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Mapping the Deltex binding surface on the Notch ankyrin domain using analytical ultracentrifugation

Andrea Gayle Allgood** and Doug Barrick*

T.C. Jenkins Department of Biophysics, Johns Hopkins University, 3400 N. Charles St., Baltimore, MD 21218

Abstract

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 (NICD), which enters the nucleus and assembles transcriptional activation complexes that drive expression of Notch-responsive genes. The most conserved region of NICD 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, and also a weaker Nank self-association, using sedimentation velocity analytical ultracentrifugation (AUC). 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 three and four as key for mediating heteroassociation. An alanine scan across the interaction loops of Nank identify a conserved hot-spot in repeats three and four, centered at R127, as critical for Dx binding. In addition, we were able to detect weak but reproducible Nank homodimerization (K_d in the mM 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 Deltex interaction appear to be compatible with tetrameric Notch₂:Dx₂ assembly.

Keywords

Notch signaling; Deltex; ankyrin repeats; analytical ultracentrifugation; protein-protein interactions

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

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*Corresponding Author: TEL (410) 516-0409; barrick@jhu.edu.

**Current Address: Department of Chemistry, East Carolina University, Science and Technology Building, Greenville, NC 27858.

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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 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 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 (Figure 1).^{17–19} Ankyrin repeat proteins are composed of thirty-three residue tandem repeats that 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.^{20; 21} Indeed, in structures of the CSL:NICD complexes with the co-activator mastermind (MAML) bound,^{22; 23} the Notch ankyrin domain makes extensive contacts with CSL through this canonical surface (Figure 1B). In addition, a novel contact has been identified crystallographically between Nank domains that appears to facilitate cooperative assembly at paired head-to-head CSL binding sites.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,^{19; 26; 27} indicating a very weak mode of interaction.

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} Deltex contains two tandem N-terminal WWE modules (the WWE₂ domain, Figure 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* WWE₂ tandem of Deltex.³⁵ Treating the binding reaction as a homotypic dimerization (the two constructs used in equilibrium AUC had nearly identical molecular weight), the dissociation constant was estimated to be 5 μ M at physiological salt concentrations.³⁵

The role of Deltex in Notch signaling remains unclear. Biochemically, there is some evidence that Deltex 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 WWE₂ domain acts as a recognition module, selecting targets (in this case NICD) for ubiquitination. There is mounting evidence Deltex may regulate Notch endocytosis.^{38–41}

Genetically, Deltex activates Notch signaling in some contexts,^{29; 30; 42} but inhibits it in others.⁴³ In *Drosophila*, homozygous and hemizygous Deltex loss-of-function alleles are nonlethal, but result in a number of morphological perturbations.⁴⁴ In humans, Deltex 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:Deltex heteroassociation and Nank self-association using sedimentation velocity analytical ultracentrifugation. 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 SEDANAL⁴⁸ to quantify the affinity of the interaction. This approach confirmed heterodimerization of Nank and Dx, and yielded a K_d of 13 μ M. In addition, we found that both Nank and Deltex WWE₂ weakly self-associate (with K_d 's in the mM range). The R107A point substitution in the third repeat decreases this self-association, indicating a weak but functionally relevant Nank self-association, in agreement with the higher order transcription factor complexes reported by Nam *et al.*²⁴ Using both wild-type Nank and the R107A variant to suppress this weak homodimerization, we mapped the binding surface of Nank involved in the heteroassociation with the Deltex WWE₂ domain. A combination of Nank deletion constructs and point substitutions identify Nank residues R127, R85, and M115, which are clustered in repeats 3 and 4 of the canonical ankyrin binding surface, as important for Nank heteroassociation with Deltex.

RESULTS

In this study, we used sedimentation velocity AUC to monitor the types of associations between the Notch ankyrin domain and the Deltex WWE₂ domain. This experimental approach to probing association in solution is well-suited to detect weak associations,⁴⁹ and with direct boundary analysis, can accurately test different stoichiometric models for association and provide quantitative estimates of binding constants.⁵⁰ Though the primary goal of this study is to map the Deltex interaction surface onto the Notch ankyrin domain, we began by characterizing the association state of the two domains in isolation. Previous methods with lower sensitivity have indicated these domains to be monomeric, although the higher sensitivity of sedimentation velocity AUC allows weak associations to be detected. Characterizing these weak associations separately allows us to more tightly constrain our analysis of mixtures of the two domains.

Nank1-7* displays weak self-association during sedimentation velocity ultracentrifugation

To determine the sedimentation behavior of the *Drosophila* Notch ankyrin domain in isolation, we sedimented a construct containing seven ankyrin repeats (Nank1-7*) over a 27-fold range of concentrations (from 2.5 to 65 μ M at the start of the run¹) in a Beckman XL-I analytical ultracentrifuge at 50,000 rpm and 20°C. Concentrations were monitored during sedimentation by interferometry. In all cases, samples sedimented as a single boundary (see, for example, Figure 2A).

To better quantify the sedimentation properties of Nank1-7* and to detect weak homotypic association, $g(s^*)$ and $c(s)$ sedimentation coefficient distributions were computed for each protein concentration (Figure 3). These two distributions have different advantages and disadvantages.⁵¹ Both methods remove time-invariant noise, although through different approaches. The $g(s^*)$ distribution² (Stafford, 1992; Philo, 2000), which is generated by numerical transformation of the primary data, includes both sedimentation and diffusion components. In contrast, the $c(s)$ distribution (Schuck, 2000), which is generated by best-fit solution of the Lamm equation, attempts to account for (and thus remove the effects of) diffusion by assuming all species to have the same frictional ratio f/f_0 .

¹Note that as described in Materials and Methods section, these concentrations are lower than the initial loading concentrations, partly due to dilution from meniscus matching centerpieces.

²The s^* notation is used because the sedimentation coefficient is an apparent coefficient, and can be influenced by solute-solute interactions as concentrations are increased (Stafford, 1992).

