The Folding and Structural Integrity of the First LIN-12 Module of Human Notch1 Are Calcium-Dependent†

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ABSTRACT: Notch1 is a member of a conserved family of large modular type 1 transmembrane receptors that control differentiation in multicellular animals. Notch function is mediated through a novel signal transduction pathway involving successive ligand-induced proteolytic cleavages that serve to release the intracellular domain of Notch, which then translocates to the nucleus and activates downstream transcription factors. The extracellular domain of all Notch receptors have three iterated LIN-12 modules that appear to act as negative regulatory domains, possibly by limiting proteolysis. Each LIN-12 module contains three disulfide bonds and three conserved aspartate (D) or asparagine (N) residues. To begin to understand the structural basis for LIN-12 function, the first LIN-12 module of human Notch1 (rLIN-12.1) has been expressed recombinantly in *Escherichia coli* and purified in a reduced form. In redox buffers, rLIN-12.1 forms only one disulfide isomer in the presence of millimolar Ca^{2+} concentrations, whereas multiple disulfide isomers are observed in the presence of Mg^{2+} and EDTA. Further, mutation of conserved residues N1460, D1475, and D1478 to alanine abolishes Ca^{2+}-dependent folding of this module. Mass spectrometric analysis of partially reduced rLIN-12.1 has been used to deduce that disulfide bonds are formed between the first and fifth (C1449−C1472), second and fourth (C1454−C1467), and third and sixth (C1463−C1479) cysteines of this prototype module. This arrangement is distinct from that observed in other modules, such as EGF and LDL-A, that also contain three disulfide bonds. One-dimensional proton nuclear magnetic resonance shows that Ca^{2+} induces a dramatic increase in the extent of chemical shift dispersion of the native rLIN-12.1 amide protons, as seen for the Ca^{2+}-binding LDL-A modules. We conclude that Ca^{2+} is required both for proper folding and for the maintenance of the structural integrity of Notch/LIN-12 modules.

Signaling through Notch receptors has been implicated in the control of cellular differentiation in animals from nematodes to humans. The fundamental role inferred from genetic studies of LIN-12/Notch proteins is that they coordinate inductive or lateral cell−cell interactions that specify cell fate (*1*). Transmembrane protein ligands containing a conserved Delta/Serrate/LAG-2 motif activate LIN-12/Notch signal transduction (*2*). In response to ligand, the intracellular domain of Notch gains access to the nucleus and acts as a transcriptional co-activator (*3*). Signaling by Notch appears to be regulated through both positive and negative feedback loops, perhaps due to cell type-specific differences in other signaling pathways (*4*).

The human Notch1 (TAN-1) gene, one of four known human homologues of the *Drosophila* gene *Notch*, was originally discovered at the breakpoint of a recurrent t(7;9)-(q34;q34.3) chromosomal translocation found in a subset of human acute T-cell lymphoblastic leukemias (T-ALL)[1] (*5*, *6*). The normal Notch1 gene product is initially synthesized in a precursor form as a 350 kDa (p350) modular transmembrane glycoprotein. During transport to the cell surface, p350 is proteolytically processed by a furin-like protease into two subunits, an extracellular p230 subunit and a transmembrane p120 subunit (*5*, *7*, *8*), which remain associated and constitute the mature heterodimeric cell surface receptor (Figure 1A). p230 contains 36 N-terminal EGF-like modules, followed by three tandemly repeated LIN-12 modules which precede the furin cleavage site by 88 residues. The p120 subunit consists of an extracellular region of 69 amino acids

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[1] Abbreviations: CDAP, 1-cyano-4-(dimethylamino)pyridinium tetrafluoroborate; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; GSH, reduced glutathione; GSSG, oxidized glutathione; IPTG, isopropyl β-D-thiogalactopyranoside; LDL, low-density lipoprotein; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; NMR, nuclear magnetic resonance; NTA, nitrilotriacetic acid; 1D, one-dimensional; PCR, polymerase chain reaction; RP-HPLC, reverse-phase high-pressure liquid chromatography; T-ALL, acute T-cell lymphoblastic leukemia; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; TFA, trifluoroacetic acid.

