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Molecular Basis for Jagged-1/Serrate Ligand Recognition by the Notch Receptor*

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Background: The site of Jagged/Serrate ligand recognition by Notch is unknown.

Results: Two critical residues involved in an intramolecular hydrophobic interaction across the central β-sheet of EGF12 form a ligand-binding platform.

Conclusion: The ligand-binding region is adjacent to a Fringe-sensitive residue involved in modulating Notch activity.

Significance: The results have implications for understanding receptor/ligand recognition, Notch regulation by O-glycosylation, and the development of paralogue-specific antibodies.

We have mapped a Jagged/Serrate-binding site to specific residues within the 12th EGF domain of human and Drosophila Notch. Two critical residues, involved in a hydrophobic interaction, provide a ligand-binding platform and are adjacent to a Fringe-sensitive residue that modulates Notch activity. Our data suggest that small variations within the binding site fine-tune ligand specificity, which may explain the observed sequence heterogeneity in mammalian Notch paralouges, and should allow the development of paralogue-specific ligand-blocking antibodies. As a proof of principle, we have generated a Notch-1-specific monoclonal antibody that blocks binding, thus paving the way for antibody tools for research and therapeutic applications.

The Notch receptor mediates a critical short range signal that is deployed in many developmental and adult contexts to refine cell fate choices (1). Genetic diseases affecting Notch pathway components give rise to a wide range of congenital and adult-onset disorders, and Notch dysregulation has been found to contribute to many types of human cancer (2). The Notch receptor is a single pass trans-membrane protein that, during its biosynthetic pathway, may be cleaved by a furin-like convertase (S1) in the trans-Golgi to generate a noncovalently associated heterodimer at the cell surface (3, 4). Canonical Notch signaling is initiated when a cell surface-expressed Delta/Serrate/LAG-2 (DSL)3 ligand binds in trans to the Notch receptor expressed on an opposing cell surface (5). Various studies have localized the binding determinants to EGF1–12 of the receptor and the DSL and EGF1–2 of the ligands (6, 7). Endocytosis of the Notch ligand complex by the ligand-expressing cell leads to a second proteolytic cleavage event at S2, which removes the extracellular moiety of the heterodimer (8). This leaves the membrane-tethered stalk, which is then cleaved by γ-secretase at S3 (see Fig. 1a) (9, 10). Following this proteolysis event, an intracellular fragment of Notch is released (11, 12). This translocates to the nucleus and assembles into a transcriptional activation complex, which includes a DNA-binding protein of the CBF-1/Suppressor of Hairless/LAG-1 (CSL) family and its co-activator Mastermind. This new assembly relieves repression and activates Notch target genes such as Hairy/Enhancer of split (Hes) (13).

We have previously shown that the 12th EGF (EGF12) domain of Notch is directly implicated in ligand binding (14). To investigate this region further, we have made a series of single amino acid substitutions in human and Drosophila Notch designed to probe ligand recognition, while retaining Notch calcium binding, which is already known to be critical for this interaction (15). Using flow cytometry and cell aggregation assays, we have identified a globally conserved hydrophobic binding site that is adjacent to the residue that forms part of the O-fucosylation consensus sequence (C2–X5–C2–X7–C2) in EGF12, where C2 and C3 represent the second and third conserved cysteines (16). This residue is reported to be subjected to further modification by the Fringe enzymes (17), resulting in changes to Notch activity (18). We discuss the functional significance of sequence variability within this region and show

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§ Serrate/LAG-2 (DSL) ligand binds in trans to the Notch receptor expressed on an opposing cell surface (5). Various studies have localized the binding determinants to EGF1–12 of the receptor and the DSL and EGF1–2 of the ligands (6, 7). Endocytosis of the Notch ligand complex by the ligand-expressing cell leads to a second proteolytic cleavage event at S2, which removes the extracellular moiety of the heterodimer (8). This leaves the membrane-tethered stalk, which is then cleaved by γ-secretase at S3 (see Fig. 1a) (9, 10). Following this proteolysis event, an intracellular fragment of Notch is released (11, 12). This translocates to the nucleus and assembles into a transcriptional activation complex, which includes a DNA-binding protein of the CBF-1/Suppressor of Hairless/LAG-1 (CSL) family and its co-activator Mastermind. This new assembly relieves repression and activates Notch target genes such as Hairy/Enhancer of split (Hes) (13).