As a result, $c(s)$ distributions have significantly sharper peaks than $g(s^*)$ distributions (Figure 3). In principle, peaks can be distinguished in $c(s)$ distributions that are not resolved in corresponding $g(s^*)$ distributions. Furthermore, the $c(s)$ distribution benefits from being able to use the entire data set instead of being limited to a small time range like the $g(s^*)$. However, there is some inherent ambiguity in the $c(s)$ distribution because of the approximations and fitted parameters. In particular, there is the possibility of finding slightly different distributions that fit the data equally well.⁵¹

Both $g(s^*)$ and $c(s)$ sedimentation coefficient distributions support a weak self-association and/or aggregation model for Nank1-7*. The $g(s^*)$ distributions show a single broad peak that shifts slightly (but reproducibly) to higher sedimentation coefficient values as concentration is increased, suggesting a weak association (Figure 3A). s^* values averaged from $g(s^*)$ distributions reflect this modest increase (Figure S1). At low concentration, the weight-average s^* value is 2.28, which is consistent with a slightly nonspherical monomer of this molecular weight. Even at highest concentration, the weight-average s^* value (2.489) is significantly less than the range expected for a dimer ($2^{2/3} s_{\text{monomer}}$), indicating that although association can be detected, it is quite weak. The $c(s)$ distribution shows an additional peak at a higher sedimentation coefficient value that is consistent with a homodimer ($s = 3.3$).

To confirm the presence of weak self-association and attempt to quantify the strength of the association, the primary data were fitted directly with the Lamm equation using the SEDANAL program.⁴⁸ In contrast to $g(s^*)$ and $c(s)$ distributions, globally fitting to the primary data takes full advantage of the information present in the reaction boundary⁵² and thus can provide more detailed information about macromolecular dissociations than sedimentation coefficient distribution analysis. Fitting with a single-species monomer model had a comparatively high standard deviation (M model, Table 1) and showed substantial systematic residuals (Figure 4A). These nonrandom residuals reflect the fact that the fitted $\Delta c/\Delta t$ curves are narrower than the experimentally observed difference curves.

Including a self-association term ($2A \xrightleftharpoons{K_A} A_2$) lowers the standard deviation of the fit (SA model, Table 1) and reduces the amount of systematic deviation, but the fit is still poor at higher radial values (Figure 4B). The fitted dissociation constant for homodimer formation is extremely high ($K_d = 61.3$ mM), and the fitted sedimentation coefficient for the homodimer is significantly higher than that possible for a protein of this size ($s = 84.4$). Restraining the s_{A_2} -value to a more physically reasonable range (i.e., $s_{A_2} < 2^{2/3} s_A$) significantly decreased the fitted K_d , but had a higher residual than the unconstrained SA model. Combined with the poor fit of the SA model, the very high fitted values for s_{A_2} and K_d ³ indicates that, like the single-component model, the SA model is missing species (and perhaps timescales) that contribute to the sedimentation of Nank1-7*.

In contrast, a model that includes an incompetent (i.e., irreversible) dimer (A_2') as well as a reversible association

fits the data reasonably well (SA+ID model, Table 1; Figure 4C), and gives fitted parameters that are well-determined (Table 1). In this model, the molecular weight and sedimentation

³In addition, the high values of s_{A_2} and K_d may also reflect strong covariance (and thus high uncertainty) between these two parameters.

coefficient of this incompetent dimer are constrained to be the same as those of the reversible dimer; the amount of incompetent dimer is estimated by adjusting the ratio of the incompetent dimer relative to the total amount of Nank ($r_{A2'}/A$). The fitted value of $r_{A2'}/A$ is fixed at the start of the run, and the fraction of the total material in the incompetent dimer is held constant over the time course of the experiment. This incompetent dimer is likely to be capturing a small amount of irreversible aggregate present in the solution. Such an aggregated species may be contributing to the peak at higher s -value in the $c(s)$ plot (Figure 3B, although these peaks may also be influenced by the reversible association in a way that is non-trivial). Although aggregation would be expected to include higher order incompetent multimers, because the range of fitted $\Delta c/\Delta t$ curves is relatively narrow, only a small range of sedimentation coefficients (biased to lower values) will be detected.

The fitted self-association + incompetent dimer model (Equation 1) fits the Nank1-7* sedimentation data with lower standard deviation than either the single species or self-association only models (Table 1). Furthermore, the residuals are more uniform, reflecting a better match of the fitted model to the data (Figure 4). The value of the fitted homodimer sedimentation coefficient is reasonable for a protein of this size, and only a small amount of incompetent dimer (5.2%) is needed to fit the data well. The fitted homodimer association equilibrium constant is still quite weak ($K_d = 1.62$ mM), but is substantially stronger than from the self-association model lacking incompetent dimer. Moreover, the sedimentation coefficient for the dimer (3.61 for both A_2 and A_2') are in the range expected for a dimer of this molecular weight. Error analysis using F-statistics,⁵³ which accounts for parameter covariance, indicates that both K_d and s_{A2} are tightly bounded in fits of equation 1 to the data (Table 1). These characteristics confirm the self-association + incompetent dimer model as the best fit for the primary sedimentation velocity data.

Point substitution R107A eliminates reversible self-association

The weak self-association ($K_d \sim 1.6$ mM) observed above for Nank1-7* may be related to the mode of association described by Nam *et al.*²⁴ where higher order complexes assemble on head-to-head paired DNA binding sites. This complex involves an interface between ankyrin domains in which an arginine residue (R1985 in human Notch1; Figure S2) in the third ankyrin repeat of Notch (R107 in the Nank1-7* construct) plays a pivotal role. To determine if this residue influences self-association, we substituted this arginine with alanine; Nam *et al.* reported that this substitution in human Notch1 abrogates transcriptional activation from sequence-paired binding sites.²⁴ We find that this point substitution has no effect on protein folding or stability (Figure 6A, B).

Unlike wild type Nank1-7*, the $g(s^*)$ distribution of R107A (Figure 3C) does not display concentration shift towards higher s^* values, indicating that this substitution substantially disrupts self-association. Moreover, the s^* values averaged from these $g(s^*)$ distributions are independent of concentration (Figure S1). Although a putative dimer peak is detected in the $c(s)$ distribution of R107A, it is significantly decreased compared to wild type Nank1-7* (Figure 3D; 2.3% of R107A compared to 15% of wild type Nank1-7*). To quantify the decrease in homodimerization strength resulting from R107A substitution, the sedimentation velocity boundary data were fitted directly using SEDANAL. When fitted using the self-association + incompetent dimer model, the fitted K_a is reduced to numerical values around zero, indicating that self-association is largely eliminated at these concentrations (74 μ M; Table 1). Including an incompetent dimer term, which is modeling small amounts of aggregation, makes minor improvements to the single species fit, but no improvements occur with the addition of an equilibrium dimer. The sedimentation coefficient of the monomer agrees well with that of wild type Nank1-7* (2.33 vs. 2.38). The value for the incompetent dimer sedimentation coefficient is similar, but not identical, to that of wild type (3.95 vs. 3.61). Given that this value is likely to be influenced by the extent of

heterogeneous, irreversible aggregate, its numerical value is likely to be sensitive to small differences in sample preparation and history. Additionally, these parameters are likely to be poorly determined, despite the narrow 95% confidence interval, owing to the low percentage of incompetent dimer present.

