Localization of the Delta-like-1-binding Site in Human Notch-1 and Its Modulation by Calcium Affinity

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The Notch signaling pathway plays a key role in a myriad of cellular processes, including cell fate determination. Despite extensive study of the downstream consequences of receptor activation, very little molecular data are available for the initial binding event between the Notch receptor and its ligands. In this study, we have expressed and purified a natively folded wild-type epidermal growth factor-like domain (EGF) 11–14 construct from human Notch-1 and have used flow cytometry and surface plasmon resonance analysis to demonstrate a calcium-dependent interaction with the human ligand Delta-like-1. Site-directed mutagenesis of three of the calcium-binding sites within the Notch-(11–14) fragment indicated that only loss of calcium binding to EGF12, and not EGF11 or EGF13, abrogates ligand binding. Further mapping of the ligand-binding site within this region by limited proteolysis of Notch wild-type and mutant fragments suggested that EGF12 rather than EGF11 contains the major Delta-like-1-binding site. Analysis of an extended fragment EGF-(10–14), where EGF11 is placed in a native context, surprisingly demonstrated a reduction in ligand binding, suggesting that EGF10 modulates binding by limiting access of ligand. This inhibition could be overcome by the introduction of a calcium binding mutation in EGF11, which decouples the EGF-(10–11) module interface. This study therefore demonstrates that long range calcium-dependent structural perturbations can influence the affinity of Notch for its ligand, in the absence of any post-translational modifications.

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deletion experiments performed in S2 cells demonstrated the importance of Notch domains EGF11 and EGF12, which were required to form aggregates with Delta expressing S2 cells (18). When EGF12 has calcium bound, both coordination of the metal ion and interdomain hydrophobic packing confer a rod-like conformation on this region, which is expected to be conserved in other regions containing tandem repeats of EGF domains with calcium-binding motifs (19). Experiments by Shao et al. (20) and Xu et al. (21) confirmed a major ligand-binding site within these domains of Notch but also suggested that O-fucosylation of sites throughout the extracellular region, and subsequent modification of these sites by Fringe, serve to modulate the interaction. Furthermore, studies with ligands demonstrated a requirement of the ligand DSL domain and EGFs1 and -2 for binding to Notch (22).

Despite extensive genetic and cellular studies of Notch and its ligands, molecular characterization of these two core components of the signaling pathway remains relatively poor. This is in part because of the disulfide-rich nature of each component, which has hindered large scale expression of native material for biophysical and biochemical studies. Following on from our structure determination of Notch EGF-(11–13) (19), we have studied more closely the binding characteristics of this region with the ligand human Delta-like-1 (hDll-1). We demonstrate, by both flow cytometry analysis and surface plasmon resonance (SPR), a specific calcium-dependent interaction between these two components. Following site-directed mutagenesis of three of the calcium-binding sites in Notch EGF-(11–14), we use our binding assays and the subsequent analysis of each mutant protein by limited proteolysis to demonstrate the crucial importance of EGF12 for binding to hDll-1, allowing further refinement of the ligand-binding region. Finally, we demonstrate that Notch EGF10 has a modulatory role on the ability of this region to bind ligand, suggesting that regulation of both this module’s interface with EGF11 and the calcium affinity of EGF11 may play a key role in controlling Notch function.

EXPERIMENTAL PROCEDURES

Cloning and Expression of Notch Constructs—DNA encoding each wild-type Notch fragment was amplified by PCR from Tan1 (human Notch-1) cDNA and cloned into the prokaryotic expression vectors pQE30 (Qiagen) and pQE30-BirA (a modified version of pQE30 containing nucleotides encoding the recognition sequence of the site-specific biotinylation enzyme BirA (19)). Calcium-binding mutant fragments were cloned through PCR-based site-directed mutagenesis of wild-type constructs using primers containing mismatched bases. The amino acid substitutions introduced into EGF11, -12, and -13 to abrogate calcium binding were N431G, D469G, and D507G, respectively. All clones were confirmed to be correct by automated DNA sequencing. Protein production and purification were carried out as described previously (19, 23), as was biotinylation of BirA-tagged constructs. The identity of the purified proteins was confirmed by electrospray ionization mass spectrometry.