The abbreviations used are: DSL, Delta/Serrate/LAG-2; J1, Jagged-1; N-1, Notch-1; hN-1, human Notch-1; dN, Drosophila Notch; hNotch, human Notch.
that it can be exploited to raise paralogue-specific antibodies that block ligand binding.

EXPERIMENTAL PROCEDURES

Protein Production—hN-111–13 was bacterially expressed and refolded in vitro as described previously and contains a 17-amino acid birin ligase (Bir A) tag at the C terminus, which was shown to increase the efficiency of refolding (19). Calcium binding by hN-111–13 wild type and mutants was measured by one-dimensional and two-dimensional NOE NMR spectroscopy, as described previously (20, 21).

Flow Cytometry of J-1/N-1 Interaction—Prokaryotically expressed and biotinylated hN-111–13 wild-type and mutant constructs were coupled to purple fluorescent avidin-coated beads (Spherotech), as described previously (14). A negative control of calcium-binding EGF12-14, from human fibrinogen-1, an unrelated protein with a similar domain organization, was used in all experiments. Coupled beads were washed with 100 μl of HBSS/BSA (Hanks’ buffered saline solution without phenol red, 1% BSA), resuspended in 50 μl of HBSS, 10% fetal calf serum (FCS) and kept on ice before being incubated with the cells. Stably transfected B16 mouse melanoma cells expressing full-length mouse Jagged-1 (J-1) were grown in T75 flasks to 10% confluency before being detached with 5 ml of PBS, 10 mM EDTA at 37 °C for 5 min. Pelleted cells from each flask were washed three times and resuspended in 1 ml of ice-cold HBSS, 10% FCS. After 1 h, 50 μl of the beads were added to 50 μl of cells and incubated on ice for 1 h prior to being resuspended in 500 μl of ice-cold HBSS for flow cytometry analysis. hN-1-binding antibodies (see below) were screened for their ability to block binding of hN-111–13 to HEK293 cells expressing full-length human J-1 by the addition of 10 μl of hybridoma supernatant to the coupled beads prior to incubation with cells. Flow cytometry was performed using a FACSCalibur machine (BD Biosciences). 10,000 cells were counted, and fluorescence intensity was monitored in FL3 at >670 nm, with excitation at 488 nm.

Drosophila Cell Aggregation Assay—For Serrate expression, the 4.2-kb cDNA sequence from pBSK+SerFL (22) was amplified by PCR to eliminate the stop codon, fused in-frame to a V5-His tag at its C terminus, and inserted into the pMT expression vector (23) to generate pMT Ser-V5. Schneider S2 cells (Invitrogen) were transfected with pMT plasmids, Notch Response Element (NRE):Firefly (gift from S. Bray), and actin:Renilla (gift from G. Merdes). After 24 h, cells were transfected and seeded into white 96-well plates (Nunc 136101), and CuSO4 was added after a further 24 h to induce expression. Cells were reseeded into white 96-well plates on top of fixed Delta-expressing S2 cells (S2-Mt-DI; Drosophila Genomics Resource Center (DGRC)). 24 h after induction, luciferase activity was assayed with Dual-Glo Luciferase (Promega) and quantified by luminometer (Berthold Technologies), and the Firefly/Renilla ratio was calculated for triplicate samples. Experiments were repeated a minimum of three times.