Dx1A* also demonstrates weak self-association and slight aggregation at high concentration

Sedimentation coefficient distributions for the WWE₂ domain of Deltex (protein construct Dx1A*) behave similarly to those of Nank1-7* (Figure 3E, F). The $g(s^*)$ distribution shifts slightly towards a higher weight-averaged sedimentation coefficient with increasing concentration (and average s^* values increase modestly with concentration, Figure S1), and the $c(s)$ distribution shows peaks at higher sedimentation coefficient values, consistent with weak self-association and/or slight aggregation. As with the wild type Nank1-7*, the best-fit model using SEDANAL is self-association + incompetent dimer. The fitted sedimentation coefficients are not unreasonable for a protein of this size in monomeric and dimeric form (Table 1), although the sedimentation coefficient of the monomer is somewhat low, consistent with the disordered loops identified in the crystal structure of Dx1A*. Like Notch, the Deltex WWE₂ domain demonstrated self-association using yeast two-hybrid experiments²⁹ but was monomeric in solution using *in vitro* experiments.³⁵

Unlike Nank, there is as yet no evidence that suggests Dx1A* self-association has functional relevance. Previous *in vivo* studies showed that WWE₂ domain self-association, mediated by fusion to a C-terminal dimerization domain (GST), did not have any impact on Deltex activity, despite being observed with yeast two-hybrid studies. Further studies will be required to determine what, if any, functional significance is associated with the weak Dx1A* self-association seen here.

The Notch ankyrin domain and Deltex WWE₂ domain heterodimerize with moderate affinity

Compared to the largely monomeric nature of Nank1-7* and Dx1A* when each domain is isolated, the heteroassociation when these domains are present in equimolar mixtures is evident even in the raw data: the boundary of a 1:1 Nank1-7*:Dx1A* mixture sediments significantly faster than either Nank1-7* or Dx1A* alone (Figure 2). Furthermore, compared to the modest shifts in the $g(s^*)$ profiles of Nank1-7* and Dx1A* alone, the $g(s^*)$ distributions of the 1:1 mixture show large shifts with increasing concentration, consistent with concentration-dependent association (Figure 5A, Figure S1). Likewise, the $c(s)$ distribution demonstrates a reduction in the monomer peak area and the appearance of a large peak at higher sedimentation coefficient value (Figure 5B).

Utilizing the findings above about the behavior of Nank1-7* and Dx1A* individually, the heteroassociation of Nank1-7* and Dx1A* was analyzed using SEDANAL. The models used to describe Nank1-7* (A) and Dx1A* (B) separately were incorporated into a heteroassociation model (Equation 2) that included a 1:1 heteroassociation for Nank1-7*:Dx1A* (AB):

The value of the heteroassociation equilibrium constant and the sedimentation coefficient of the heterodimer were allowed to float freely; all other parameters were constrained to the

95% confidence intervals of the parameters determined by the individual fits. This model fitted reasonably well to a series of sedimentation velocity data at loading concentrations ranging from 1.27 to 35.6 μM Nank1-7* and Dx1A* (maintained at a 1:1 ratio). A sedimentation coefficient of 4.781 was obtained for the Nank:Dx1A* heterodimer, which is at the upper limit of the range expected for a heterodimer of this molecular weight. A dissociation constant of 12.8 μM was obtained for the heterodimer.

The R107A substitution has no impact on the heterodimerization between Nank and Deltex

Using a two-component (monomer + incompetent dimer) model to describe the properties of unbound R107A, we obtained a fitted R107A:Dx1A* heterodimer K_d of 12.9 μM (Table 2), indicating that while R107A eliminates the weak self-association seen for wild-type Nank, it does not impact Nank:Deltex heteroassociation. This observation suggests that a different surface is used to interact with Deltex than is used in homotypic Notch self-association. This result is consistent with the self-association surface identified by Nam *et al.*²⁴, which is opposite to the surface typically involved in ankyrin repeat protein interactions.^{20; 21}

Deletion studies identify which Notch ankyrin repeats are involved in Deltex binding

Because repeat proteins lack long-range contacts, individual repeats can be removed without disrupting the overall protein fold. Previous work by Mello and Barrick studied the folding of various deletion constructs of the Notch ankyrin domain.⁵⁴ Here we use these whole-repeat deletion constructs (repeat boundaries indicated in Figure S2) to identify which repeats are critical for binding to Dx1A*.

As with the full-length (seven repeat) Notch ankyrin domain, all deletion constructs exhibited weak self-association except for Nank4-7*, which was best fitted by a single species model (Table 1). This result agrees with the result that R107, which is in the third repeat, is mediating self-association. Of all the ankyrin deletion constructs studied here, Nank4-7* is the only construct that lacks the third repeat.

The C-terminal deletion constructs (Nank1-6*, Nank1-5*, and Nank1-4*) appeared to aggregate more readily, as rapidly sedimenting species were observable in the early scans through means of two observable boundaries in the primary data (data not shown). However, the scans selected for $g(s^*)$ distributions and SEDANAL fitting were late enough that this first boundary had already cleared the plateau region. To further minimize this effect, these experiments were repeated using protein taken directly from a size-exclusion column without concentrating. In the resulting samples, the amount of rapidly sedimenting material was reduced drastically, to the point where the early boundary was not observable. The sedimentation coefficients for the monomeric forms of Nank1-6*, Nank1-5*, and Nank1-4* were unchanged, although reversible association was not needed to fit the data. This difference seems likely to be a result of the greatly reduced concentrations of the starting material caused by the inevitable dilution (to 10 – 30 μM) associated with size-exclusion chromatography.

The interactions of these deletion constructs with equimolar mixtures of Dx1A* were quantified by fitting the sedimentation velocity data with Equation 2 using SEDANAL (Table 2). Information from the individual fits was incorporated into lines 1 and 2 of Equation 2. N-terminal deletion of repeats one and two have minimal effects on Deltex binding, whereas deletion of repeat three significantly diminishes binding affinity. C-terminal deletion of repeat seven also decreases affinity although this effect is less than that observed for repeat three; further deletion of repeats six and five have only modest effects on binding, although the confidence intervals for the fitted binding constants are somewhat large for these constructs.⁴ Although we were unable to directly test the contribution of

repeat four (Nank deletion constructs with only three repeats are not folded), the observation that binding is detected both for Nank1-4* and Nank4-7* indicates that repeat four also contributes to binding (Table 2). Taken together, the deletion data suggest that repeats three and four are important for binding, but that some affinity is derived from repeats six and seven.