Limited Proteolysis of Wild-type and Mutant cbEGF Domain Constructs—Proteolysis with endoprotease Glu-C (1:100, w/w) was performed as described previously (24). N-terminal sequencing was used to characterize the proteolytic digestion products. Proteolysis performed in 10 mM EGTA or 10 mM CaCl2 was terminated after 60 min by acidification to pH 2. Samples were purified under nonreducing conditions by reverse-phase HPLC. After lyophilization, aliquots of HPLC fractions were analyzed by SDS-PAGE and N-terminal sequencing on an Applied Biosystems 494A Procise sequencer (PE Biosystems). Comparison of wild-type and mutant digest products obtained in the presence of EGTA confirmed that the introduced amino acid substitutions had not had further reaching consequences than the intended abrogation of calcium binding in a single cbEGF domain.

Flow Cytometry-based Notch-Ligand Binding Assay—Prokaryotically expressed biotinylated Notch fragments were coupled to avidin-coated fluorescent beads (Spherotec Inc., Libertyville, IL). 10 μl of beads were washed in 100 μl of HBSS/BSA (Hanks’ buffered saline solution without phenol red, 1% bovine serum albumin; Invitrogen). Pelleted beads were resuspended in 50 μl of HBSS/BSA, and 1 μg of biotinylated Notch protein was added prior to incubation on ice for 1 h. For the negative control, beads were either left with an avidin-only surface or they were coupled with a construct containing cbEGFs 12–14 from human fibrillin-1. Coupled beads were washed with 100 μl of HBSS/BSA, resuspended in 50 μl of HBSS, 10% fetal calf serum, and sonicated at 20% power for 1 min (Heat Systems, Sonicator) prior to addition to cells. Stably transfected Chinese hamster ovarian cells expressing full-length extracellular hDll1 (CHOΔ cells, kindly supplied by Lorantis Ltd.) were grown in T75 flasks to 90% confluency. Cells were detached via trypsinization, washed in ice-cold HBSS, and plated to avidin-coated fluorescent beads (Spherotec Inc., Libertyville, IL). Cells were kept on ice for at least 1 h prior to mixing with prepared beads. Cells were thoroughly resuspended, and 50 μl was placed into the required number of wells of a 96-well plate (Nunc). 50 μl of prepared beads were added to the wells, and the cell/bead mixture was incubated on ice for 1 h. The mixture was resuspended by pipetting once during the incubation. To demonstrate calcium dependence of the Notch-ligand interaction, either 5 mM EGTA was added to the cell/bead binding solution prior to incubation or cell/beads were incubated in HBSS containing 1.26 mM calcium chloride. Following incubation the cell/bead mixture was taken up and diluted into 500 μl of ice-cold HBSS, 10% fetal calf serum giving a concentration of ~10⁷ cells/ml. Cells were kept on ice for at least 1 h prior to mixing with prepared beads. Cells were thoroughly resuspended, and 50 μl was placed into the required number of wells of a 96-well plate (Nunc). 50 μl of prepared beads were added to the wells, and the cell/bead mixture was incubated on ice for 1 h. The mixture was resuspended by pipetting once during the incubation. To demonstrate calcium dependence of the Notch-ligand interaction, either 5 mM EGTA was added to the cell/bead binding solution prior to incubation or cell/beads were incubated in HBSS containing 1.26 mM calcium chloride. Following incubation the cell/bead mixture was taken up and diluted into 500 μl of ice-cold HBSS in preparation for flow cytometry. Flow cytometry was performed on a FACSCalibur machine (BD Biosciences). 10,000 cells were counted per analysis, and the fluorescence intensity in FL1 was recorded.