mAb Generation and Validation—Mouse monoclonal antibodies (N1-96A, isotype IgG2a and N1-39E, isotype IgG1) were raised, using MF1 mice and NS0 cells as the fusion partner (24). The antigen was bacterially expressed recombinant hN-111–13 as described above, using TiterMax Gold as adjuvant. Fifteen hybridoma cell lines, generated from three fusions, were positive for antibody reactivity against hN-111–13 by ELISA assay against the immunogen. Two hybridoma lines (N1-39E and N1-96A) were found to be positive for the detection of cell surface N-1 by FACS analysis of transient HEK293T transfectants. For epitope mapping, wild-type and mutant hN-111–13 constructs were prepared at 20 ng/μl, and 1 μl of each was spotted onto nitrocellulose. The proteins were detected with the noninhibitory antibody N1-96A (1:200) and the blocking antibody N1-39E (1:400) followed by anti-mouse HRP conjugate (1:2000) and visualized by chemiluminescence (Amer sham Biosciences ECL plus Western blotting detection system).

Flow Cytometry and Immunocytochemistry to Test Paralogue Specificity—Full-length human Notch-1, -2, -3, and -4 transfectants were generated. Cells were washed three times with FACS Wash Buffer (2% FBS in PBS + 0.01% NaN3) and then incubated on ice for 20 min with primary antibody, i.e. undiluted hybridoma supernatant. After two washes with FACS Wash Buffer, cells were incubated for 20 min at 4 °C (protected from light) with secondary conjugated antibody, i.e. alkaline phosphocyanin-conjugated anti-mouse Ig (H+L) (Invitrogen, A10539) at 1:50 dilution. Cells were washed with FACS Wash Buffer and resuspended in 500 μl of 1% formaldehyde in PBS before analysis using a FACSCalibur flow cytome-
Site-directed Mutagenesis of Human N-111–13—Mutations in hN-1 were made in the context of the soluble hN-111–13 construct (Fig. 1a), which we have previously demonstrated to be ligand binding-competent (19). To assay these hN-111–13 mutants, we have used flow cytometry to study binding to full-length J-1 expressed on the surface of B16 cells. Initially, two mutants containing alanine substitutions were constructed on the basis of sequence conservation across Notch from multiple species (Fig. 1b). Of these, one was similar to wild type (WT) in its ability to bind J-1 (M479A), but the other, L468A, was completely unable to bind J-1 (Fig. 2). Mapping Leu-468 to the structure of hN-111–13 (PDB 2VJ3) has previously shown that it lies within the central β-hairpin of EGF12. The Notch-binding region previously identified in the J-1 DSL domain makes it unlikely that Leu-468 is the only residue to contribute to the ligand-binding interface (19); we therefore made a second round of alanine substitutions at residues that were seen to lie close to this region and to have surface-exposed side chains not involved in calcium coordination (Fig. 1b). This allowed us to identify one further mutant, I477A, which abrogated binding similarly to L468A, two other mutants, E473A and Q475A, which showed a more modest reduction in binding, and one mutant, Q470A, which bound at least as well as WT hN-111–13 (Fig. 2a). Loss of binding to L468A and I477A was not due to misfolding or indirect effects on calcium binding (in contrast to a previously studied D469G substitution) because two-dimensional NOESY analysis showed that a marker for calcium binding to EGF12 (Tyr-444) gave a similar change in chemical shift upon the addition of Ca2+ to that observed in the WT (Fig. 2b) (14). The crystal structure of hN-111–13 (PDB 2VJ3) has previously shown that Leu-468 and Ile-477 side chains form a hydrophobic interaction across the central β-hairpin of EGF12 (19); our data suggest that this structural feature may serve as a stable platform for ligand interactions (Fig. 2c).