Mapping residues on Nank involved in Deltex binding using point substitutions

To further map the Nank binding interface to Deltex, site directed mutagenesis was used to substitute surface residues with alanine in all repeats, and the binding of these variants to Dx1A* was monitored during sedimentation velocity. Residues were selected from the canonical ankyrin binding surface, which encompasses the surface between the loops and the first helix of the ankyrin repeat structure (Figure 1). To narrow the list of substitution sites further, we identified residues within the canonical ankyrin binding surface with high sequence conservation among Notch homologues. Based on these two criteria, we identified 19 residues for alanine substitution (Figures 6, S2). In most cases, single point substitutions were tested, but in some cases, two or three neighboring substitutions were initially combined.⁵ The monomeric R107A variant was used as a background for these point substitutions to eliminate self-association and simplify the fitted models for binding. Far-UV CD and urea-denaturation experiments were performed to verify that all variants were well folded under experimental conditions (Figure 6A, B).

Sedimentation velocity experiments of all Nank variants in the absence of Deltex were analyzed and most were best fitted by a monomer + incompetent dimer model in SEDANAL, similar to R107A (Table S1). Two aspartate to alanine mutations (D81A and D117A), however, exhibited weak self-association despite the R107A substitution. These substitutions were the most destabilizing by urea-denaturation (although all constructs appeared to retain a folded native state; Figure 6A, B), so it is possible that the self-association could be coupled to partial unfolding. Fits of R85A (R107A) and H116A (R107A) were not improved by the addition of an incompetent dimer, so a single species model was used for these fits.

Fitted values obtained from the analysis of sedimentation velocity data of Nank point substitutions in the absence of Deltex were used as constraints in the analysis of binding in equimolar mixtures of Dx1A* with the substituted Nank variants (Table 3). One protein, D117A (R107A), does not fit well without incorporating an additional incompetent heterodimer term into the fitting model. Three point substitutions in the third and fourth repeats (R85A, M115A, and particularly, R127A) significantly weaken the Nank:Dx1A* interaction. The other nine variants, all in repeats 1, 2, 6, and 7, have no significant effect on binding (Table 3).

The greatest change in binding is observed by the R127A (R107A) substitution (fourth repeat), which shows no detectable binding to Dx1A* (Figures 7, S1). Equimolar mixtures of Dx1A* with the R127 (R107A) variant are best fitted by a model that only incorporated the parameters of the individual proteins and does not include a heteroassociation equilibrium constant or a sedimentation coefficient for the heterodimer. The R85A (R107A) substitution (third repeat) increases the dissociation constant by a factor of five. The M115A (R107A) substitution (third repeat) increases the dissociation constant by a factor of two. These results are consistent with the deletion construct results that indicate the third repeat is

⁴Broader confidence intervals for Nank1-6*, Nank1-5*, and Nank1-4* may reflect the dilution resulting from the additional size exclusion chromatography step.

⁵If variants with multiple substitutions were found to disrupt binding, each substitution was to be studied separately. However, we found that none of the variants with multiple substitutions (which were made in repeats 1, 6, and 7) affected binding.

important for binding. A fourth substitution, T83A (R107A), also located in the third repeat near R85 and M115, results in a modest increase in the K_d , although the change is less than two-fold, it is significant according to the 95% confidence intervals determined by F-statistics.

DISCUSSION

The primary motivation for this study was to obtain a structural map of the interaction surface for the Deltex WWE₂ domain on the Notch ankyrin domain. This required a quantitative biophysical technique that could measure affinity over a range of K_d values from micromolar to millimolar and could be applied to Nank and Deltex constructs that differed from each other in molecular weight (as is required for the ankyrin deletion series). The method we use here, sedimentation velocity AUC, appears to be able to detect weak associations in which complexes are only partly formed. Examining the concentration dependence of $g(s^*)$ ^{45; 46} provides a sensitive and unbiased test for association, although the resulting sedimentation coefficient distributions are broad, owing to the high diffusion rates of these moderately sized macromolecules. Analysis of the data in terms of $c(s)$,⁴⁷ especially at high concentrations, provides a rough correction for diffusional spreading and gives a more detailed picture of the species formed and their sedimentation coefficients, although in some instances, peaks can result from reversible association rather than discrete, sedimenting species. Together, these complementary distributions provide a strong set of constraints for the types of interaction models to be used in direct boundary analysis with SEDANAL.^{48; 55}

The structural basis for the interaction of the Notch ankyrin domain with Deltex

Unlike globular proteins, ankyrin domains are tolerant to removal of one (33 residues) or more whole repeat units, particularly when repeats are removed from the N- or C-termini.⁵⁴ Our previously characterized terminal deletion constructs of Nank provided a convenient means to quantitatively probe which repeats are important for binding to Deltex.⁶ Based on changes in binding energy of this deletion series, it appears that ankyrin repeats three and four contribute significant binding free energy, but additional contributions extend to the C-terminal (but not N-terminal) repeats. Point substitution confirms the importance of repeats three and four in binding to Deltex, identifying a “hot spot”^{57; 58} for binding on the canonical recognition surface of these repeats (Figure 6E). Most notably, substitution of a single arginine with an alanine residue in the fourth repeat (R127A) completely disrupts binding, as detected by sedimentation velocity AUC (Figure 7). In addition, a point substitution (*N^{Su42c}*; substitution of a conserved alanine with a valine) has previously been reported in the fifth repeat that results in a *Deltex* loss-of-function phenotype. This alanine is near the Deltex binding residue R127 (Figure 6E) and may also contribute to the Deltex binding surface of Notch.²⁸ Although the deletion series did not implicate the fifth repeat in binding per se, it did identify a contribution from the C-terminal repeats; thus, close contacts with repeat five seem likely. The identification of a hot spot in recognition of Deltex by the Notch ankyrin domain is reminiscent of a hot spot found in binding of the Ets transcription factor GABP- α by GABP- β , an unrelated ankyrin repeat protein.⁵⁹

This picture is somewhat at odds with an earlier mapping using the yeast two-hybrid assay.²⁹ The two-hybrid experiments show a strong interaction with repeats 1–5 and the entire Notch intracellular domain, whereas in the AUC study presented here, deleting repeats 6 and 7 weaken the interaction. Deleting repeats 1 and 2 disrupts the two-hybrid

⁶The effects of deletion of internal repeats was not examined since previous studies have shown that such deletions are less stable than constructs missing terminal repeats.⁵⁶

signal, but contributes to the interaction detected by AUC. The major determinant of binding here is repeats 3–5, which in the two-hybrid assay is insufficient to activate transcription. However, it is likely that repeats 3–5 are not folded without stabilizing interactions with either N- or C-terminal repeats, which may explain the lack of interaction in the two-hybrid assay.²⁹