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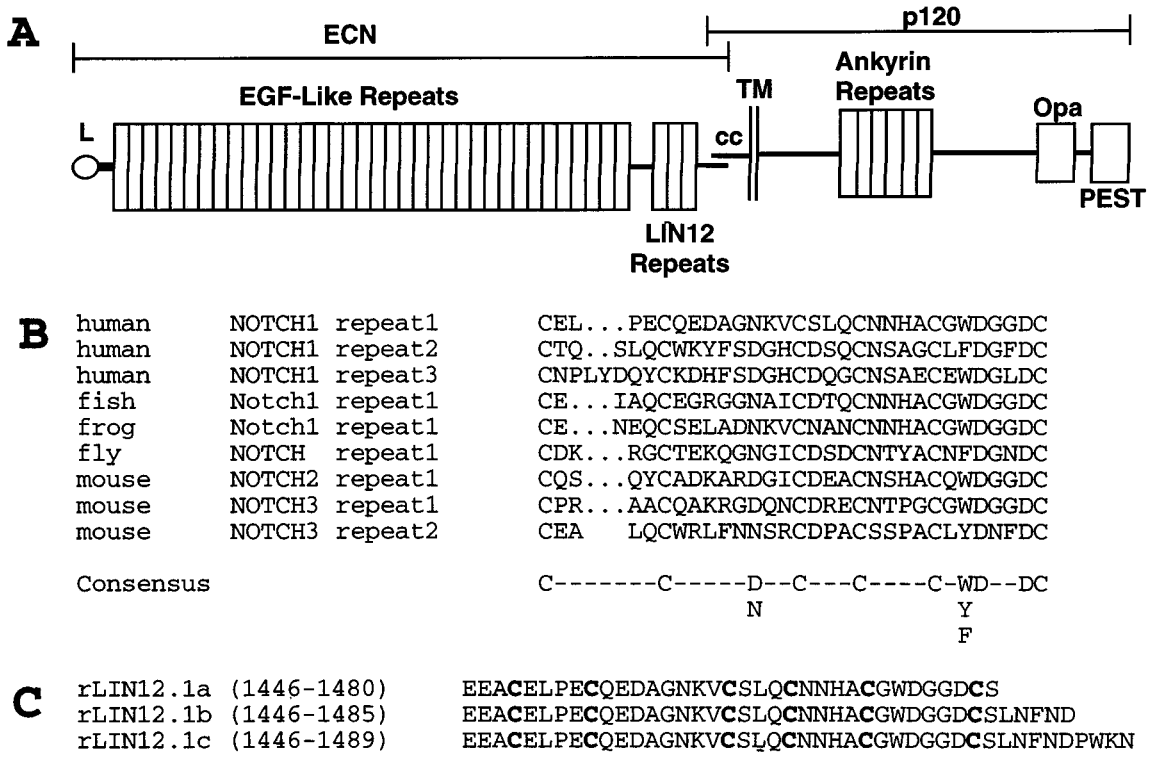

FIGURE 1: Structure of receptors containing Notch/LIN-12 modules. (A) Structure of human Notch1. Depicted are the mature Notch1 receptor, a heterodimer composed of ECN, an extracellular subunit containing 36 EGF modules and three Notch/LIN-12 modules, and p120, a noncovalently associated transmembrane signaling subunit. (B) Sequence alignment of Notch/LIN-12 modules from evolutionarily distant animal species. (C) Sequences of various forms of recombinant Notch/LIN-12 module from human Notch1 (rLIN-12.1) used in the experiments described in this report. The N-terminal residue of rLIN-12.1 corresponds to amino acid E1446 of human Notch1; the C-termini of the recombinant constructs lie at position 1480 (rLIN-12.1a), 1485 (rLIN-12.1b), or 1489 (rLIN-12.1c).

that includes a pair of conserved cysteine residues, followed by a single transmembrane segment and an intracellular region containing six ankyrin-like repeats flanked by two nuclear localization signal sequences, an Opa sequence, and a PEST sequence.

The current working model for Notch receptor activation proposes that ligand binding to the EGF domain of p230 triggers additional successive proteolytic cleavages (for a review, see ref *9*). Although the details of the proteolytic events that lead to activation of Notch receptors have not yet been completely defined, previous work has shown that the *Drosophila notch* genetically interacts with kuzbanian (*10*), a transmembrane metalloprotease belonging to the ADAM family of proteases. Ultimately, proteolysis occurs at a juxtamembranous intracellular site (*11*), releasing the 110 kDa Notch intracellular domain from its membrane attachment. This domain is then free to translocate to the nucleus of the cell, where it may bind to and activate downstream transcription factors (*3*, *12*).

Review of the sequence database indicates that LIN-12 domains are restricted to members of the Notch/LIN-12 gene family, all of which have a LIN-12 domain consisting of three tandemly repeated LIN-12 modules. Each LIN-12 module is approximately 35−40 residues long and contains six cysteine residues engaged in three disulfide bonds (Figure 1B). A high-resolution X-ray or NMR structure has not been determined for any LIN-12 module; indeed, even its native disulfide bond connectivity remains unknown.