Site-directed Mutagenesis of Full-length Drosophila Notch—To extend this analysis to full-length Notch, we then substituted the equivalent amino acids in the evolutionarily distant dN and tested these mutants for interaction with Drosophila Serrate using cell aggregation assays. Results were very similar to those seen with the hN-111–13/J-1 interaction, with dN L504A showing the strongest reduction of binding, and as expected, a concomitant reduction in ligand-induced signaling (Fig. 3). An exception was dN V513A, which reduced but did not abrogate binding as had the equivalent hN-111–13 I477A. This most likely reflects the more conservative nature of a valine to alanine change than that of the more bulky isoleucine to alanine. Collectively, our data suggest that the location of the ligand-binding site for the Jagged/Serrate family ligands is conserved across human and Drosophila Notch (Table 1) despite the observed sequence divergence within this region (Fig. 1b).

RESULTS AND DISCUSSION

Site-directed Mutagenesis of Human N-111–13—Mutations in hN-1 were made in the context of the soluble hN-111–13 construct (Fig. 1a), which we have previously demonstrated to be ligand binding-competent (19). To assay these hN-111–13 mutants, we have used flow cytometry to study binding to full-length J-1 expressed on the surface of B16 cells. Initially, two mutants containing alanine substitutions were constructed on the basis of sequence conservation across Notch from multiple species (Fig. 1b). Of these, one was similar to wild type (WT) in its ability to bind J-1 (M479A), but the other, L468A, was completely unable to bind J-1 (Fig. 2). Mapping Leu-468 to the structure of hN-111–13 shows that it lies within the central β-hairpin of EGF12. The Notch-binding region previously identified in the J-1 DSL domain makes it unlikely that Leu-468 is the only residue to contribute to the ligand-binding interface (19); we therefore made a second round of alanine substitutions at residues that were seen to lie close to this region and to have surface-exposed side chains not involved in calcium coordination (Fig. 1b). This allowed us to identify one further mutant, I477A, which abrogated binding similarly to L468A, two other mutants, E473A and Q475A, which showed a more modest reduction in binding, and one mutant, Q470A, which bound at least as well as WT hN-111–13 (Fig. 2a). Loss of binding to L468A and I477A was not due to misfolding or indirect effects on calcium binding (in contrast to a previously studied D469G substitution) because two-dimensional NOESY analysis showed that a marker for calcium binding to EGF12 (Tyr-444) gave a similar change in chemical shift upon the addition of Ca2+ to that observed in the WT (Fig. 2b) (14). The crystal structure of hN-111–13 (PDB 2VJ3) has previously shown that Leu-468 and Ile-477 side chains form a hydrophobic interaction across the central β-hairpin of EGF12 (19); our data suggest that this structural feature may serve as a stable platform for ligand interactions (Fig. 2c).

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These mutational data now implicate a different face of Notch in ligand binding from our earlier studies based on NMR data derived from mapping intensity changes in the spectrum of hN-111–13 upon titration of an hJ-1DSL-EGF3 fragment (19) (the more informative chemical shift mapping was not possible due to a combination of the poor solubility of hJ-1DSL-EGF3 and the kinetics/affinity of the interaction). The most perturbed residues in this assay were hN-1 Val-453 and Gly-472, which contribute to the EGF12 calcium binding by donation of a backbone carbonyl ligand and to an EGF11–12 packing interaction, respectively. The spatial distance and orientation of Val-453 and Gly-472, when compared with the Leu-468 and Ile-477 hydrophobic patch identified as being important for ligand binding in this study, preclude both sites making direct contacts to the ligand. To try and resolve this contradiction, we mutated the Val-453 equivalent residue, Ile-489, in dN. This had no effect on ligand binding (data not shown), implying that the intensity changes observed previously (19) reflect an indirect effect on the residues stabilizing calcium binding to EGF12 by ligand binding within the central β-hairpin (because β-hairpin stability and calcium binding affinity are closely coupled).

This conservation of ligand-binding site between human and evolutionary distant Drosophila Notch proteins, despite a relatively high level of sequence diversity (Fig. 1b) and differences in the specific ligands assayed, suggests that this region of Notch will be involved in ligand binding in all species. It is also interesting to note that the threonine/serine residue (Thr-466 in hN-111–13/Ser-502 in dN), implicated in regulation by O-fucosylation, and Fringe extension are indicated.