The Deltex-binding surface of Nank, including residue R127, maps to the canonical binding face for ankyrin repeats.^{20; 21} Moreover, this canonical surface of Nank has been shown crystallographically to interact directly with CSL.^{22; 23} This is shown in Figure 1A and B, where the subset of canonical binding residues shown here to interact with Deltex are colored black. Thus, the binding of CSL and Deltex to the Notch ankyrin domain are likely to be mutually exclusive. Since the interaction between Notch and CSL occurs in the nucleus, and Deltex is believed to mediate endocytosis of Notch in the cytosol, this overlap is not likely to result in direct competition (note, however, that Yamamoto *et al.* found significant nuclear localization for mammalian Deltex-1, although transcriptional inhibition appeared to be mediated by p300 rather than by Notch⁶⁰). However, recognition of the canonical interaction surface by both CSL and Deltex may tightly constrain sequence variation at the canonical interaction surface of the Notch ankyrin domain.

Weak homotypic interactions detected by sedimentation velocity AUC

In many cases, protein-protein interactions are identified in biologically based assays such as the yeast two-hybrid assay or coprecipitation from cell lysates, but cannot be detected by biophysical methods such as ITC, gel filtration, or light scattering. These discrepancies are often rationalized by dismissing the biologically based interaction as a “false-positive”; indeed, false positives are known to occur commonly with such approaches^{61; 62}. However, the alternative possibility that the interactions are both real and biologically important, but are too weak to detect by these biophysical methods, is difficult to rule out.

In addition to the comparatively strong interaction between the Notch ankyrin domain and Deltex, both individual domains appeared to interact by the two-hybrid studies, but association could not be detected by equilibrium AUC and MASLS.^{19; 27} The fact that weak homotypic interaction can be detected for both proteins by sedimentation velocity AUC highlights the high sensitivity of this technique for exploring weak interactions. Although these associations cannot easily be observed in the primary data (unlike the stronger heteroassociation between Nank and Deltex, see Figure 2), they are detected by three complementary modes of data analysis: $c(s)$, $g(s^*)$, and direct boundary fitting. Moreover, the direct boundary fitting method was able to give tightly constrained estimates of K_d values for these complexes, even though the constants are clearly in the low affinity range (~1 mM; Table 1). Indeed, this high sensitivity was no doubt key to our ability to determine quantitative changes in heterodimeric association between Nank and Dx1A* resulting from whole-repeat deletion and point-substitution.

We cannot be sure that the modes of interaction detected by sedimentation velocity are the same as those detected by two-hybrid and colocalization studies.²⁵ However, for Nank, disruption of homodimerization by the R107A point substitution provides a potential link between these two approaches. The observations that this substitution has no effect on protein stability (Figure 6) or Deltex binding (Table 1) indicates that this protein is folded and binding competent. Thus, the homotypic interaction we detected here is likely to be a specific (albeit weak) mode of association, rather than a nonspecific aggregation. Moreover, the fact that this substitution disrupts cooperativity in binding to (and subsequent activation from) paired CSL:DNA complexes²⁴ demonstrates that the interface we detect by AUC is of functional importance. It would be interesting to test whether the R107A substitution disrupts the Nank interaction detected in two-hybrid assay.

Like the canonical CSL:Nank interaction, our mapping indicates that this weak Nank homotypic interaction should be compatible with Nank:Deltex heterodimerization via the canonical recognition surface containing R127, R85, and R115. In other words, both interactions (Nank homodimerization and Nank:Deltex heterodimerization) should be able to form simultaneously, leading to heterotrimers and heterotetramers (Dx:Nank₂ and Dx:Nank₂:Dx, respectively). Consistent with this, *c(s)* distributions reveal a minor, concentration-dependent species in equimolar mixtures of Dx1A* with wild-type Nank1-7* at high sedimentation coefficient (Figure 5B) that is not detected (or is greatly diminished) in mixtures of the R107A variant. Likewise, fitted values for the Nank:Dx1A* heterodimer sedimentation coefficient (*s*_{AB}, Table 2) from SEDANAL are higher for wild-type Nank1-7* than for R107A, and *s** values averaged from *g(s*)* distributions of equimolar mixtures of Nank1-7* with Dx1A* are larger than those from mixtures with R107A (Figure S1).

In the same way that proximity of CSL sites is believed to promote the Nank homotypic interaction, Nank dimerization may be promoted by tethering to the plasma membrane prior to membrane cleavage. Thus, to the extent that Deltex interacts with membrane-bound Notch,^{39; 40} Deltex may recognize a dimeric population of Nank. The degree to which this dimerization may contribute to the Deltex:Nank interaction will require further studies.

MATERIALS AND METHODS

Site-directed mutagenesis and protein purification

The constructs for Nank1-7*, Nank deletion constructs, and Dx1A* are as previously described.^{19; 35; 54} All point substitutions were generated by site-directed mutagenesis performed using the Stratagene QuikChange II® kit. All proteins were expressed in either LB or TB with 0.1 mg/mL ampicillin. Prior to induction, cultures were grown at 37°C to an OD₆₀₀ appropriate for induction with 1 mM IPTG. Cultures grown in LB were induced at an OD₆₀₀ of ~0.7 and were subsequently grown at 37°C for four hours after induction. Cultures grown in TB were induced at an OD₆₀₀ of ~1.3 and grown at 20°C overnight. Bacteria were collected by centrifugation and cell pellets were stored at -80°C. Cell pellets were resuspended in 300 mM NaCl, 25 mM Na phosphate, pH 7.0 and lysed using a French press. After lysis, 0.2 mg/mL DNase I and 10 mM MgCl were added, and the lysate was stirred at 4°C for at least half an hour. Samples were clarified by centrifugation at 16,000 rpm in a Beckman JA-20 rotor for 20 minutes.

Nank1-7*, Nank2-7*, Nank4-7*, and all single-residue variants were purified from the lysis supernatant, which was applied to a 5mL bench-top Ni-NTA column (Qiagen) that had been equilibrated in lysis buffer. Protein-bound Ni-NTA columns were washed with lysis buffer containing 20 mM imidazole and eluted in lysis buffer with 500 mM imidazole. Proteins were dialyzed to 300 mM NaCl, 25 mM Tris, pH 8.0 and further purified by chromatography on a HiPrep™ 26/60 Sephacryl™ S-100 size-exclusion column (GE Healthcare Life Sciences) using an ÄKTApurifier 10 (GE Healthcare Life Sciences).