SPR Analysis of Notch-Ligand Binding—Interaction analysis was performed on a BLACore 2000 instrument (BLACore AB, Stevenage, UK). HBSS was used as running buffer in all experiments at a flow rate of 10 μl/min. For amine coupling of streptavidin (SA) to chip surfaces, 30 μl of 1-ethyl-3(3-dimethylamino-propyl)-carbodiimide hydrochloride/N-hydroxysuccinimide was injected followed by 30 μl of 0.1 mg/ml SA in 10 mM sodium acetate, pH 5.0. Unreacted carboxymethyl groups were blocked through injection of 40 μl of 1 M ethanolamine. Notch fragments were captured on SA-coupled surfaces at den-
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A. Notch receptors are represented here by human Notch-1. B. Notch ligands homologous to Drosophila Serrate are represented here by hJagged1; C, those homologous to Drosophila Delta are represented here by hDll-1. The regions of Notch-1 and hDll-1 produced in this study are indicated.

FIGURE 1. Schematic illustrations showing Notch-receptor and ligand-domain organizations. A, Notch receptors are represented here by human Notch-1. B, Notch ligands homologous to Drosophila Serrate are represented here by hJagged1; C, those homologous to Drosophila Delta are represented here by hDll-1. The regions of Notch-1 and hDll-1 produced in this study are indicated.

sities of 2000–3000 RU by injection of 30 μl of ~10 μg/ml Notch solution (diluted in HBSS + 300 mM NaCl).

The analyte used for equilibrium binding analysis was eukaryotically expressed hDll-1 fragment containing the N-terminal (NT) domain, DSL domain, and EGFs 1–3 (NTEGF3, kindly provided by Lorantis Ltd.; see Fig. 1). Studies of truncated forms of hDll-1 in a functional Notch signaling assay showed the fragment to be the smallest fully active fragment of this Notch ligand.6 Purification on a Superdex 75 gel filtration column (Amersham Biosciences) was performed prior to use. 10-μl samples of doubling dilutions of a concentrated (~300 μM) NTEGF3 stock were injected over Notch-immobilized sensor surfaces. The actual binding response at each concentration of NTEGF3 (RUAct) was calculated by subtracting the response seen with the same concentration of NTEGF3 on the control surface from the response seen on the test surface. Initially the control surface used was SA alone, but in subsequent experiments N1 11–14 12DG mutant was used because this showed negligible binding to ligand (see supplemental Fig. S3). Each binding response was normalized for the amount of Notch immobilized on the test surface. The molar concentration of Notch immobilized ([N]) was calculated from the number of response units of Notch immobilized (RUIm) (1000 RU is equivalent to a protein concentration of 10 mg/ml). From this the binding response per 100 μM of immobilized Notch (RU100mMIm) was calculated. Equations 1 and 2 were used,

\[
[N] = \frac{RU^{\text{Im}}}{(100 \times M_i)} \quad (\text{Eq. 1})
\]

\[
RU^{100\mu\text{MIm}} = RU^{\text{Act}}/(N/[100 \times 10^{-6}]) \quad (\text{Eq. 2})
\]

To demonstrate the calcium dependence of Notch-NTEGF3 binding, EGTA to a final concentration of 2.5 mM with or without CaCl2 or MgCl2 to 5 mM final concentration was added to NTEGF3 injections.

NMR Spectroscopy—1H NMR experiments were performed at 500 MHz. N1 11–13, N1 11–13 12DG, and N1 10–13 samples were dissolved in 550 μl of matrix solution (99.9% D2O containing 5 mM Tris-HCl and 150 mM NaCl, pH 7.5). Calcium titrations were performed by adding small aliquots (5–20 μl) of CaCl2 solutions in D2O in 100 μM to 1 mM increments. One-dimensional NMR data were collected with a spectral width of 5494.51 Hz, 4096 complex points, and 512 acquisitions at 25°C.

