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Jagged-1 juxtamembrane region: Biochemical characterization and cleavage by ADAM17 (TACE) catalytic domain

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ABSTRACT

Ectodomain shedding of membrane receptors and ligands carried out by ADAMs (A disintegrin and metalloprotease) plays a major role in several signaling pathways, including Notch. The grounds of substrate recognition, however, are poorly understood. We demonstrate that a recombinant protein corresponding to the juxtamembrane region of Jagged-1, one of the Notch ligands, behaves as a structured module and is cleaved by ADAM17 catalytic domain at E1054. A short synthetic peptide is cleaved at the same site but at a much higher rate, implying that the structure of the cleavage site in the native protein is a key determinant for substrate recognition. We also show that an Alagille syndrome-associated mutation near E1054 increases the cleavage rate, which suggests that this mutation may lead to an unbalance in Notch signaling due to a higher level of Jagged-1 shedding.

1. Introduction

Jagged-1, one of the ligands of Notch receptors, is a type I membrane-spanning protein with a large extracellular region made of a poorly characterized N-terminal region, a Delta/Serrate/Lag-2 (DSL) domain, which is required for receptor binding, a series of tandem epidermal growth factor-like (EGF) repeats, a cysteine-rich domain that bears some similarity with von Willebrand factor type C (VWC) domains, and a ~130 residue juxtamembrane region (Fig. 1). Jagged-1, like Notch receptors, undergoes regulated intramembrane proteolysis and shedding of the extracellular region [1]. Ectodomain shedding and intramembrane cleavage are carried out by ADAMs (A disintegrin and metalloprotease) [2] and by the presenilin/γ-secretase complex [3], respectively. It was shown that Jagged-1 shedding is carried out by ADAM17 (also known as TACE, TNFα converting enzyme) [4] but the precise position of the cleavage is not known. Ectodomain shedding of membrane receptors and ligands carried out by ADAMs plays a major role in several signaling pathways mediated by membrane-bound receptors such as ErbB, TNFR, IL-6R, and Notch [5]. The grounds for specific substrate recognition by ADAMs, however, are not well understood. Modeling studies predict an extended conformation of the polypeptide chain in peptide substrates bound to the active site of ADAMs zinc metalloproteases [6]. Accordingly, specific substrate recognition by ADAM17 compared to ADAM10 depends both on the preference for certain amino acid types at selected positions of the cleaved sequence and on subtle differences in the residues forming the enzyme catalytic pocket [7]. ADAM17, however, cleaves a number of different substrates [8] and the cleavage sequence shows indeed a high variability [7,9–11]. It was suggested that, rather than a strict consensus, a stalk – a short segment between the membrane and the cleavage site – of 10–15 residues is required for shedding [12]. Other studies showed that the length of the stalk plays an important role, but is not the only determinant of shedding [13]. Because of the importance of ligand and receptor shedding in different signaling pathways, mutations that affect either ADAM17 or its substrates have the potential to interfere with the correct signal processing. Indeed, a mutation, p.V1055_R1056delinsG (VR→G), falling within the juxtamembrane region was found in a patient with Alagille syndrome [14], an autosomal dominant disorder (OMIM ID: 118450) that can affect the liver, heart, skeleton, eye, and kidneys in a complex manner [15].

To address the grounds of Jagged-1 recognition and cleavage by ADAM17, we undertook the expression, refolding, and purification of a recombinant protein, J1X, corresponding to the previously uncharacterized juxtamembrane region of human Jagged-1, and of a mutant, J1Xm, associated with Alagille syndrome, and report here their biochemical characterization, the identification of the cleavage site and the analysis of the processing rates for the recombinant proteins and for short synthetic polypeptides comprising the cleavage sequence.

2. Materials and methods

2.1. Sequence analysis

Secondary structure predictions were run on the Phyre web server [16] (http://www.sbg.bio.ic.ac.uk/phyre2/) using different

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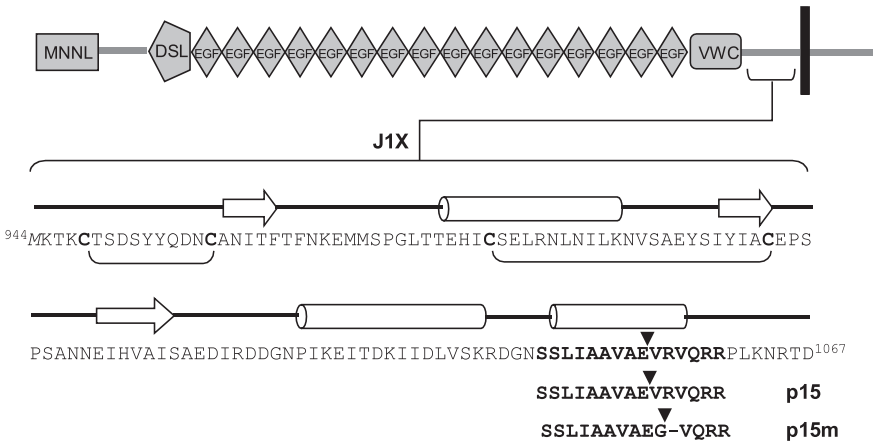

Fig. 1. Domain architecture. Sketch of Jagged-1 domain architecture (MNNL, N-terminal domain of Notch ligands; DSL, Delta/Serrate/Lag2 domain; EGF, epidermal growth factor repeat; VWC, von Willebrand factor type C-like domain; the transmembrane region is shown as a black rectangle), predicted secondary structure, amino acid sequence, disulfide bond connectivities of the recombinant J1X protein (M944 is a cloning artifact); the position of the cleavage site and the sequences of the p15 and p15m peptides are also shown.

methods (PsiPred, JNet, and SSPro) and the consensus results used to calculate the overall secondary structure content (%).