Nank1-6*, Nank1-5*, Nank1-4*, and Dx1A* partitioned primarily into the lysis pellets, which were resuspended in 6 M urea, 300 mM NaCl, 25 mM Na phosphate, pH 7.0. Resuspended pellets were then clarified by centrifugation at 16,000 rpm in a Beckman JA-20 rotor for 20 minutes. The resulting urea supernatant was applied to a urea buffer-equilibrated Ni-NTA column, washed with urea buffer containing 20 mM imidazole, and eluted in urea buffer with 500 mM imidazole. After the Ni-NTA column, urea was removed by two-step dialysis: dialyzed to 300 mM NaCl, 25 mM Tris, pH 8.0, then to 50 mM NaCl, 25 mM Tris, pH 8.0. After dialysis, Dx1A* was applied to a 5 mL HiTrap™ SP HP cation exchange column (GE Healthcare Life Sciences) equilibrated in Buffer A (50 mM NaCl, 25

mM Tris, pH 8.0) using an ÄKTApurifier 10 (GE Healthcare Life Sciences). Protein was washed with 2% Buffer B (1M NaCl, 25 mM Tris, pH 8.0) and then eluted with a linear gradient from 2% B to 10% B.

Nank3-7* was expressed as a Maltose Binding Protein (MBP) fusion, as described by Mello and Barrick.⁵⁴ MBP-Nank3-7* was purified from the supernatant with a Ni-NTA column as described above, and MBP-Nank3-7* was then cleaved by Tobacco Etch Virus (TEV) protease at 4°C. Cleaved Nank3-7* was purified by loading onto a 5 mL HisTrap™ Ni column (GE Healthcare Life Sciences) equilibrated in 300 mM NaCl, 25 mM Na phosphate, pH 7.0 using an ÄKTApurifier 10 (GE Healthcare Life Sciences). Protein was eluted by a linear gradient of running buffer containing 250 mM imidazole. Nank3-7* was dialyzed to 300 mM NaCl, 25 mM Tris, pH 8.0 and then further purified with a size-exclusion column as described above.

Protein purity was assessed by SDS-PAGE. Proteins were dialyzed after purification to 300 mM NaCl, 25 mM Tris, pH 8.0, and were concentrated using Vivaspin 6 centrifugal concentrators (Sartorius).

Circular Dichroism

Circular dichroism (CD) spectra were collected on an Aviv Model 400 CD Spectrometer in 300 mM NaCl, 25 mM Tris, pH 8.0 at 25°C. Far-UV spectra were collected in an 0.1 cm cell at protein concentrations of 20 μM. For shape comparison, spectra were scaled by shifting baselines (280 nm) to a value of zero and were divided by the ellipticity value at 222 nm. Urea-induced unfolding titrations were generated using a Hamilton Microlab 500 automated titrator. Unfolding transitions were monitored by CD at 222 nm in a 1 cm cell with protein concentrations of 2 μM. Free energies of unfolding were extrapolated to zero denaturant concentration, assuming a linear dependence of unfolding free-energy on denaturant,^{63; 64}

Sedimentation velocity analytical ultracentrifugation

All proteins were extensively dialyzed to 300 mM NaCl, 25 mM Tris, pH 8.0 before analytical ultracentrifugation. The resulting dialysate was used as a reference. For equimolar mixtures of Nank constructs and Dx1A*, proteins were dialyzed separately in the same buffer to ensure buffer matching. For sedimentation studies involving a single protein, samples were prepared at 90 μM by dilution in dialysis buffer. The 90 μM sample was then diluted 1:3, 1:9, and 1:27 with dialysis buffer to cover a concentration range from 90 μM to 3.33 μM. For equimolar mixtures, proteins were prepared at 45 μM Nank construct and 45 μM Dx1A*. These samples were diluted in the same three-fold series as the individual protein samples to make four samples for each construct at the same total protein concentration.

Analytical ultracentrifugation cells were assembled using SedVel60K 1.2 cm Meniscus Matching Centerpieces (SpinAnalytical) and sapphire windows. Sample and buffer were loaded into the cells and spun at 6,000 rpm until the menisci of sample and buffer matched. Concentrations were diluted somewhat as a result of the meniscus matching. The rotor and cells were then removed from the AUC and inverted multiple times to remix the samples. The rotor and cells were returned to the Beckman XL-I and allowed to equilibrate under vacuum at 20°C for at least two hours. Sedimentation velocity experiments were run for 8 hours at 50,000 rpm using interference optics. Interference scans were collected every 30 seconds for each cell.

Sedimentation coefficient $g(s^*)$ distributions were generated from a subset of the primary data where the sedimenting boundary curves had completely cleared the meniscus and were located approximately in the middle of the centrifuge cell using SEDANAL.⁴⁸ 150 curves

were used for individual proteins and 100 curves were used for mixtures. $c(s)$ distributions were generated using SEDFIT.⁴⁷ $c(s)$ distributions used every 25th scan, starting with the 100th scan and proceeding to the end of the run. Direct boundary fitting using the Lamm equation was performed in SEDANAL. Parameter confidence intervals were determined at the 95% level using F-statistics. The same scans used in the $g(s^*)$ distributions were used in the SEDANAL fits. Partial specific volumes were calculated from the protein sequence using SEDNTERP (<http://www.jphilo.mailway.com/>).⁶⁵ The $\Delta c/\Delta t$ curves for all four protein concentrations were fitted simultaneously. The protein molecular weight, partial-specific volume, and refractive index increment were held constant during the fitting process. The equilibrium constant and sedimentation coefficients were fit, as were the protein concentrations of the individual cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Structure of the Notch ankyrin domain, and interaction with its partners

(A) The *Drosophila* Notch ankyrin domain (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^{20; 21}. Positions involved in Deltex 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 panel (A), the surface of CSL is colored grey. The extended helix of MAML and bound DNA are colored brown; R1985 (the equivalent to R107) is shown in pink. (C) The *Drosophila* Deltex WWE₂ domain (pdb code 1A90).

Figure 2. Sedimentation velocity data for Nank1-7*, Dx1A*, and an equimolar mixture of the two proteins

Neighboring interference boundaries are offset by five minutes each over a 250 minute interval, at a rotor speed of 50,000 RPM. Curves are aligned from the meniscus to the cell base. (A) 64.9 μ M Nank1-7*. (B) 67.3 μ M Dx1A*. (C) 35.6 μ M Nank1-7* + 35.6 μ M Dx1A*.

