doi:10.1016/j.jmb.2011.09.050 J. Mol. Biol. (2011) 414, 243–259

Contents lists available at www.sciencedirect.com

Journal of Molecular Biology

journal homepage: http://ees.elsevier.com.jmb

Mapping the Deltex-Binding Surface on the Notch Ankyrin Domain Using Analytical Ultracentrifugation

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Received 28 July 2011;
received in revised form
28 September 2011;
accepted 29 September 2011
Available online
6 October 2011

Edited by J. E. Ladbury

Keywords:
Notch signaling;
Deltex;
ankyrin repeats;
analytical ultracentrifugation;
protein–protein interactions

The Notch signal transduction pathway controls cell fate determination during metazoan development. The *Notch* gene encodes a transmembrane receptor that is cleaved upon activation, liberating the Notch intracellular domain, which enters the nucleus and assembles transcriptional activation complexes that drive expression of Notch-responsive genes. The most conserved region of the Notch intracellular domain is an ankyrin domain (Nank), which binds directly to the cytosolic effector protein Deltex (Dx), controlling intracellular Notch activity. However, the structural and energetic basis for this interaction remains unknown.

Here, we analyze the thermodynamics and hydrodynamics of the Nank:Dx heteroassociation, as well as a weaker Nank self-association, using sedimentation velocity analytical ultracentrifugation. By comparing $g(s^*)$ and $c(s)$ distributions, and by direct fitting of sedimentation boundaries with thermodynamic association models, we were able to characterize the Nank:Dx heterodimer, measure its affinity, and map the interaction on the surface on Nank. N- and C-terminal deletions of whole ankyrin units implicate repeats 3 and 4 as key for mediating heteroassociation. An alanine scan across the interaction loops of Nank identifies a conserved hot spot in repeats 3 and 4, centered at R127, as critical for Dx binding. In addition, we were able to detect weak but reproducible Nank homodimerization (K_d in the millimolar range). This association is disrupted by substitution of a conserved arginine (R107) with alanine, a residue previously implicated in a functionally relevant mode of interaction within dimeric transcription complexes. The distinct binding surfaces on Nank for homotypic *versus* Dx interaction appear to be compatible with teterameric $Notch_2$:Dx_2 assembly.

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Introduction

The Notch signal transduction pathway comprises an evolutionarily conserved network of interactions that controls cell fate determination in a broad distribution of tissues during metazoan development.[1–3] In humans, the Notch pathway is involved in the differentiation of T cells and the maintenance of progenitor states, including stem cells.[4–6] Mutations in the Notch signaling pathway have been linked to multiple types of cancers[7,8] and inherited diseases.[9–12] Moreover, some viruses, such as the Epstein–Barr virus, use misactivation of the Notch signaling pathway as part of their viral life cycle.[13]

The *Notch* gene encodes a large single-pass transmembrane receptor. In the canonical mode of signaling, the binding of transmembrane protein ligands from the surfaces of adjacent cells activates

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Abbreviations used: Dx, Deltex; NICD, Notch intracellular domain; AUC, analytical ultracentrifugation; MASLS, multi-angle static light scattering; SA model, self-association model; SA+ID model, self-association, incompetent dimer model; MBP, maltose binding protein.

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the Notch receptor by stimulating a pair of proteolytic cleavage reactions. The second of these cleavage reactions liberates the Notch intracellular domain (NICD), which travels to the nucleus. Once inside the nucleus, NICD dissociates repressor proteins from Notch-responsive promoters through the DNA-binding protein CSL in what is believed to be a direct displacement mechanism.[14–16] Moreover, NICD directly participates in the assembly of transcriptional activation complexes by recruiting coactivator proteins to CSL complexes bound to Notch-responsive promoters, thereby driving expression of Notch-responsive genes.