RESULTS

Expression, Purification, and Characterization of Notch-1-(11–14) Constructs—A wild-type human Notch-1 fragment containing EGFs 11–14 (Fig. 1) was expressed as a His tag fusion protein using the previously described NM554 bacterial expression system (19, 23). The fragment was expressed with and without a 15-amino acid biotinylation tag (BirA tag) at the C terminus to facilitate binding studies (see “Experimental Procedures”). A previously described N1 11–13 fragment was also expressed for comparative studies (19).

All constructs underwent in vitro refolding using well-established methods developed for the production of disulfide-rich proteins (23) and were subsequently purified by reverse-phase and anion-exchange chromatography (19, 23). Evidence suggesting native oxidation of each reduced fragment during refolding was given by a characteristic change in reverse-phase HPLC elution profile. The presence of a single band on nonreducing SDS-PAGE and a single peak on electrospray ionization (ESI) mass spectrometry also gave evidence for the presence of a single oxidized species in each refolded preparation as opposed to a heterogeneous mixture of differently disulfide-bonded forms (supplemental Fig. S1 and supplemental Table S1).

Additional evidence for the production of natively folded material was provided through limited proteolysis studies. Because calcium binding to EGF domains has been demonstrated to rigidify the region in the vicinity of the calcium ion, these domains show protection from proteolysis in the presence of the ion (19). Notch constructs were digested in the presence of EGTA or calcium, and aliquots were taken from each digestion over a 1-h time course. Upon analysis by SDS-PAGE, both N1 11–14 (Fig. 2) and N1 11–13 (data not shown) displayed protection from proteolysis when in the presence of calcium suggesting each one’s capacity to bind this divalent cation. Calcium-binding ability is taken as evidence for the possession of a native chEGF fold, because it is dependent upon the

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**Flow Cytometry and SPR Demonstrate That Human Notch-1 (11–13) and Notch-1-(11–14) Bind to Human-delta-like-1**—Consistent with previous work reporting that EGF domains 11 and 12 are necessary and sufficient for Notch-ligand interaction, we initially demonstrated binding of prokaryotically expressed and in vitro refolded N1 11–13 and 11–14 fragments to full-length extracellular hDll-1. Upon analysis by flow cytometry, CHO cells expressing full-length extracellular ligand (CHOΔ cells) showed increased fluorescence intensity when incubated with Notch-coated fluorescent beads compared to when incubated with beads coated with a control protein (control beads) (Fig. 3A and Fig. 4A). Also in accordance with previous studies, this interaction was demonstrated to be calcium-dependent (Fig. 3A). Both Notch-(11–13) and Notch-(11–14) gave very similar profiles, indicating that the addition of EGF14 did not significantly enhance binding.

An SPR-based assay was used to derive more detailed binding data for the Notch-hDll-1 interaction. In this assay, 11–13 (data not shown) and 11–14 Notch fragments (Fig. 3B) were immobilized to a BiACore chip surface via their biotinylation tags (see “Experimental Procedures”). A purified fragment of hDll-1 encompassing the N terminus, the DSL domain, and EGFs1–3 was used as analyte (NTEGF3). Equilibrium binding analysis was used to determine a mean K_d value of 130 ± 14 μM (S.D., n = 3) for the N1 11–14-NTEGF3 interaction (Table 1). The addition of 2.5 mM EGTA to the analyte prior to injection abrogated all interactions above background. In the presence of EGTA, a binding response could be re-gained upon addition of excess calcium. This was not the case upon addition of excess magnesium thus confirming the calcium specificity of Notch-ligand binding (Fig. 3B).