Figure 3. Sedimentation coefficient distributions of separate Nank1-7* and Deltex constructs (A, B) Nank1-7*, (C, D) R107A, (E, F) Dx1A*. Left panels show normalized $g(s^*)$ distributions; right panels show $c(s)$ distributions. For $g(s^*)$ distributions, fitted concentrations and weight-averaged sedimentation coefficient values are as follows: (A, Nank1-7*) circles (●): 64.9 μM , $s_w=2.489$; squares (■): 22.7 μM , $s_w=2.441$; diamonds (◆): 7.25 μM , $s_w=2.369$; triangles (▲): 2.41 μM , $s_w=2.282$. (C, R107A) circles (●): 72.4 μM , $s_w=2.252$; squares (■): 24.4 μM , $s_w=2.226$; diamonds (◆): 8.01 μM , $s_w=2.242$. (E, Dx1A*) circles (●): 67.3 μM , $s_w=2.259$; squares (■): 24.9 μM , $s_w=2.139$; diamonds (◆): 8.39 μM , $s_w=2.109$; triangles (▲): 3.08 μM , $s_w=2.058$. The $c(s)$ distributions (B, D, F) are calculated for the highest protein concentration. For R107A (D), the $c(s)$ distribution for wild-type Nank1-7* (dashed line, — —) is shown for comparison.

Figure 4. Direct boundary fits of Nank1-7* using SEDANAL

Out of 75 $\Delta c/\Delta t$ curves fitted at each concentration, five are displayed, including the first and last curve. Residuals are shown below. Red: 64.9 μM ; blue: 22.7 μM ; green: 7.25 μM ; orange: 2.41 μM . Fitted curves are shown in black. (A) one-species fit. (B) self-association fit. (C) self-association + incompetent dimer fit.

Figure 5. Sedimentation coefficient distributions and direct boundary fit of equimolar mixtures of Nank and Dx1A*

(A–C) wild-type Nank1-7*, (D) R107A. (A) Normalized $g(s^*)$ distribution of Nank1-7* and Dx1A*. Circles (●): 35.6 μM Nank1-7* + 35.6 μM Dx1A*, $s_w=3.517$; squares (■): 12.5 μM Nank1-7* + 12.5 μM Dx1A*, $s_w=3.114$; diamonds (◆): 4.22 μM Nank1-7* + 4.22 μM Dx1A*, $s_w=2.856$; triangles (▲): 1.27 μM Nank1-7* + 1.27 μM Dx1A*, $s_w=2.804$. (B) $c(s)$ distributions of Nank1-7* and Dx1A*. Dotted line (.....): 64.9 μM Nank1-7*; dashed line (---): 67.3 μM Dx1A*; solid line (—): 35.6 μM Nank1-7* + 35.6 μM Dx1A*. (C) Direct boundary fit of Nank1-7* and Dx1A* using SEDANAL. Out of 50 $\Delta c/\Delta t$ curves fitted at each concentration, five are displayed, including the first and last curves. Residuals are shown below. Fitted curves are shown in black. Red: 35.6 μM Nank1-7* + 35.6 μM Dx1A*; blue: 12.5 μM Nank1-7* + 12.5 μM Dx1A*; green: 4.22 μM Nank1-7* + 4.22 μM Dx1A*; orange: 1.27 μM Nank1-7* + 1.27 μM Dx1A*. (D) $c(s)$ distributions of 32.4 μM R107A and 32.4 μM Dx1A* (solid line, —). For comparison, the $c(s)$ distribution of 35.6 μM wild-type Nank1-7* + 35.6 μM Dx1A* is shown (dashed line, ---).

Figure 6. Alanine scan of the binding surface of Nank1-7* to probe Deltex binding

(A) Normalized far-UV CD spectra and (B) urea-induced equilibrium unfolding transitions, monitored at 222 nm of Nank1-7* point-substitutions. Colors for (A) and (B) are as follows: orange, D81A (R107A); cyan, D117 (R107A); magenta, H215A/M216A/R218A (R107A); light blue, K152A (R107A); red, T50A/E52A/R60A (R107A); green, R127A (R107A); pink, M115A (R107A); purple, E226A/R227A (R107A); dark blue, K183A/R193A/E194A (R107A); light gray, R107A; dark gray, Nank1-7*; black, H116A (R107A); brown, T83A (R107A); yellow, R85A (R107A). (C) Ribbon representation and (D) surface image of the canonical recognition surface of Nank1-7*, with residues substituted in this study shown in stick representation, colored by mutation. Red: T50, E52, R60 (repeat 2); orange: D81 (repeat 2); brown: T83 (repeat 3); yellow: R85 (repeat 3); salmon: M115 (repeat 3); black: H116 (repeat 3); cyan: D117 (repeat 3); green: R127 (repeat 4); light blue: K152 (repeat 5); dark blue: K183 (repeat 5), R193 (repeat 6), E194 (repeat 6); magenta: H215 (repeat 6), M216 (repeat 7), R218 (repeat 7); purple: E226 (repeat 7), R227 (repeat 7). (E) Nank

substitutions that perturb Deltex binding. Green: R127 (repeat 4); yellow: R85 (repeat 3); salmon: M115 (repeat 3). In addition, A160, which has been implicated in interaction with Deltex through genetic studies²⁸ is shown in tan.

Figure 7. Sedimentation coefficient distributions of equimolar mixtures of R127A (R107A) and Dx1A*

(A) $g(s^*)$ distributions. Circles (●): 32.7 μM of each protein; squares (■): 10.7 μM each; diamonds (◆): 3.34 μM each. (B) $c(s)$ distributions. Dotted line (.....): 58.8 μM R127A (R107A) alone; dashed line (---): 67.3 μM Dx1A* alone; solid line (—): equimolar mixture of R127A (R107A) and Dx1A* (32.7 μM each).

Table 1

Fitted parameters for single-component sedimentation velocity boundary analysis.