Our interest in the LIN-12 domain stems from studies suggesting that their normal function is crucial for appropriate regulation of Notch receptor signaling. Certain point mutations within the LIN-12 repeats of the lin-12 gene of *Caenorhabditis elegans* confer a dominant gain of function phenotype consistent with ligand-independent activation (*13*), and mutant forms of Notch having deletions that span the LIN-12 domain are also constitutively active (*3*, *14*). In addition, forms of Notch1 lacking the LIN-12 repeats are potent inducers of T-ALL in a murine model (*5*, *15*). Conversely, variants of Notch that lack the amino-terminal EGF modules, but retain the LIN-12 domain, behave as loss of function alleles (*3*, *11*). On the basis of the current model of receptor activation, the LIN-12 domain may regulate access of the p230−p120 heterodimer to activating proteases; however, the details of how this might occur are unknown.

Insights into the structure and function of domains within large proteins have emerged from the strategy of protein dissection, in which large modular proteins are trimmed into smaller fragments capable of folding autonomously (see ref *16* for a recent review). Here, we have used protein dissection to investigate the first LIN-12 module (LIN-12.1) within the LIN-12 domain of human Notch1. We show that recombinantly expressed LIN-12.1 (rLIN-12.1) folds autonomously to a unique disulfide isomer. We have deduced the disulfide connectivity of rLIN-12.1, established that rLIN-12.1 requires calcium for folding to a unique disulfide isomer, and determined that calcium is required to maintain the structural integrity of rLIN-12.1, as evaluated by 1D ^{1}H NMR. Mutant forms of the LIN-12 module in which conserved polar and acidic residues have been substituted with alanine fail to fold to a single disulfide isomer, suggesting that these residues may coordinate a calcium ion and assist with module folding.

MATERIALS AND METHODS

rLIN-12.1 Expression Constructs. cDNAs encoding three different versions of LIN-12.1 were synthesized using PCR. The 5′ primer in all PCRs was 5′-AAAAAGCTTATGGAGGAGGCGTGCGAGCTG, which introduces a 5′ *Hin*dIII site and an in-frame ATG prior to LIN-12.1 sequences. The 3′ primers (designated a−c), which introduce a 3′ *Bam*HI site and a translational stop site, were as follows: a, 5′-AAAGGATCCCTAGGAGCAGTCACCGCCGT; b, 5′-AAAGGATCCCTAGTCATTGAAGTTGAGGGA; and c, 5′-AAAGGATCCCTAGTTCTTCCAGGGGTCAT. The a−c primer sets result in synthesis of cDNAs encoding forms of LIN-12.1 that begin at residue 1446 and end at residue 1480 (a), 1485 (b), or 1489 (c) of human Notch1 (Figure 1C). PCRs were performed for 20 cycles in a thermal cycler (MJ Research, Inc.) using 10 ng of template cDNA (*6*), 1.25 units of Taq polymerase, and the following parameters: 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s. After a final extension at 72 °C for 8 min, PCR products were purified from agarose gels on Qiaex II beads (Qiagen), cut with *Hin*dIII and *Bam*HI, and ligated to the vector pMMHb (*17*). Standard recombinant DNA techniques were used (*18*). Ligated DNA was used to transform *Escherichia coli* strain BL21(DE3)pLys(S). Clones bearing the desired cDNA insert were identified by colony PCR and confirmed by dideoxy DNA sequencing (AmpliTaq Sequencing Kit, Perkin-Elmer).

Site-Directed Mutagenesis. Site-directed mutagenesis was performed with mismatched oligonucleotides using the method of Kunkel (*19*). The following oligonuclotides were used to introduce codons for alanine at various positions in the LIN-12.1a cDNA: codon 1453, 5′-CTCCTGGCACGCGGGGCAGCTC; codon 1460, 5′-GCTGCAGACCTTGGCGCCCGCGTCCTC; codon 1475, 5′-GTCACCGCCGGCCCAGCCGCA; and codon 1478, 5′-GAGGGAGCAGGCACCGCCGTC (mismatched nucleotides are underlined). The identity of each mutant was confirmed by DNA sequencing.

Expression and Purification of rLIN-12.1 Peptides. Recombinant normal and mutated LIN-12.1 modules were expressed as trpLE fusion peptides, using a modified form of trpLE in which the methionine and cysteine residues have been replaced by leucine and alanine, respectively (*17*), and to which an amino-terminal $(His)_9$ tag has been added. As a result of the hydrophobic trpLE domain, $(His)_9$−trpLE−Met−LIN-12.1 chimeric peptides localize to inclusion bodies. BL21(DE3)pLys(S) clones were grown to an OD_{600nm} of 0.8 and then induced with 1 mM IPTG for 3 h at 37 °C. The inclusion bodies were isolated and solubilized from the cell pellet, passed over a column of Ni^{2+}−NTA agarose, lyophilized, and cleaved with cyanogen bromide as described previously (*17*, *20*). The peptides were dialyzed exhaustively against 5% acetic acid after cleavage and lyophilized. The dry protein was dissolved for 30 min at 37 °C in 10 mM Tris (pH 8) containing 8 M urea, 6 M guanidine hydrochloride, and 4 mM β-mercaptoethanol and passed again over a column of Ni^{2+}−NTA agarose. The flow-through was collected, reduced by addition of dithiothreitol (50 mM), dialyzed against 5% acetic acid, and purified to homogeneity by reversed-phase HPLC over a C-18 preparative VYDAC column. The identity of normal and mutated LIN-12.1 peptides was confirmed by MALDI-TOF mass spectrometry (Voyager Elite, PerSeptive Biosystems). All observed masses were within 1 Da of the expected value. Purified peptides were stored in a lyophilized form at 4 °C.

