed in the presence of 10 mM CaCl_2 were unsuccessful as assessed by negative-stain EM (data not shown), suggesting that filament formation is not compatible with Su(H) binding. Thus, these observations not only demonstrate the profound impact calcium ions can exert on the structure of NICD but also indicate that NICD has the potential to self-associate and oligomerize.

On the other hand, the presence of 1 mM CaCl_2 , a concentration that cannot induce the formation of filamentous NICD structures, does not affect the complex formation of NICD with Su(H). The NICD–Su(H) complex formed in the presence of this low CaCl_2 concentration eluted from the gel filtration column similar to the complex formed in the absence of CaCl_2 (data not shown). Additionally, velocity sedimentation analysis revealed that the NICD–Su(H) complex behaved identically in the presence (1 mM) and absence of CaCl_2 (Fig. 3 *b* and *c*).

The Polyglutamine Region of *Drosophila* NICD Is Involved in Filament Formation. It is known that polyglutamine domains are associated with the formation of protein filaments as well as aggregates (13, 14), and *Drosophila* NICD contains a stretch of 31 glutamine residues near its C terminus (11). Such polyglutamine regions are also present, but to a lesser extent, in mammalian Notch 1 proteins. To probe the possible involvement of the polyglutamine region of NICD in filament formation, we took advantage of the polyglutamine aggregation inhibitor C2-8, which has been shown to prevent polyglutamine aggregation in Huntington's disease neurons (15). NICD was incubated with 10 mM CaCl_2 , and filament formation was monitored over the course of 72 h in the presence and absence of the C2-8 inhibitor (50 mM) (Fig. 5). Immediately upon mixing (0 h), the two samples appeared identical, containing both compact and extended NICD molecules (Fig. 5 *a* and *d*). After 4 h, the NICD sample containing C2-8 was similar to the 0-h mixture (Fig. 5*b*), whereas the sample without C2-8 showed the presence of thin filaments (Fig. 5*e*). After 24 h, the NICD sample lacking C2-8 formed thicker filaments and filamentous aggregates (Fig. 5*f*), and after 72 h, the sample contained excessive plaques consisting of tangled thick filaments (data not shown). By contrast, in the sample containing C2-8, no NICD filaments could be observed after 24 h (Fig. 5*c*) or 72 h of incubation in 10 mM CaCl_2 . These results strongly indicate that the polyglutamine region is responsible for filament formation, a region that may be naturally occluded by the N terminus in the compact NICD conformation.

To determine whether the C2-8 inhibitor is able to revert preformed filaments back to its constituent NICD particles, two separate aliquots from the NICD sample containing thin filaments (Fig. 5*e*) were removed. To one aliquot, 50 mM C2-8 was added, whereas the other aliquot served as a control for filament formation. After 24 h (Fig. 5*g*) or 48 h (Fig. 5*h*) of incubation in the presence of 50 mM C2-8 (Fig. 5*g*), only compact and extended NICD particles were observed. In contrast, the sample

that did not receive C2-8 contained thick, tangled filaments (Fig. 5*i*), and these thick filaments did not disassemble upon incubation with 50 mM C2-8 (Fig. 5*j*). These experiments demonstrate that the C2-8 polyglutamine inhibitor prevents the progression of filament formation and that the inhibitor can disassemble preformed thin filaments but not tangled thick filaments, suggesting that the polyglutamine repeats remain accessible only within the thin filaments.

Discussion

The results we report here suggest that the interaction of NICD with other proteins may be regulated by factors that influence NICD conformation. The data we obtained from analytical ultracentrifugation and EM analysis all point to NICD being a highly flexible monomer that can exist as an extended, linear molecule or in a U-shaped, more compacted state. Importantly, our data demonstrate that the ability of NICD to interact with Su(H) is conformation-dependent. The single particle averages indicate that binding is compatible only with a molecule that is at least partially extended (see *Results*), and this conclusion is supported by the observation that Su(H) does not interact with NICD in a low-ionic-strength environment, in which NICD adopts the compact, U-shaped state, but readily interacts with NICD in 1 mM CaCl_2 , a condition favoring the extended form of NICD. Thus, any factor that regulates NICD conformation could potentially regulate its association with Su(H) and, consequently, its activity. Importantly, these results imply that protein interaction assays between NICD and other protein species must be carefully controlled.

The role monovalent and divalent cations play in regulating NICD conformation and signaling *in vivo* remains to be determined. Our attempts to address this issue have not been conclusive, as we have found that manipulating intracellular calcium levels in cultured insect cells appears to induce apoptosis. Nonetheless, it must be emphasized that the cellular response to Notch signaling seems to be uniquely sensitive to signal dosage (3), and therefore changes in ion concentrations, which could modulate the availability of NICD to interacting proteins, may play a significant role in influencing the biological output of Notch activity. In this regard, it is important to note that changes in both extracellular and intracellular calcium concentrations appear to be important in localized Notch signaling within the developing organism (16, 17).