FIGURE 2. Characterization and interaction studies of hN-1 variants. a, flow cytometry analysis of the interaction between J-1 and biotinylated wild-type (WT) and mutant N-111–13 constructs. Left-hand panel, flow cytometry of B16 cells expressing J-1 after interaction with biotinylated hN-1 mutants (red line) bound to avidin-coated fluorescent beads (see "Experimental Procedures"); in each case, a negative control (fibrillin calcium-binding EGF12–14, gray shading) and positive control (WT hN-111–13, black line) are shown. Three representative mutant constructs are shown; M479A is indistinguishable from WT, L468A overlays negative control, and Q475A shows intermediate binding. Right-hand panel, quantification of flow cytometry data for all EGF12 mutants expressed relative to WT control (WT = 1) (bars indicate mean, error bars indicate S.D., n = 3) and Western blot of hN-111–13 samples to show experiments controlled for protein loading. b, NMR analysis shows that WT and mutant constructs have native-like calcium binding properties indicative of correct folding. Aromatic regions of 1H–1H two-dimensional NOESY spectra at pH 7.5 of hN-111–13 WT, L468A, and I477A are shown. A marker for calcium binding in EGF12 (Tyr-444) is highlighted in the absence (blue) and presence (red) of Ca2+ (15 mM), and the shift is indicated by a red arrow. Quantification of data indicates relative chemical shift change on addition of Ca2+.

Red, WT; blue, L468A; green, I477A. The structure of hN-1 EGF12 (PDB 2VJ3) is shown; residues implicated in binding by flow cytometry (red), non-binding residues (blue), position of residue Thr-466 that is implicated in regulation by O-fucosylation, and Fringe extension are indicated.
Jagged-1/Serrate Recognition by Notch

Production of Paralogue-specific Antibodies—To test our hypothesis, we have used our soluble hN-111–13 containing the ligand-binding site to generate a panel of mAbs. Of these, one mAb (N1-39E) is seen to block receptor/ligand complex formation between hN-111–13 and cell surface expressed J-1 in our flow cytometry assay, whereas another mAb N1-96A has no effect on the interaction (Fig. 4a). Epitope mapping localizes the binding site of both N1-39E and N1-96A to EGF12 and/or the C-terminal region of EGF11 because they both recognize hN-1 mutants defective in calcium binding to EGF11 and EGF13, but do not recognize a mutant defective in calcium binding to EGF12 (Fig 4b). Subsequent testing against our panel of hN-1 EGF12 mutants demonstrates that the epitope of N1-39E, but not N1-96A, encompasses residues 468–477 of hN-1, which includes Leu-468 and Ile-477, directly implicated in flow cytometry analysis. The N1-39E epitope thus lies within the EGF12 Notch ligand-recognition site, explaining the molecular mechanism underlying its blockade of the hN-1/J-1 interaction. To test whether or not N1-39E is paralogue-specific, as suggested by the residues that form its epitope (Figs. 1b and 4c), we assayed whether or not it could recognize HEK 293 cells transfected individually with the different hNotch paralogues (N-1, N-2, N-3, and N-4). Flow cytometry analysis demonstrated that N1-39E specifically recognized only HEK293 cells expressing N-1 (expression of hNotch by each transfected cell line was confirmed by immunocytochemistry (Fig. 4d)). Recent work has demonstrated that selective interference of Notch paralogue activity by antibody binding can inhibit tumor growth in mouse xenograft models with reduced intestinal toxicity (30). Such reagents are likely to be valuable in the treatment of

