Protease Sensitivity Map of the Highly Structured Heterodimerization Domain (HD) of the Human Notch2 Receptor in the Presence and Absence of the Furin Cleavage Loop

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Abstract

Notch receptors of the highly conserved signaling pathway regulate cell fate decisions in metazoans. Normal activation of the receptor depends on a tightly regulated, ligand dependent, proteolytic cleavage within the extracellular Heterodimerization Domain (HD). Aberrant Notch signaling is associated with a multitude of diseases. The HD consists of an intertwined α/β sandwich around a conserved hydrophobic core, a hotspot for many disease- inducing point mutations. HD harbors two of the three Notch specific proteolytic cleavage sites: S1, cleaved by a furin-like protease during receptor maturation, and S2, by an ADAM-type metalloprotease rendering the receptor susceptible to the activating-secretase cleavage in the membrane. Here, we present our study on the accessibility of specific protease sites within the human Notch2 HD (hN2 HD) in the presence and absence of the unstructured loop around the S1 site. We used limited proteolysis with trypsin and chymotrypsin followed by Matrix-assisted Laser Desorption Ionization-Time of Flight mass fingerprinting and C18 reverse phase Liquid Chromatography to create a relative protease susceptibility map for HD and compared the experimental results to the structure based predictions. Together with other stability data on specific HD mutants, the presented information provides further insight into the mechanism of Notch activation both in the normal and diseased states.

Background and Objectives

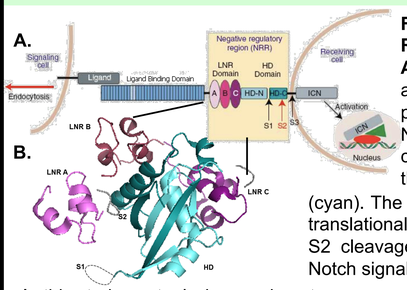

Figure 1. Domain organization of the Notch Receptor and Notch Signaling
A. Notch receptors exhibit a highly conserved modular architecture, which include in their extracellular portion a ligand binding domain (blue), and a Negative Regulatory Region (NRR) (tan box) composed of three Lin-Notch Repeats (LNR's) (pink) that fold around the Heterodimerization Domain (HD) (cyan). The HD harbors both the S1 cleavage site where a post-translational processing by a furin-like protease occurs and the S2 cleavage site where a ligand-dependent cleavage initiates Notch signaling. **B.** Crystal Structure of hN2NRR[1] (PDBID:2OO4)

- In this study, proteolysis experiments were conducted on hN2HDWT and hN2HDdel (HDWT missing the loop around the S1/ furin cleavage site) as a function of time and protease concentration using trypsin and chymotrypsin that targeted different regions throughout the HD. Experimental results were compared to cleavage predictions based on sequence and crystal structure of the NRR.
- **Hypotheses:**
  1. The presence of the furin loop results in differential accessibility of the HD by trypsin and chymotrypsin.
  2. HD harbors protease-accessible hotspots throughout the domain.

Materials and Methods

Sample Preparation:
Protein expression: hN2 HDWT and HDdel constructs were expressed as N-terminal His-tag proteins with a custom TEV cleavage site from a modified pET15b vector in pLysS cells. Bacterial cells were lysed with sonication and soluble HD protein was recovered after centrifugation.
Protein purification: The His-tagged HDWT and HDdel proteins were Ni^{2+} affinity purified and eluted with 250 mM imidazole in 50 mM Tris, pH 8.0, 300 mM NaCl and 5 mM $CaCl_2$. The proteins were subsequently purified on a S75 column and the monomeric species were pooled and concentrated.
Protease Digestion Experiments:
HDWT and HDdel were digested in parallel at 37°C with either trypsin (Promega) or chymotrypsin (Roche Diagnostics) at using a concentration range of 0.05 (0.5x)-1 mg/ml (10x) for 15min-4 hours. Control proteolytic digest experiments with no addition of enzyme for each condition were performed simultaneously. Proteolytic activity was terminated by addition of aprotinin (2μg/ml) for 20 min. prior to heat inactivation (10 min. 100°C).
Mass Spectrometry Data Collection:
The trypsin- and chymotrypsin-digested HDWT and HDdel were purified from salts and simultaneously concentrated with ZipTipC18 (Millipore) prior to MALDI-TOF mass spectrometry (MS) analysis. 1 μl of protein sample was mixed with 1 μl of HCCA matrix (4 mg/ml) and directly spotted on the target. Final MS spectra were acquired from the sum of 5000 laser shots in reflector mode with a method covering a mass window of 500-6000Da.
Data Analysis:
Bioinformatics: Trypsin and chymotrypsin cleavage sites and probabilities were predicted with the ExPasy PeptideCutter program[2]. Surface accessibility of these sites based on the crystal structure of NRRdel was calculated using DSSP[3].
Molecular Visualization: The pdb file for HDdel was derived from hN2NRR crystal structure (PDB ID: 2OO4) by removing the coordinates for the LNR Domain. PyMOL[4] was used to visualize the HDdel structure and create all the figures.
Mass Spectra Analysis: Trypsin and chymotrypsin digest spectra were analyzed and annotated using Bruker FlexAnalysis software.