Our structural studies also reveal a potential of NICD for self-association. In the presence of high calcium concentrations, NICD forms filament-like structures, and polyglutamine aggregation inhibitors can reverse this physical state of NICD. Polyglutamine stretches have been shown to play important roles in protein function and have been associated with a number of neurodegenerative diseases. Moreover, it is thought that polyglutamine-mediated protein interactions can influence the oli-

Fig. 5. NICD in the presence of CaCl_2 and C2-8 polyglutamine inhibitor. Shown are raw images of NICD incubated with 10 mM CaCl_2 and the C2-8 polyQ aggregation inhibitor (50 μM) for 0 h (initial addition) (a), 4 h (b), and 24 h (c). Also shown are raw images of NICD incubated with only 10 mM CaCl_2 for 0 h (d), 4 h (e), and 24 h (f). After 4 h of incubation in only 10 mM CaCl_2 (e), equal aliquots of sample were removed and further incubated in either 50 μM C2-8 (g) or buffer (control) (i) for up to 24 h and reexamined on continuous carbon grids using EM. In g, both compact (white circles) and extended (white arrows) monomers were present. After an additional 24-h incubation period (h) the structural distribution of the population did not change. (j) Filamentous networks formed in only calcium (i) were further incubated with 50 μM C2-8 for 24 h. (Scale bars: 100 Å.)

gomerization state of proteins, affecting their activity (18, 19). One such example in *Drosophila* occurs in the case of Groucho, which is a member of a family of metazoan corepressors that is not only linked to Notch-dependent signaling but has widespread roles in development (20). Although a cellular role of NICD filaments we observe in the presence of calcium has yet to be determined, it is attractive to speculate that formation of such structures could be used as a means to sequester NICD in a nonactive state by virtue of our finding that they cannot bind Su(H).

This study emphasizes the utility of EM analysis in addressing aspects of Notch signaling that have hitherto been unexplored and raises the hypothesis that conformational modulation of NICD may, in fact, define an important and fundamental mechanism in Notch pathway control.

Methods

Protein Expression and Purification. The entire intracellular domain of *Drosophila* Notch (amino acids 1765–2703) was cloned into pFastBac1 with sequences encoding a Flag tag at its C terminus and a 6xHis tag at its N terminus. Similarly, the entire Su(H) protein was cloned into pFastBac1 with sequences encoding myc and Flag tags at its C terminus. NICD and Su(H) were expressed in Sf9 cells using a baculovirus overexpression system and purified using M2-affinity (Sigma, St. Louis, MO) chromatog-

raphy. Flag-tagged proteins were eluted from the M2-column in 0.8 mg/ml Flag peptide in BC300 (20 mM Hepes, pH 7.9/300 mM KCl/10% glycerol/0.2 mM EDTA). Affinity-purified Flag-tagged proteins were further purified over a Superdex 200 column by using an ÄKTA FPLC.

Analytical Ultracentrifugation. Velocity sedimentation experiments were performed by using a Beckman (Fullerton, CA) Optima XL-I analytical ultracentrifuge with an An-60 Ti rotor operating at a speed of 50,000 rpm at 20°C. For the NICD and the NICD–Su(H) complex, 400 μl of sample was loaded into a double-sector quartz cell and blanked against the sample buffer containing either (i) 50 mM Hepes (pH 7.5) and 150 mM KCl, (ii) 50 mM Na_2PO_4 (pH 7.5) and 150 mM KCl, or (iii) 50 mM Na_2PO_4 (pH 7.5) and 30 mM KCl. Because Su(H) at concentrations of ≈ 1 mg/ml tends to form aggregates at KCl concentrations < 100 mM, for the experiments performed in 30 mM KCl, we first centrifuged the Su(H) sample (0.3 mg/ml) at $100,000 \times g$ for 30 min and examined the protein by negative-stained EM. The Su(H) sample appeared to be free of aggregates and remained so even after incubation in 30 mM KCl for up to 24 h. Absorbance data were collected in triplicate by using a step size of 0.005 cm in continuous mode at either 230 nm or 280 nm depending on the protein concentration. Absorbance data were analyzed by using the SEDFIT program (21).

Electron Microscopy and Image Processing. Three milliliters of sample solution were adsorbed to glow-discharged, carbon-coated, 200-mesh copper grids. Grids were washed with five drops of Milli-Q water and stained with two drops of 0.75% uranyl formate as described (22). Negatively stained specimens were examined by using an FEI (Hillsboro, OR) Tecnai 12 electron microscope operating at an accelerating voltage of 120 kV. Images were recorded on imaging plates under low-dose conditions at a magnification of $\times 67,000$ and a defocus value of approximately $-1.5\ \mu\text{m}$. Imaging plates were scanned by using a Ditabis scanner (Pforzheim, Germany) at a step size of $15\ \mu\text{m}$ and binned over 2×2 pixels for a final sampling of $4.48\ \text{\AA}$ per pixel on the specimen level.

Particles of each sample were selected from the images by using the WEB display program associated with the SPIDER software package (12). For NICD in EGTA, 7,301 particles were selected from 36 images, and for the NICD–Su(H) complex, 7,708 particles were selected from 55 images. For each data set, the selected particles were windowed into individual images of 70×70 pixels, and the particle images were subjected to 10 rounds of multireference alignment and K-means classification, specifying 30 output classes.

PolyQ Inhibitor Experiments. For the experiments using the C2-8 polyQ aggregation inhibitor (Calbiochem, San Diego, CA), a

100-ml sample of purified NICD (0.3 mg/ml in 50 mM Hepes, pH 7.5/150 mM KCl) was mixed with 10 mM CaCl_2 (final concentration) in the presence and absence of C2-8 (50 mM) and incubated for 72 h at 4°C . At time points of 0, 4, 24, and 72 h, negatively stained specimens were prepared of both mixtures and examined by EM. Additionally, at the 4-h time point, equal volumes of sample (25 ml each) were removed from the NICD mixture containing 10 mM CaCl_2 but not C2-8. To one of the aliquots, 25 ml of the above calcium buffer was added, and to the other aliquot, 25 ml of buffer containing C2-8 (50 mM final concentration) was added. Negatively stained specimens were prepared of each mixture after 24 h of incubation. After this step, we also incubated the sample that received the additional calcium buffer with 50 mM C2-8 for 24 h. Negatively stained specimens were again prepared after this final incubation step.

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