Disulfide Exchange Experiments. The calcium dependence of folding was investigated for rLIN-12.1 peptides under several sets of conditions permitting disulfide exchange. In analytical experiments, purified, reduced rLIN-12.1 peptides were resuspended at a concentration of 10 μM in 1 mL of 50 mM Tris (pH 8.0) containing reduced glutathione (2 mM), oxidized glutathione (1 mM), and various concentrations of $CaCl_2$, $MgCl_2$, or EDTA in an anaerobic chamber (Coy Laboratory Products) under 95% N_2 and 5% H_2 for 2−5 days at 20 °C. For large scale preparation of rLIN-12.1 peptides for ^{1}H NMR (see below), oxidation was also performed by dialysis against the same redox buffer in the presence of 50 mM $CaCl_2$. Complete oxidation of purified peptides was confirmed with Ellman's reagent. Disulfide exchange was stopped by addition of acetic acid to a final concentration of 5% (v/v). Samples were analyzed by reversed-phase HPLC on a VYDAC C-18 column using a linear gradient of 0.1%/min of solvent B. Solvent reservoirs contained water with 0.1% trifluoroacetic acid (A) and 90% acetonitrile with 0.1% trifluoroacetic acid (B).

Assignment of the Disulfide Connectivity of the rLIN-12.1 Module. Disulfide connectivity was determined as described by Wu and Watson (*21*) with minor modifications. Briefly, after partial reduction with TCEP and cyanylation by CDAP, partially reduced forms of rLIN-12.1c were purified by reversed-phase HPLC on an analytical C-18 VYDAC column using a linear gradient of acetonitrile in the mobile phase. Three partially reduced and cyanylated species were isolated, and the mass of each of these species was determined by mass spectrometry (MALDI-TOF, Voyager Elite, PerSeptive Biosystems) to be that of the parent rLIN-12.1c recombinant peptide plus 52 Da, corresponding to a doubly cyanylated peptide. Each doubly cyanylated peptide was dried under vacuum overnight, and was cleaved under basic conditions for 1 h at room temperature by addition of a 1 M solution of ammonium hydroxide (10 μL). After each peptide was again dried under vacuum, each was reduced in ammonium bicarbonate buffer (10 mM, pH 7.5) containing β-mercaptoethanol (15 mM) for 30 min at 37 °C. Each sample was then dried under vacuum again, and redissolved in a final volume of 50 μL of 0.1% TFA for analysis by MALDI-TOF mass spectrometry.

One-Dimensional ^{1}H NMR Spectroscopy. ^{1}H NMR spectra were obtained at 11.75 T using a Varian Unity spectrometer at 25 °C. Samples of rLIN-12.1a were prepared at protein concentrations of ∼1 mM in 90% H_2O/10% D_2O (pH 6.5) in the absence of added buffer or salt. Spectra were acquired before and after the addition of 10 mM $CaCl_2$. Sixty-four transients of 2048 points with a 7000 Hz sweep width were acquired for each sample using WATERGATE water suppression (*22*). The residual unsuppressed solvent signal was removed by convolution; 1 Hz of line broadening was applied to the data before zero filling to 4096 points. Spectra were referenced to water at 4.76 ppm.

RESULTS

Calcium Requirement for the Formation of Native Disulfide Bonds. Inspection of the consensus sequence for LIN-