Results

Figure 1. Sequence Alignment of hN2 HD: HDdel (red) is shown below HDWT (black) sequence. Dashes indicate deleted S1/furin loop. Arrows and coils below the sequences represent β-strands and α-helices, respectively. Boxed Tag: MHHHHHHGSENLYFQG. Pink and blue arrows above the sequences indicate predicted chymotrypsin and trypsin cleavage sites respectively.

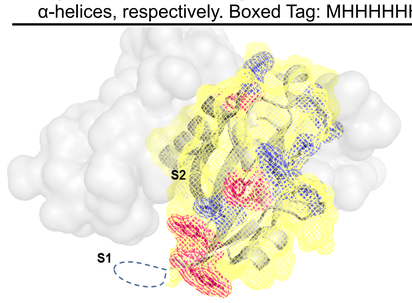

Figure 2. Predicted cleavage sites mapped onto the HDdel Structure LNR and HD domains are shown in solid gray surface and cartoon representation with a yellow mesh surface, respectively. Dashed loop indicates the missing S1/furin loop that is present in HDWT. Chymotrypsin and trypsin cleavage sites are highlighted in pink and blue, respectively. S1 and S2 sites are indicated on the figure.

FR. ID (HDWT HDdel)	Resulting peptide sequence	Peptide mass [Da]	C.P.	DSSP Accessibility[1]
C1/C1	MHHHHHHSFENLY	**1635.7**	74.7	--
C2/C2	F	**165.2**	63.5	--
C3/C3	QGENLAEGTLVIVVLMPPEQLLQDARSF	**3068.5**	67.8	0.0 (0.0,0.0)
C4/C4	LRALGTLLHTNLRIKRDSQELMVYPY	**3158.7**	44.3	26.1 (25.7,0.3)
C5/C5	Y	**181.9**	90.3	55.6 (46.8, 8.7)
C6/C6	GEKSAAMKKQRMTRRSLPGEQEQEVAGSKVFGEVAGSKVF	**3465.0** **893.0**	67.8	- 8.2 (8.2, 0.0)
C7/C7	LEIDNRQCVQDSDHCF ⌐ LEIDNRQCVQDSDHCF	**1922.0** S-H **1920.0** S-S	85.0	12.8 (12.2, 0.5)
C8/C8	KNTDAAAALLASHAIQGTLSYPLVSVVSESLTPERTQL	**3953.5**	--	--

Table 1. Chymotrypsin and trypsin cleaved HDWT and HDdel fragments Fragments and masses unique to HDdel are shown in red. Fragment are numbered consecutively with fragment identification (FR. ID) C for chymotrypsin and T for trypsin. Cleavage probability (C.P.) values are in percentages. Surface accessibility calculated for HDdel is shown as: total (side chain, backbone).

FR. ID (HDWT HDdel)	Resulting peptide sequence	Peptide mass [Da]	C.P.	DSSP Accessibility[1]
T1/T1	MHHHHHHGSENLYFQGENLAEGTLVIVVLMPPEQLLQDAR	**4599.2**	100	62.8 (60.4,2.5)
T2/T2	SFLR	**521.6**	95.7	59.5 (56.0,3.5)
T3/T3	ALGTLLHTNLR	**1208.4**	100	24.7 (24.1,0.0)
T4/T4	IK	**259.3**	89.9	12.7 (12.7,0.0)
T5/T5	R	**174.2**	100	44.3 (38.6,5.7)
T6	DSQGELMVYPYYGEK	**1779.0**		-
T7	SAAMK	**506.6**		-
T8	K	**146.2**		-
T9	Q	**302.3**	100	-
T10	MTR	**406.5**		-
T11	R	**174.2**		-
T12	SLPGEQEQEVAGSK	**1458.5**		21.3 (21.3,0.0)
T12	DSQGELMVYPYYGEVAGSK	**2093.3**		
T13/T13	VFLEIDNR	**1005.1**	100	18.6 (18.4 0.3)
T14/T14	QCVQDSDHCFK ⌐ QCVQDSDHCFK	**1309.4** S-H **1307.4** S-S	100	42.4 (37.2,5.2)
T15/T15	NTDAAAALLASHAIQGTLSY PLVSVVSESLTPER	**3482.9**	100	N/A
T16/T16	TQL	**360.4**	-	-