**Site-directed Mutagenesis of Calcium-binding Sites in Notch-(11–14) Region**—To identify the molecular basis of the calcium dependence of the Notch-ligand interaction, we systematically introduced calcium-binding mutations into individual cbEGF domains in the 11–14 construct. Mutant fragments were therefore produced containing calcium-binding mutations in either EGF11, EGF12, or EGF13. In each case a glycine residue was substituted for the calcium-binding consensus residue that forms part of the major β-hairpin in the cbEGF structure (Asn-431 in EGF11, Asp-469 in EGF12, and Asp-507 in EGF13). Substitution of this residue in cbEGF domains from fibrillin-1 has been shown previously to eliminate calcium binding or reduce significantly (K_d for calcium is in the millimolar range) the calcium affinity of the mutant domain without disrupting its native fold (27, 28), in accordance with its role as a side-chain ligand for calcium in all calcium-binding EGF domain structures solved to date.

Following in vitro refolding, each mutant fragment gave comparable HPLC and fast protein liquid chromatography purification profiles to those of the wild-type fragment. For all constructs, possession of the amino acid substitution, formation of the correct number of disulfide bonds, His tag cleavage, and site-specific biotinylation were confirmed by ESI mass spectrometry of the final product. All mass spectra showed single peaks indicating that the final product of each mutant construct contained the expected fully oxidized species. Nonreducing SDS-PAGE showed single bands for each construct suggesting the presence of a single conformer (supplemental Fig. S1 and supplemental Table S1).

Within each mutant construct only one calcium-binding site was mutated; therefore, every fragment contained three wild-type cbEGF domains. Limited proteolysis, as performed for the wild-type fragments, was used to demonstrate retention of calcium-dependent protection against proteolysis, consistent with the presence of some native calcium-bound structure (supplemental Fig. S2).

**Binding of Mutant Notch-1 Fragments to Human Delta-like-1**—Having established that the mutant Notch constructs possessed natively folded cbEGF domains, we assessed the ligand-binding ability of these fragments by both flow cytometry and SPR. All mutants with the exception of N1 11–14 12DG, gave a comparable shift in CHOΔ cell fluorescence to that observed for the wild-type 11–14 fragment (Fig. 4A). The N1 11–14 12DG mutant, however, bound to CHOΔ cells no better than control protein. SPR analysis showed a similar trend in that all fragments (Fig. 4B and Table 1), except the N1 11–14 12DG mutant, bound well to the hDll-1 NTEGF3 fragment. For this mutant, binding was slightly greater than the background control, indicating that the interaction was not completely abrogated by the calcium-binding substitution (supplemental Fig. S3). This most likely reflects the high sensitivity of this method, which is often used to measure weak protein-protein interactions (29). Collectively, these data suggest that the ligand-binding region of the 11–14 fragment resides predominantly within EGF12 and the interfacer region with EGF11, and that EGF13 does not contribute directly or indirectly to the interaction.

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A.  

B.

FIGURE 3. N1 11–13 shows calcium-dependent binding to full-length hDil-1. A, in a flow cytometry assay CHO cells expressing full-length extracellular hDil-1 (CHOQ cells) were incubated with N1 11–13–coated fluorescent beads in the presence of calcium (see "Experimental Procedures"). The increased fluorescence intensity of a proportion of cells incubated with N1 11–13–coated beads compared with cells incubated with beads coated in a triple cbEGF domain construct from fibrillin-1 (control beads) represents the specific binding of N1 11–13 to full-length extracellular hDil-1 (red arrow). When 10 mM EGTA is added to the cell/bead incubation buffer, the fluorescence intensity of CHOQ cells shifts back to the level of cells incubated with control beads (blue arrow) demonstrating the calcium dependence of the N1 11–13–interaction with hDil-1. B, surface plasmon resonance was used to study in greater detail the interaction between N1 11–14 and a recombinant hDil-1 fragment (NTEGF3). An NTEGF3 binding response above background (streptavidin alone) is only seen when free calcium is present in the running buffer (I and IV). Chelation of the calcium with 2.5 mM EGTA abrogates the binding response (II). Binding cannot be restored through addition of excess (5 mM) MgCl2 (III), but it can be restored through addition of 5 mM CaCl2 (IV).