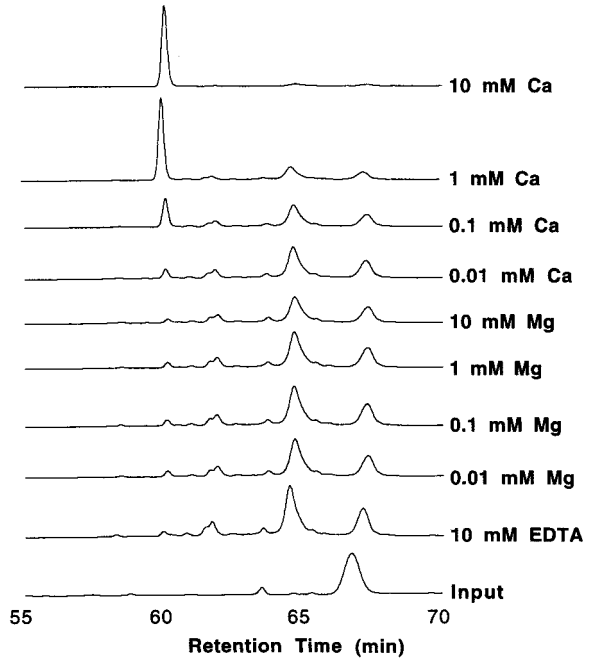

FIGURE 2: Folding of LIN-12.1 is Ca^{2+}-dependent. Reduced rLIN-12.1a (10 μM) was incubated for 48 h in a redox buffer containing the indicated concentration of Ca^{2+}, Mg^{2+}, or EDTA. Peptide isomers were resolved by reverse-phase HPLC. The elution profile of peptides was determined by measuring the absorbance at 229 nm.

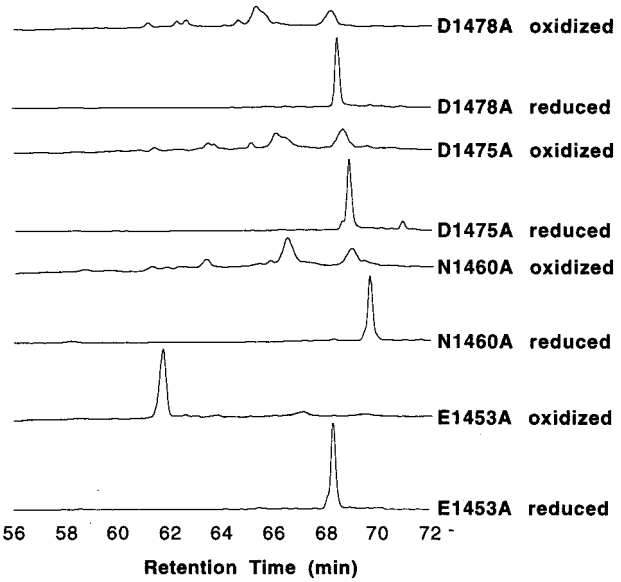

FIGURE 3: Mutation of conserved N and D residues abolishes calcium-dependent folding of LIN-12.1. Various forms of reduced rLIN-12.1a (10 μM) harboring the indicated amino acid substitutions were incubated for 48 h in a redox buffer containing 10 mM Ca^{2+}. Input-reduced peptides and oxidized peptide isomers were resolved by reverse-phase HPLC. The elution profile of peptides was determined by measuring the absorbance at 229 nm.

12 modules from diverse species revealed conservation of acidic or polar residues at three positions (Figure 1B). In the LIN-12.1 module of human Notch1, these residues correspond to N1460, D1475, and D1478. Acidic and polar residues have previously been shown to coordinate Ca^{2+} in EGF (*23*) and LDL-A protein modules (*24*), which also contain three disulfide bonds. Because calcium binding mediates proper folding of LDL-A modules (*17*, *25*), we hypothesized that Ca^{2+} would guide the formation of native disulfide bonds in LIN-12 modules.

To test this hypothesis, purified reduced rLIN-12.1 was allowed to equilibrate in the presence of varying concentrations of Ca^{2+}, Mg^{2+}, or EDTA in a redox buffer under conditions permitting disulfide exchange. The resulting distribution of disulfide-bonded isomers was resolved by RP-HPLC (Figure 2). Because it was possible that sequences lying between LIN-12.1 and LIN-12.2 might contribute to the structural integrity of LIN-12.1, three peptides, rLIN-12.1a, -b, and -c, were made that differed only in the length of their C-terminal sequences (Figure 1C). Identical results were obtained with all three, and data are shown only for the smallest peptide, rLIN-12.1a, which has only one residue C-terminal to the sixth cysteine of LIN-12.1. Prior to disulfide exchange, reduced rLIN-12.1a elutes as a single, fully reduced peak (Figure 2). After disulfide exchange for 48 h in the presence of 10 mM EDTA or up to 10 mM Mg^{2+}, a mixture of new species appears that reflects the presence of multiple disulfide isomers and/or partially oxidized intermediates. In contrast, in the presence of a millimolar concentration of Ca^{2+}, a single novel disulfide isomer is observed (Figure 2). The same distribution of isomers is observed after 5 days of redox exchange (not shown), indicating that equilibrium is reached by 48 h. When reduced peptides are oxidized by extensive dialysis against 2 mM GSH/1 mM GSSG at 4 °C under room air, a single peak is again observed when a millimolar concentration of Ca^{2+} is included in the buffer, whereas multiple peaks are seen in its absence (not shown). The peptide that folded in the presence of Ca^{2+} also fails to react with Ellman's reagent, indicating that it is fully oxidized. Hence, one disulfide isomer (among 15 possibilities) is thermodynamically favored when rLIN-12.1 oxidation is performed in the presence of Ca^{2+}.