Protein	MW ^a	Model ^b	RMSE ^c	s _A ^d	s _{A2} s _{A2} ^d	r _{A2} /r _A (%) ^d	K _d (mM) ^d
Nank1-7*	28.8	M	2.79E-02	2.56	--	--	--
		SA	1.57E-02	2.38	84.4	--	61.3
		SA+ID	6.79E-03	2.38 (2.37–2.38)	3.61 (3.58–3.64)	5.24 (5.01–5.48)	1.62 (1.51–1.75)
R107A	28.8	M	1.04E-02	2.34	--	--	--
		SA+ID	8.09E-03	2.32	3.60	1.47	K _d = 0
		M+ID	7.68E-03	2.33 (2.33–2.33)	3.95 (3.83–4.08)	1.35 (1.21–1.54)	--
Nank2-7*	22.6	SA+ID	5.74E-03	2.16 (2.15–2.16)	3.94 (3.78–4.13)	2.81 (2.45–3.33)	13.3 (8.62–29.5)
Nank3-7*	19.2	SA+ID	4.59E-03	1.88 (1.86–1.89)	3.07 (2.89–3.39)	0.386 (0.13–1.06)	1.01 (0.794–1.41)
Nank4-7*	15.6	M	5.76E-03	1.71 (1.71–1.72)	--	--	--
Nank1-6* ^e	23.9	M+ID	3.00E-03	1.99 (1.98–1.99)	3.20 (3.10–3.32)	3.09 (2.80–3.40)	--
Nank1-5* ^e	20.2	M+ID	4.48E-03	1.89 (1.89–1.90)	3.78 (3.60–4.00)	2.89 (2.49–3.51)	--
Nank1-4* ^e	16.6	M	3.33E-03	1.52 (1.51–1.53)	--	--	--
Dx1A*	28.4	M	3.12E-02	2.35	--	--	--
		SA	1.54E-02	2.15	48.7	--	30.9
		SA+ID	8.49E-03	2.13 (2.12–2.13)	3.70 (3.65–3.76)	3.26 (3.10–3.41)	1.01 (0.955–1.07)

^aMolecular weight, in kDa, calculated from primary sequence.

^bM, single-species monomer; SA, equilibrium self-association to a homodimer; ID, incompetent dimer.

^cRoot mean square deviation of the fit.

^d95% Confidence intervals from F-stat analysis for final models are shown in parentheses.

^eParameters for Nank1-6*, Nank1-5*, and Nank1-4* were for samples that had been eluted from a size-exclusion column immediately before AUC.

Table 2

Fitted parameters for heteroassociation models for equimolar mixtures of Deltex with Nank full-length terminal deletion constructs.^a

Sample	RMSD ^b	s_{AB} ^c	K_d (μ M) ^c	ΔG° (kcal/mol) ^c
Nank1-7*+Dx1A*	1.40E-02	4.781 (4.767–4.797)	12.8 (11.6–13.0)	6.56 (6.55–6.61)
R107A+Dx1A*	7.98E-03	4.309 (4.304–4.378)	12.9 (12.7–14.3)	6.56 (6.49–6.56)
Nank1-7* + Dx1A*	1.40E-02	4.78 (4.77–4.80)	12.8 (11.6–13.0)	6.55 (6.55–6.61)
Nank2-7* + Dx1A*	1.27E-02	4.30 (4.28–5.72)	20.9 (20.8–67.2)	6.27 (5.59–6.27)
Nank3-7* + Dx1A*	7.95E-03	3.08 (3.07–3.09)	28.7 (25.7–29.3)	6.09 (6.07–6.15)
Nank4-7* + Dx1A*	7.40E-03	3.36 (3.25–3.39)	282 (252–295)	4.76 (4.73–4.82)
Nank1-6* + Dx1A*	6.54E-03	2.94 (2.77–2.98)	54.8 (44.3–62.7)	5.72 (5.63–5.83)
Nank1-5* + Dx1A*	6.40E-03	2.76 (2.56–2.80)	104 (52.5–108)	5.34 (5.31–5.73)
Nank1-4* + Dx1A*	3.89E-03	2.13 (2.04–2.16)	66.8 (50.9–79.8)	5.59 (5.49–5.75)

^aData were fitted by the a heterodimer model (Scheme 2) that includes homodimer formation from each component (Nank* and Dx1A*), constraining homodimer K_d and s values with values from single-component fits (Table 1). Parameters fitted using SEDANAL.

^bRoot mean square deviation of the fit.

^c95% Confidence Intervals from F-stat analysis shown in parentheses.

Table 3

Fitted parameters for heteroassociation models for equimolar mixtures of Deltex with Nank point substitutions.^a

Sample	RMSD ^b	s _{AB} ^c	r _{AB'/A} (%) ^c	K _d (μM) ^c	ΔG ^d (kcal/mol) ^c
R107A + Dx1A*	7.98E-03	4.309 (4.304–4.378)	--	12.9 (12.7–14.1)	6.55 (6.50–6.56)
T50A/E52A/R60A (R107A) + Dx1A*	1.22E-02	3.542 (3.534–3.557)	--	12.7 (12.5–14.6)	6.50 (6.48–6.57)
D81A (R107A) + Dx1A*	1.89E-02	4.318 (4.305–4.636)	--	7.88 (7.83–15.1)	6.84 (6.46–6.84)
T83A (R107A) + Dx1A*	1.19E-02	4.164 (4.154–4.372)	--	17.6 (16.8–23.7)	6.37 (6.20–6.40)
R85A (R107A) + Dx1A*	7.65E-03	2.984 (2.976–4.355)	--	56.7 (55.2–97.8)	5.69 (5.37–5.70)
M115A (R107A) + Dx1A*	8.10E-03	3.963 (3.915–3.985)	--	23.9 (20.6–24.0)	6.19 (6.19–6.28)
H116A (R107A) + Dx1A*	1.63E-02	3.976 (3.659–3.987)	--	14.0 (4.64–14.0)	6.50 (6.50–7.15)
D117A (R107A) + Dx1A*	1.96E-02	4.305 (4.300–4.378)	3.54 ^d (1.33–5.65)	5.71 (5.25–5.89)	7.02 (7.01–7.07)
R127A (R107A) + Dx1A*	6.87E-03	--	--	--	--
K152A (R107A) + Dx1A*	9.31E-03	3.397 (3.349–3.414)	--	9.91 (8.45–10.3)	6.70 (6.68–6.80)
K183A/R193A/E19A (R107A) + Dx1A*	8.03E-03	3.433 (3.385–3.437)	--	5.13 (4.39–5.18)	7.09 (7.08–7.18)
H215A/M216A/R218A (R107A) + Dx1A*	1.26E-02	3.792 (3.551–3.796)	--	20.7 (10.1–20.8)	6.28 (6.27–6.69)
E226A/R227A (R107A) + Dx1A*	9.92E-03	3.425 (3.419–3.439)	--	13.0 (12.6–13.2)	6.55 (6.54–6.56)

^aData were fitted by the a variant of the heterodimer model (Scheme 2) that includes homodimer formation for Dx1A* but not Nank1-7*. K_d and s values for Dx1A* homodimerization were constrained to values from single-component fits (Tables 1 and S1). Parameters fitted using SEDANAL.

^bRoot mean square deviation of the fit.

^c95% Confidence intervals from F-stat analysis shown in parentheses.

^dFor D117A (R107A) + Dx1A*, incompetent heterodimer (AB') was included in the fit.