TABLE 1

The NTEGF3-binding properties of prokaryotically expressed Notch constructs as measured through equilibrium binding analysis using SPR are shown

N-terminal Analysis of Notch Proteolysis Products—N-terminal analysis was performed on HPLC-purified protease digestion products prepared from the Notch mutants N1 11–14 12DG and N1 11–14 11NG to identify the regions destabilized through the loss of calcium binding. This well-established method has been used previously to determine the structural consequences of various disease-causing mutations in other EGF-containing proteins, because abrogation of calcium binding leads to a loss of calcium-dependent protection against proteolysis (28). Previous studies have indicated that in the majority of cases calcium binding to multiple tandem repeats of EGF domains is noncooperative; therefore, this method can be used in conjunction with binding data to define the region of Notch to which ligand binds (30). In 10 mM EGTA, both mutants were seen to contain the same cleavage sites as the wild-type construct. As expected, quantitative analysis showed that all sites were cleaved in the presence of EGTA to a similar extent in each fragment (Table 2).

N-terminal analysis of cleavage products from the 12DG mutant, obtained in the presence of calcium, demonstrated a local destabilizing effect of the amino acid substitution on the mutant domain (Table 2 and Fig. 5). The two cleavage sites within cbEGF12, 473FQCIC and 488VNTDE, both showed increased proteolytic susceptibility in the 12DG mutant construct compared with the wild type. This result suggests that calcium binding to wild type cbEGF12 causes stabilization of the entire domain right up to its most C-terminal point. However, the absence of increased cleavage at the site present in cbEGF13, 511FQCEC, in the 12DG mutant, suggests that this stabilization does not extend into the flanking C-terminal domain. Additionally the results from the 12DG mutant construct suggest that calcium binding to cbEGF12 causes structural stabilization in an N-terminal direction, as has been observed in other tandem cbEGF domains. The 437CQCLQ and 450IDVNE cleavage sites within cbEGF11 were seen to be more susceptible to proteolysis in the 12DG mutant construct than they were in the wild-type constructs (Table 2). This is expected for the 450IDVNE site located in the minor β-sheet of cbEGF11 because it is spatially close to the calcium-binding site within cbEGF12 (Fig. 5) and contains the packing residue which, along with calcium bound to cbEGF12, is important in stabilizing interactions between the two domains (19, 31). However, the increased susceptibility seen at the 437CQCLQ cleavage site located in the major β-sheet of cbEGF11 indicates a longer range N-terminal effect of calcium binding beyond the packing interaction and has been observed previously in multiple tandem repeated cbEGF domains in fibrillin-1 (32). One-dimensional NMR analysis of N1 11–13 12DG shows the expected calcium-dependent shifts from residues sensitive to calcium binding within EGFl1 and EGF13, but not EGF12, indicating that the structural destabilization caused by the loss of calcium binding to EGF12 does not significantly affect calcium-binding sites in EGFl1 or -13, in accordance with previous studies (supplemental Fig. 54) (30).

N-terminal analysis of the N1 11–14 11NG mutant following digestion in 10 mM calcium indicated that the 11NG substitu-
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N-terminal Linkage of EGF10 Significantly Reduces Notch-
hDII-1 Binding—Because we and others have reported previously that N-terminal cbEGF domains do not possess native-like calcium-binding affinity, we cloned and expressed an N1 10–14 construct for use in ligand-binding assays (Fig. 1). EGF10 is itself a noncalcium binding EGF domain; however, it contains a conserved packing residue at its C terminus and is therefore expected to form a rigid interface with EGF11 raising the calcium affinity of this domain. To our knowledge, this five domain construct is the largest EGF/cbEGF fragment to have been produced using an in vitro refolding system to date. Successful production of a natively folded single species was indicated from HPLC and fast protein liquid chromatography profiles, limited proteolysis (data not shown), ESI mass spectrometry, and nonreducing and reducing SDS-PAGE ((supplemental Fig. S1 and supplemental Table S1).