Site-directed mutagenesis was used to assess the role of conserved N and D residues in peptide folding. rLIN-12.1a harboring the substitutions N1460A, D1475A, and D1478A is insensitive to the presence of Ca^{2+}, as they form a distribution of several disulfide isomers and/or partially oxidized species (Figure 3). In contrast, a mutated form of rLIN-12.1 in which the nonconserved acidic residue E1453 is changed to alanine folds efficiently to a single predominant disulfide isomer (Figure 3). Thus, conserved N and D residues are necessary for Ca^{2+}-dependent folding.

Assignment of Native LIN-12 Module Disulfide Connectivity. To determine the disulfide bond connectivity of rLIN-12.1, we modified a previously published method in which the peptide is partially reduced under acidic conditions, followed by rapid cyanylation of free cysteines (*21*). The partially reduced, cyanylated species are isolated by RP-HPLC, cleaved at the cyanylated cysteine residues with base, reduced fully, and identified by MALDI-TOF mass spectrometry. In the final reduction step, we substituted a volatile reducing solution containing β-mercaptoethanol in place of TCEP in guanidine hydrochloride. This adaptation permitted the use of less starting material and caused less interference during subsequent mass spectrometric analysis.

On the basis of the results shown in Figure 4, disulfide bonds are formed between the first and fifth (C1449−C1472), second and fourth (C1454−C1467), and third and sixth (C1463−C1479) cysteines of rLIN-12.1. A detailed

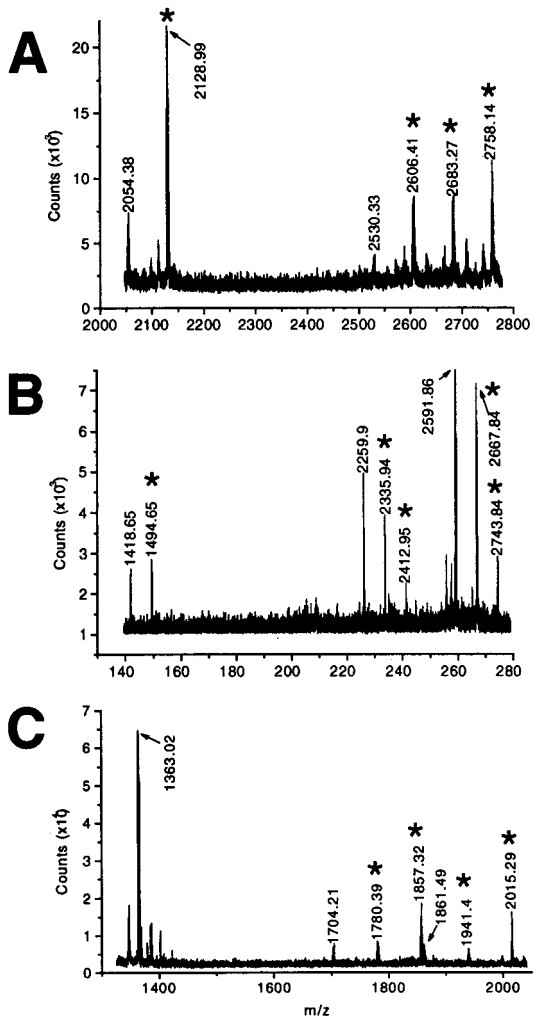

FIGURE 4: Determination of the disulfide connectivity of rLIN-12.1. rLIN-12.1c (amino acid residues 1446−1489 of Notch1) folded in the presence of Ca^{2+} was partially reduced, cyanylated, cleaved at cysteine residues, and reduced as described in Materials and Methods. A mass spectrum is shown for each of three singly reduced species derived from native LIN-12.1c, permitting unambiguous assignment of the C1449−C1472 disulfide bond (A), the C1454−C1467 disulfide bond (B), and the C1463−C1479 disulfide bond (C). Detailed comparisons of the observed masses of fragments with those predicted for disulfide pairs between the first and fifth (C1449−C1472), second and fourth (C1454−C1467), and third and sixth (C1463−C1479) cysteines are listed in Table 1. A ladder of peaks differing in mass by 76 Da is seen for all fragments containing internal cysteine residues, representing mixed disulfides formed with β-mercaptoethanol in the final reduction step (see Table 1). These β-mercaptoethanol derivatives of the parent peptide fragments are denoted with asterisks.

summary of the observed peptide fragments and the basis for these assignments are presented in Table 1. Note that a variable fraction of all reduced species containing free cysteine(s) formed mixed disulfide(s) with β-mercaptoethanol (mass of 76 Da) during the final reduction step. For a peptide with mass m containing n reduced cysteines, therefore, additional species that correspond to $m + 76(1)$, $m + 76(2)$, ..., $m + 76(n)$ are observed. This ladder of peaks thus helps to confirm the identity of each parent fragment.