The intracellular domain includes a highly conserved ankyrin domain (Nank), composed of seven tandem ankyrin repeats (Fig. 1).[19–21] Ankyrin repeat proteins are composed of 33 residue tandem repeats that each adopt two short α-helices and an extended β-hairpin. Adjacent repeats are stacked in a linear array. Ankyrin repeat domains typically mediate protein–protein interactions through sequence-specific interactions involving residues in the β-hairpin and adjacent helix.[17,18] Indeed, in structures of the CSL:NICD complexes with the coactivator mastermind (MAML) bound,[22,23] the Notch ankyrin domain makes extensive contacts with CSL through this canonical surface (Fig. 1b). In addition, a novel contact that appears to facilitate cooperative assembly at paired head-to-head CSL binding sites has been identified crystallographically between Nank domains.[24] Although yeast two-hybrid and colocalization studies supported homotypic association of Nank,[25] physical studies using equilibrium analytical ultracentrifugation (AUC) and multi-angle static light scattering (MASLS) were unable to detect association,[21,26,27] indicating a very weak mode of interaction.

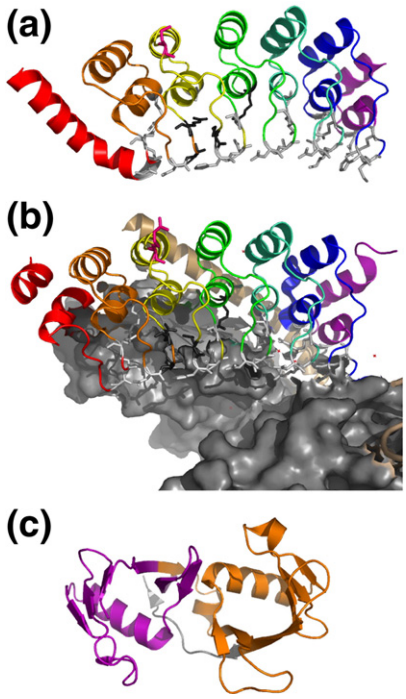

Fig. 1. Structure of the Notch ankyrin domain and interaction with its partners. (a) The *Drosophila* Notch ankyrin domain [Protein Data Bank (PDB) code 1OT8]. Ankyrin repeats are colored from red (first repeat, largely disordered) to purple (seventh repeat). Positions typically involved in direct contacts with target proteins in other ankyrin repeat complexes (excluding Nank) are shown in white.[17,18] Positions involved in Dx interaction (Table 3) are shown in black. (b) Complex between the human Notch1 ankyrin domain and CSL (PDB code 2F8X). The color code for the Notch1 ankyrin domain is the same as in (a); the surface of CSL is colored gray. The extended helix of MAML and bound DNA are colored brown; R1985 (the equivalent to R107) is shown in pink. (c) The *Drosophila* Dx WWE_2 domain (PDB code 1A90).

The ankyrin domain of Notch has also been implicated in interaction with Deltex (Dx),[28–30] a protein identified in a *Drosophila* screen for genes that modify Notch signaling.[31–33] Dx contains two tandem N-terminal WWE modules (the WWE_2 domain, Fig. 1c)[34] and a C-terminal RING finger motif.[29] Equilibrium AUC and MASLS experiments demonstrated the formation of a heterodimer between Nank and the *Drosophila* WWE_2 tandem of Dx.[35] Treating the binding reaction as a homotypic dimerization (the two constructs used in equilibrium AUC had nearly identical molecular weight), we estimated the dissociation constant to be 5 μM at physiological salt concentrations.[35]

The precise role of Dx in Notch signaling remains unclear. Biochemically, there is some evidence that Dx has E3 ubiquitin ligase activity,[36,37] consistent with the C-terminal RING finger. In analogy to other E3 ligases, it is possible that the WWE_2 domain acts as a recognition module, selecting targets (in this case, NICD) for ubiquitination. There is mounting evidence that Dx may regulate Notch endocytosis.[38–41]

Genetically, Dx activates Notch signaling in some contexts[29,30,42] but inhibits it in others.[43] In *Drosophila*, homozygous and hemizygous Dx loss-of-function alleles are nonlethal but result in a number of morphological perturbations.[44] In humans, Dx mutations have been linked to Noonan's syndrome,[12] an autosomal dominant congenital disorder.

To better understand the mechanisms by which Notch signaling is regulated, we have quantified and structurally dissected both Nank:Dx heteroassociation and Nank self-association using sedimentation velocity AUC. We used a combination of $g(s^*)$[45,46] and $c(s)$[47] analysis to identify association schemes most appropriate to different constructs and their mixtures and used direct boundary fitting with SEDANAL[48] to quantify the affinity of the

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