Surprisingly we found that the N1 10–14 fragment gave a negative result in the flow cytometry binding assay with CHOΔ cells. Cells incubated with N1 10–14-coated beads gave a comparable fluorescent profile to cells incubated with control beads (Fig. 6A). Furthermore, when assayed by SPR, the binding characteristics of the interaction of N1 10–14 with hDll-1 were significantly different when compared with the wild-type 11–14 fragment, giving both reduced maximal saturation and a reduced affinity $K_d$ 202 μM ± 22 (S.D., $n = 3$) (Fig. 6B and Table 1). Because all analyses demonstrated that the new Notch construct was natively folded, we reasoned that this negative result may result from a steric effect of EGF10 on the EGF11/12 region, preventing effective binding of ligand. Because the hydrophobic packing interaction between EGF10 and -11 both rigidifies the domain interface and raises the affinity of EGF11 for calcium (Fig. 7), as shown for other EGF-cbEGF pairs (33), we cloned and expressed an N1 10–14 fragment containing a calcium-binding mutation in cbEGF11 (N1 10–14 11NG) to decouple the two domains ((supplemental Fig. S1 and supplemental Table S1). In contrast to the

![Image](image-url)

**FIGURE 4.** Calcium binding by cbEGF12 is required for ligand binding. A, hDll-1 binding abilities of the N1 11–14 WT, N1 11–14 11NG, N1 11–14 12DG, and N1 11–14 13DG constructs were assessed in comparative flow cytometry experiments in the presence of calcium (see “Experimental Procedures”). Ligand binding by all N1 11–14 constructs except the 12DG mutant was seen. This suggests that calcium binding by cbEGF12 is an essential requirement for Notch-hDII-1 binding. Control beads are coated in avidin only. B, binding curves obtained following SPR analysis of the interaction between NTEGF3 and N1 11–14 wild-type and mutant constructs are shown. The N1 11–14 12DG mutant was used as the control surface because it showed very weak interaction with NTEGF3 (supplemental Fig. S3), and a binding curve is therefore not shown for this fragment. The curves were obtained through nonlinear curve fitting of the raw BIAcore data to the Langmuir binding isotherm.

**TABLE 2**

Quantitation of digestion products from calcium-binding mutant Notch-1-(11–14)-BirA constructs reveals reduced calcium-dependent protection from proteolysis compared with that seen in wild-type constructs

Digestion products identified through N-terminal analysis were quantitated in picomoles, and the levels of each relative to the true N terminus (SAQDV) were calculated as a percentage. For each fragment, comparison of the amount of cleavage in EGTA with that seen in CaCl₂ reveals the level of calcium-dependent protection from proteolysis at each site. At certain sites mutant constructs show reduced protection compared with the wild-type fragment (see "Results").

| Fragments | SaqvDV | 437CQCLQ | 450IDVNE | 473FQCIC | 488VNTDE | 513FQCEC |
|-----------|--------|-----------|----------|----------|----------|----------|
| N1 11–14 WT | 680 | 100 | 467 | 100 | 763 | 100 | 547 | 100 | 677 | 100 | 582 | 100 |
| CaCl₂ | pmol/ % | pmol/ % | pmol/ % | pmol/ % | pmol/ % | pmol/ % |
| EGTA | | | | | | |
| N1 11–14 11NG | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| CaCl₂ | pmol/ % | pmol/ % | pmol/ % | pmol/ % | pmol/ % | pmol/ % |
| EGTA | | | | | | |
| N1 11–14 12DG | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| CaCl₂ | pmol/ % | pmol/ % | pmol/ % | pmol/ % | pmol/ % | pmol/ % |
| EGTA | | | | | | |
| N1 11–14 13DG | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| CaCl₂ | pmol/ % | pmol/ % | pmol/ % | pmol/ % | pmol/ % | pmol/ % |
| EGTA | | | | | | |
Notch-(11–13) and Notch-(11–14) WT fragments were shown to bind to CHO cells expressing Dll-1 similarly to that seen previously when Notch-(11–13) was demonstrated to bind to L-cells expressing murine Dll-1 (19). Furthermore, in a novel SPR assay both WT Notch fragments were demonstrated to interact specifically in a calcium-dependent manner with Dll-1 NTEGF3. A $K_d$ of $\sim 130 \mu M$ was calculated for the N1 11–14–NTEGF3 interaction based on a one to one binding model. This is considerably weaker than binding constants reported for Notch-ligand interactions previously (22). This may reflect the lack of post-translational modifications on our Notch constructs, the monomeric presentation of each Notch construct in SPR, or the absence of accessory regions outside the 11–14 region that facilitate binding. Nevertheless, the establishment of a quantitative assay for binding allowed us to probe for the first time the structural features of the interaction.