Site-directed mutagenesis of residues in hN-1 and Drosophila Notch and their respective effects in flow cytometry, ligand binding, and cell aggregation assays

| hN-1 residue and substitution | EGF domain | Effect on ligand binding observed in flow cytometry analysis | Equivalent substitution in Drosophila Notch | Effect on Notch/Serrate cell aggregation |
|-------------------------------|------------|-------------------------------------------------------------|-------------------------------------------|----------------------------------------|
| Q442A<sup>a</sup> | 11 | Binds as WT | Q478A | Close to WT level |
| L468A | 12 | No binding | L506A | Strongly reduced |
| D469G<sup>a</sup> | 12 | No binding | D505A | Strongly reduced |
| Q470A | 12 | Binds as WT | ND | ND |
| E473A | 12 | Moderate binding | T509A | Moderately reduced |
| Q475A | 12 | Moderate binding | RS11A | Moderately reduced |
| I477A | 12 | No binding | V513A | Moderately reduced |
| M479A | 12 | Binds as WT | M515A | Binds as WT |

<sup>a</sup>Gln-442 in EGF1<sub>i</sub> is in close proximity to the region of ligand binding in EGF1<sub>2</sub>.
<sup>b</sup>Conserved calcium-binding residue. Loss of ligand binding is due to loss in structural integrity, rather than direct involvement in N-1/ligand interaction.
breast, melanoma, and T-cell acute lymphoblastic leukemia cancers, which are associated with aberrant Notch signaling, as well as important research tools (31). To date, potential therapeutic antibodies have targeted the negative regulatory region of Notch and have shown activity against Notch-1, -2, and -3 (30, 32, 33); antibodies to the extracellular domain of hN-1 encompassing EGF11–13 and EGF11–15 have also been reported (33, 34). We have now demonstrated a strategy for producing paralogue-specific antibodies to a region known to be critical for ligand binding. It should be noted that monoclonal antibodies raised to the ligand-binding region of Notch, and which block binding, may or may not reduce signaling (34, 35). This is

![FIGURE 4. Characterization of a ligand-blocking mAb to hN-1, which recognizes J-1-binding site.](#)

a, flow cytometry assay to show mAb N1-39E (red), but not N1-96A (blue), blocks the binding of hN-111–13 to HEK cells expressing hJ-1. b and c, epitope mapping by dot-blotting of WT and mutant hN-111–14 proteins (11NG, 12DG, 13DG) that contain a calcium-binding mutation in EGF11, EGF12, or EGF13 localizes the epitope of mAb N1-39E and N1-96A to EGF12 or the C terminus of EGF11. Dot-blotting of a panel of hN-1 EGF12 mutants demonstrates that L468A, Q470A, E473A, Q475A, and I477A are not recognized by N1-39E, and thus form part of its epitope. d, flow cytometry and immunocytochemistry (cytospin) analysis demonstrates that N1-39E is paralogue-specific. The red line indicates binding of mAb N1-39E; only 293T cells expressing hN-1 show a positive result, indicated by a rightward shift. Cytospin analysis (using monoclonal (mAb) or polyclonal (pAb) paralogue-specific antibodies) shows that each transfected cell line tested expresses a specific human Notch paralogue.

![FIGURE 5. Amino acid residues within β-hairpin implicated in ligand recognition and antibody binding in human and Drosophila Notch.](#) EGF12 of hN-1 is shown as a graphic and overlaid with a semitransparent surface. Positions that perturb activity in an assay are colored in red, those with no effect are colored in blue, and those not probed are colored in gray.
most likely due to the different affinities of the antibodies, their ability to recognize post-translationally modified forms of the receptor, or the cells used in the signaling assay expressing more than one paralogue on the cell surface. Nevertheless, given appropriate scale-up and screening of the molecular properties of such antibodies, this approach should yield reagents important for future research and therapies.

Our data demonstrate that the position and hydrophobic nature of the binding site for Jagged/Serrate ligands is conserved on the Notch EGF12 molecular surface throughout evolution (Fig. 5). Despite such global conservation, individual differences within the EGF12 site occur, suggesting that fine-tuning of the receptor/ligand interaction is possible, which may be subject to further regulation by O-glycosylation. As a consequence of the sequence variation observed, we demonstrate that it is possible to make targeted paralogue-specific, ligand-blocking mAbs (Fig. 5), which may be important for future research and therapeutics.

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