One-Dimensional ^{1}H NMR Spectroscopy. In the absence of well-defined structure, chemical shifts tend toward the values observed in random coil peptides. Conversely, well-defined structure results in an increased extent of chemical shift dispersion because protons are positioned in unique local environments.

To address the role of calcium coordination in maintaining the structural integrity of LIN-12 modules, 1D ^{1}H NMR spectra of oxidized rLIN-12.1a were acquired in the presence and absence of calcium. These studies are simplified by the removal of Ca^{2+} from oxidized peptides under the conditions used for HPLC purification. The 1D ^{1}H spectrum of rLIN-12.1a with native disulfide bonds demonstrates a dramatic increase in the extent of chemical shift dispersion upon re-exposure to Ca^{2+} (Figure 5), particularly in the amide region. This observation indicates that the LIN-12.1 module requires Ca^{2+} not only to guide formation of correct disulfide bonds (Figure 2) but also to maintain a single well-defined structure in solution.

DISCUSSION

Here we present the first biochemical characterization of a prototypical LIN-12 module, LIN-12.1, derived from the human Notch1 receptor. Our data indicate that Ca^{2+} plays an essential role in the folding and structural integrity of LIN-12 modules, which show a disulfide connectivity different from that found in other types of protein modules containing three disulfide bonds, such as EGF modules (*26*), and LDL-A modules found in the LDL receptor family (*17*, *27*, *28*). This unique arrangement, in which the first and fifth (C1449−C1472), second and fourth (C1454−C1467), and third and sixth (C1463−C1479) cysteines of this prototype module form disulfide bonds, suggests a novel fold.

Under equilibrium folding conditions in the presence of millimolar Ca^{2+} concentrations, but not Mg^{2+} or EDTA, only one of 15 possible LIN-12.1 isomers is observed, strongly supporting the thesis that the observed isomer represents the natively folded module. The role we have identified for Ca^{2+} is a recurring theme among small extracellular protein modules that contain three disulfide bonds, as the folding of LDL-A modules (*17*, *25*) and the structure of a subset of EGF modules (*23*, *26*) are also Ca^{2+}-dependent.

Ca^{2+} coordination in other proteins and protein modules is frequently mediated through the side chains of aspartate, glutamate, asparagine, and glutamine. In LDL-A and EGF modules, the consensus Ca^{2+}-binding motifs are **D**x$_3$**D**cxdxs**DE** (*24*) and **D**x**DE** (*23*) (Ca^{2+}-coordinating residues in bold), respectively. In the LIN-12.1 module of Notch1 characterized here, mutation of each of three conserved N or D residues prevents Ca^{2+}-dependent folding. Although the definitive identification of the Ca^{2+}-coordinating residues awaits high-resolution structural analysis (currently in progress; C. L. North and S. C. Blacklow, unpublished results), our mutational studies suggest that these conserved asparagine and aspartate residues are likely to be important for Ca^{2+} binding (Figure 6), and thereby contribute to formation of the native fold.

Our NMR data indicate that Ca^{2+} is also critical for maintaining the structure of natively folded LIN-12 modules (Figure 5). Clues to the potential significance of this observation for the function of the LIN-12 domain come from studies of natively folded Ca^{2+}-binding EGF (*29*, *30*) and LDL-A (*31*, *32*) modules, the structures of which are likewise dependent on Ca^{2+}. NMR studies of an adjacent pair of EGF modules from fibrillin indicate that bound Ca^{2+} maintains

Table 1: Assignment of the LIN-12.1c Disulfide Connectivity by Mass Spectrometry[a]

assignment	exp (Da)	obs (Da)	$m + 76$[c] (Da)	$m + 152$[c] (Da)	$m + 228$[c] (Da)
C1449−C1472					
EEA	347	ND[b]			
CELPECQEDAGNKVCSLQCNNHA[d]	2531	2530	2606	2683	2758
CGWDGGDCSLNFNDPWKN[d]	2053	2054	2129		
C1454−C1467					
EEACELPE	918	ND[b]			
CQEDAGNKVCSLQ[d]	1420	1419	1495		
CNNHACGWDGGDCSLNFNDPWKN[d]	2592	2592	2668	2744	
EEACELPECQEDAGNKVCSLQ[e]	2260	2260	2336	2413	
C1463−C1479					
EEACELPECQEDAGNKV	1862	1861	1941	2015	
CSLQCNNHACGWDGGD[d]	1705	1704	1780	1857	
CSLNFNDPWKN[d]	1363	1363			

[a] Expected and observed values of *m*/*z*, based on average masses, are given for each peptide. The identity of these fragments is consistent only with the disulfide bond connectivity shown, permitting unambiguous assignment. The sequence of LIN-12.1c is given in Figure 1C. [b] ND, not detected. [c] The $m + 76$, $m + 152$, and $m + 228$ masses result from formation of mixed disulfides between internal cysteines of the peptide with β-mercaptoethanol. [d] Blocked at the N-terminus with an iminothiazolidine group, resulting in a fragment with a mass 27 Da greater than that predicted for the free peptide shown. [e] β-Elimination product derived from residues E1446−C1467.