Through the production of calcium-binding defective Notch constructs, we have been able to demonstrate that the requirement of calcium for Notch-ligand binding is confined to EGF12. When calcium binding in this domain is abrogated through amino acid substitution of a conserved side-chain ligand for calcium, the Notch-Dll-1 interaction is abolished. However, when calcium binding in EGF11 or EGF13 is abrogated by the same means, the Notch-ligand interaction remains. Through the use of limited proteolysis, we have delineated the structural changes that occur to the 11–14 fragment upon loss of calcium binding to either EGF12 or EGF11. The most destabilized region of N1 11–14 12DG, as evidenced by an increase in proteolytic susceptibility, is in the vicinity of the EGF12 calcium-binding site. This is in agreement with NMR studies of N1 11–13 12DG, which indicated that, even at high calcium concentrations, EGF12 was unable to bind calcium, unlike EGF11 and -13 (supplemental Fig. S4). Given that the N1 11–14 11NG mutant binds normally to ligand, despite increased proteolytic susceptibility within EGF11, our data demonstrate that EGF12 contains the major ligand-binding site. However, an indirect contribution from the EGF11 hydrophobic packing residue Tyr-444 is also a likely requirement as it is needed to maintain the high affinity calcium binding in EGF12 on which we have shown ligand binding is dependent (19). These data explain the observed phenotypic effects of a previously characterized Drosophila Notch muta-

**DISCUSSION**

Previous studies on Notch-ligand binding have demonstrated the requirement for EGF11 and -12 of Notch as well as the presence of calcium for productive interaction (8, 18). To refine further the details of this binding event, we have developed two assay systems enabling us to study the interaction of wild-type and mutant Notch fragments with full-length extracellular hDll-1, expressed on a cell surface, and with an N-terminal hDll-1 fragment (NTEGF3). Prokaryotically expressed Notch constructs containing EGF11 and -12 were produced and rigorously assessed for integrity of fold prior to use in binding assays. Because these fragments are produced in a bacterial expression system and subsequently refolded in vitro, they lack any post-translational modifications. However, it has previously been shown by us and others that glycosylation of this region is not required for Notch binding (19, 34).
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FIGURE 7. A comparison of the aromatic regions of one-dimensional 1H NMR spectra of constructs demonstrating EGF-(10–11) interdomain packing. Spectra recorded for N1 11–13 (upper) and N1 10–13 (middle) in the absence of CaCl2 and for N1 10–13 (lower) in the presence of 0.3 mM CaCl2 are shown. N1 10–13 rather than 10–14 was used in the NMR analysis because of its smaller molecular weight (supplemental Table S1). EGF10 contains a single aromatic residue, Tyr-404; this residue is located in the sequence at the conserved aromatic interdomain packing position. The asterisk indicates a peak assigned to Tyr-404 in the spectrum of calcium-free N1 10–13; this peak is not observed in the spectrum of N1 11–13, confirming its assignment. The peak corresponding to Tyr-404 disappears upon the addition of 1 equivalent of CaCl2. This observation is consistent with the perturbation of this residue as a result of the formation of a rigid interdomain interface, between EGF10 and cbEGF11, when EGF11 binds CaCl2 with high affinity.

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