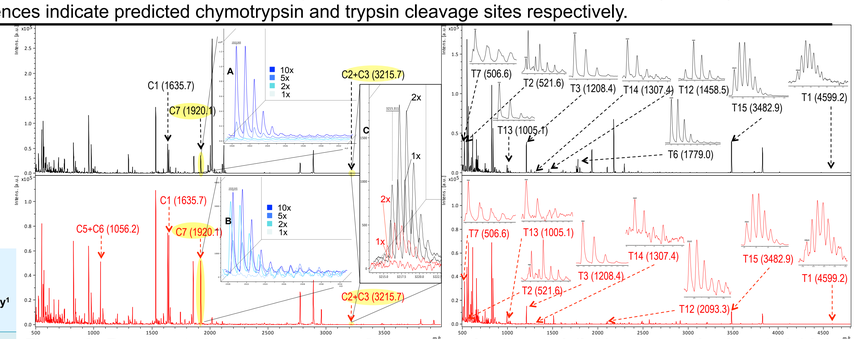

Figure 3. Representative mass spectra of chymotrypsin digested (left) and trypsin digested (right) HDWT (black) and HDdel (red) HD chymotrypsin cleavage products are shown for 4 hour/10x enzyme conditions (left). Inserts show a sample stacked view comparison of (**A**) HDWT and (**B**) HDdel peaks for highlighted fragment C7 at increasing chymotrypsin concentrations, as well as for highlighted fragment C2+C3 (**C**). Isotopically resolved peak profiles are shown for all observed trypsin cleavage products under 15min/1x enzyme conditions (right).

[E]	30 min	1 hour	2 hours	4 hours
1x	C1; C2+C3	C1; C2+C3; C4	C1; C2+C3; C4	N/A
	C1	C1	C1; C5+C6	N/A
2x	C1; C2+C3	C1; C2+C3	C1; C2+C3	N/A
	C1; C2+C3; C5+C6; C7	C1; C2+C3; C5+C6; C7	C1; C2+C3; C5+C6; C7	N/A
5x	C1; C2+C3	C1; C2+C3; C4; C7	C2+C3; C4; C7	C1; C7
	C1; C2+C3; C5+C6; C7	C1; C2+C3; C5+C6; C7	C1; C2+C3; C5+C6; C7	C1; C2+C3; C5+C6; C7
10x	N/A	C1; C2+C3; C7	C1; C2+C3; C7	C1;C2+C3; C7
	N/A	C1; C2+C3; C5+C6; C7	C1;C2+C3; C5+C6; C7	C1; C2+C3; C5+C6; C7

[E]	15 min	1 hour	2 hours
0.5x	T1; T2; T3; T6;T7; T12; T13; T14; T15	N/A	N/A
	T1; T2; T3; T12; T13; T14;T15	N/A	N/A
1x	T1; T2; T3; T6; T7; T12; T13; T14; T15	T1; T2; T3; T6; T7; T12; T13; T14; T15	T1; T2; T3; T6; T7; T12; T13; T14; T15;
	T1; T2; T3; T12; T13; T14; T15	T2; T3; T12; T13; T14; T15	T1; T2; T3; T12; T13; T14; T15
2x	N/A	N/A	T1; T2; T3; T6; T7; T12; T13; T14; T15
	N/A	N/A	T1; T2; T3; T12; T13; T14; T15
5x	N/A	T1; T2; T3; T6; T7; T12; T13; T14; T15	T1; T2; T3; T6; T7; T12; T13; T14; T15
	N/A	T1; T2; T3; T12; T13; T14; T15	T1; T2; T3; T12; T13; T14; T15

Table 2. Observed Fragments from HDWT (black) and HDdel (red) by chymotrypsin (pink panel) and trypsin (blue panel) The fragment IDs and color-coding correspond to those used in Table 1. Enzyme concentration ([E]) ranges from 0.050 mg/ml (0.5x) to 1mg/ml (10x) for trypsin and chymotrypsin. Due to matrix interference, digested fragments below 500Da (C2, C5, T4, T5, T8-T11, T16) are not detected. All observed fragment masses are within 0.5Da of the predicted masses. Conditions for which there are no matching HDWT and HDdel digest experiments are indicated with N/A.

Conclusions

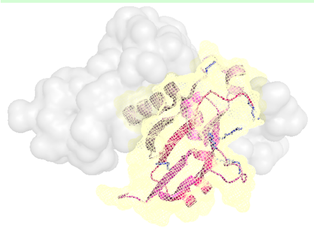

Figure 4. Protease Susceptibility map of HDdel. Differential protection of HDdel regions targeted by chymotrypsin shown in cartoon representation are indicated by varying intensities of pink. Trypsin cleavage sites are shown in blue.

- All predicted trypsin cleavage sites on both HDWT and HDdel were accessible under all experimental conditions indicating that:
  i. The S1/furin loop did not result in differential accessibility by trypsin in already highly solvent exposed HD regions.
  ii. All trypsin cleavage sites correspond to highly accessible hotspots within HD.
- Chymotrypsin cleavage profiles for HDWT and HDdel were similar with some important differences for most experimental conditions, with HDdel harboring more accessible sites in general indicating that:
  i. The S1/furin loop resulted in differential accessibility by chymotrypsin in the hydrophobic core of the HD.
  ii. There were accessible hydrophobic hotspots within HD as well as well-protected regions. For example, in both HDWT and HDdel, the C-terminus of residue Y1593, preceding the S1/ furin, was protected from chymotrypsin cleavage under all examined conditions.

Future Directions

To obtain quantitative data on the relative amount of accessible and protected fragments from both HDWT and HDdel we will perform High-Performance Liquid Chromatography analysis on all corresponding HD digests.

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Acknowledgments

This work is supported by NIH/NCI grant 1R15CA143892-01A1 (DVU and CYC) and the Wellesley College Sophomore Early Research Program (AA).