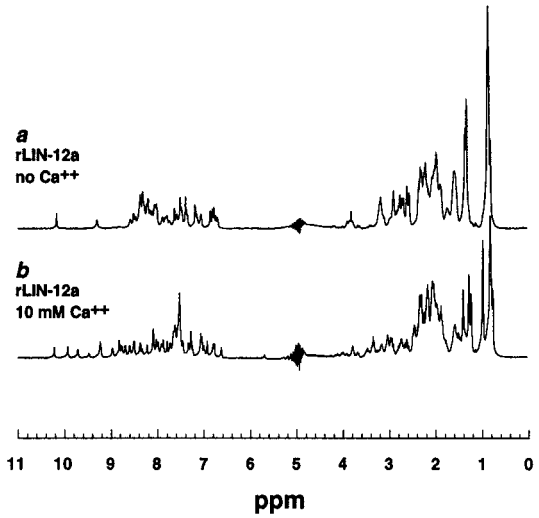

FIGURE 5: Ca^{2+}-dependent structural integrity of oxidized rLIN-12.1 with native disulfide bonds. 1D ^1H NMR spectra were obtained for LIN-12.1a (~1 mM) in the absence (a) and presence (b) of Ca^{2+} at pH 6.5 and 25 °C. A dramatic increase in the extent of chemical shift dispersion is observed in the presence of Ca^{2+}, particularly in the amide region.

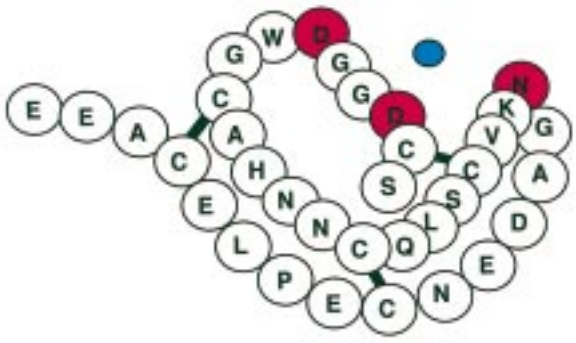

FIGURE 6: Structural model for Notch/LIN-12 modules. The model is based on the observed disulfide connectivity of rLIN-12.1 and assumes that conserved N and D residues (red) coordinate Ca^{2+} (blue).

the orientation of one EGF module with respect to the next, serving a structural role that may have important functional consequences for fibril formation (*30*). Binding of ligands by the LDL-A modules of LDL receptor family proteins, many of which transport lipoproteins into the cell, is also Ca^{2+}-dependent (*32*). Bound ligands are subsequently released in endosomes at low pH, where the affinity of the LDL-A modules for Ca^{2+} is substantially reduced (*31*).

The role of the LIN-12 modules in controlling signal transduction by Notch receptors is only beginning to be elucidated. Forms of Notch lacking the LIN-12 modules are constitutively active (*3*, *14*), whereas forms of Notch that retain the LIN-12 modules but lack the ligand-binding EGF repeats are refractory with respect to activation (*3*, *11*). These findings suggest that the LIN-12 modules function as a negative regulatory domain through either intra- or intermolecular interactions. Conceivably, ligand binding might relieve this inhibition by directly altering interactions involving the LIN-12 domain, thus permitting access of a ligand-dependent Notch-specific protease to its cleavage site (*11*).

Alternatively, ligand binding might serve as an initiation event, with receptor activation occurring several steps downstream. Genes encoding presenilin (*33*, *34*) and dynamin (*35*), proteins implicated in the regulation of vesicular transport, interact genetically with Notch, suggesting that ligand-dependent endocytosis might be involved in normal Notch receptor activation. Thus, activation might occur after movement of the receptor to acidified endosomal vesicles, with inhibitory LIN-12 interactions being relieved through changes in pH that alter Ca^{2+} binding and/or protein−protein interactions.

To investigate each of these potential mechanisms of activation, it will be important to determine whether the LIN-12 domain associates with other extracellular regions of Notch and, if it does, to evaluate whether these interactions are pH-dependent. Our ability to produce natively folded LIN-12 modules in large amounts will facilitate such studies.

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