The 43 non-identical cleavage sequences of ADAM17 substrates classified as physiologically relevant were retrieved from the MEROPS database [17] (http://merops.sanger.ac.uk/), and the frequencies of amino acid types at different positions plotted as a sequence logo [18] (http://weblogo.berkeley.edu/).

2.2. Cloning, expression, and purification

The synthetic genes encoding the juxtamembrane region of human Jagged-1 (J1X, residues 945–1067 of JAG1_HUMAN, Fig. 1) or the p.V1055_R1056delinsG mutant (J1Xm), optimized for expression in *Escherichia coli* and cloned in the pET-11a expression plasmid, were purchased from GenScript (Piscataway, NJ, USA). The J1X and J1Xm recombinant proteins were expressed in *E. coli* BL21(DE3) cells (Novagen) and purified from inclusion bodies by ion exchange chromatography in denaturing conditions, followed by oxidative folding and final purification by RP-HPLC (see Figure S1 for details). The purified proteins were analyzed by SDS–PAGE and LC–MS to assess purity, correct molecular weight, and formation of disulfide bonds.

2.3. Disulfide mapping

Protein samples (20 μg) dissolved in 50 μL of 100 mM sodium acetate buffer, pH 5.5, containing 10 mM $CaCl_2$ were digested with 1 μg of modified porcine trypsin (sequencing grade, Promega). Aliquots of 5 μL were quenched at regular intervals with 10 μL of 10% TFA. The aliquots were analyzed by RP-HPLC on a Aeris C4 4.6×150 mm column, (Phenomenex) using a 30 min linear gradient from 0.1% TFA in water to 0.1% TFA in acetonitrile. The column effluent was diverted to an ESI ion trap mass spectrometer (Amazon SL, Bruker) and the mass spectra collected in positive ion mode.

2.4. Limited proteolysis

Protein samples (20 μg) dissolved in 50 μL of 100 mM sodium acetate, pH 5.5, containing 10 mM $CaCl_2$ were digested with 0.2 μg of chymotrypsin (sequencing grade, Roche). Aliquots of 5 μL were quenched at regular intervals with 10 μL of 10% TFA. The aliquots were analyzed by RP-HPLC as described in Section 2.3.

2.5. Size exclusion chromatography

Protein samples were dissolved in the elution buffer (20 mM sodium phosphate, pH 8.0, 50 mM NaCl), loaded onto a Superdex 75 10/300 GL column (GE Healthcare) and eluted in the same buffer. The apparent molecular mass of the protein was estimated from a calibration carried out with the following molecular standards: bovine serum albumin (67 kDa), ovalbumin (43 kDa), myoglobin (17 kDa), ribonuclease A (13.7 kDa), and aprotinin (6.5 kDa).

2.6. Peptide synthesis

Peptides were synthesized on solid phase (pre-loaded NovaSyn TGT, Merck) by Fmoc/t-Bu chemistry using a home-built automatic synthesizer based on a Gilson Aspec XL SPE system. The peptide-resins were cleaved/deprotected with TFA/triisopropylsilane/water (95/2.5/2.5%, v/v) for 3 h. The crude peptides were then precipitated by diethylether, washed, and freeze-dried. Crude peptides were purified by preparative RP-HPLC on a 25×300 mm column (Load&Lock system, Varian) packed with VariTide RPC resin (Polymer Laboratories – Varian) using a gradient from 0.1% TFA in water to 0.1% TFA in acetonitrile. The purified fractions were checked by ESI–MS for molecular mass correctness, pooled, and freeze-dried.

2.7. CD spectroscopy

Samples for CD spectroscopy were prepared dissolving the freeze-dried proteins in buffer (5 mM Tris–HCl, pH 7.4). Peptides were dissolved in water to obtain stock solutions that were diluted in buffer only or buffer containing TFE to obtain 5, 10, 20, 40, and 50% TFE (v/v). Protein concentration was determined by UV absorbance at 280 nm using the calculated ε value of 6210 M^{-1} cm^{-1}. Peptide concentration was determined by amino acid analysis (Alta Bioscience, Birmingham, UK) of the stock solutions. CD spectra were recorded on a Jasco J-810 spectropolarimeter using jacketed quartz cuvettes of 0.5 mm pathlength. Typically, 4–6 scans at a scan rate of 20 nm/min were acquired for each spectrum in the range 190–260 nm. Mean residue ellipticity (MRE, deg cm^2 $dmol^{-1}$ $residue^{-1}$) was calculated from the baseline-corrected spectrum. A quantitative estimation of secondary structure content was obtained using CDSSTR run from the DichroWeb server [19]. Helix content (%) of the peptides was calculated from the MRE at 222 nm according to the formula:

$[\alpha] = -100 \cdot MRE_{222}/39{,}500 \cdot (1 - 2.57/N)$, where N is the number of peptide bonds [20].

2.8. Enzymatic cleavage

The recombinant J1X and J1Xm proteins and the related p15 and p15m synthetic peptides were assayed for cleavage by ADAM17 enzyme. J1X and J1Xm stock solutions were prepared by dissolving freeze-dried aliquots in 25 mM Tris–HCl buffer, pH 8.0 at a final concentration of 140 μM and 151 μM, respectively. Synthetic peptide stock solutions were prepared in H_2O at a concentration of 225 μM for p15 and 705 μM for p15m. Human recombinant ADAM17 catalytic domain (ADAM17C) was purchased from Enzo Life Science, Farmingdale, NY. Both protein and peptide substrates were incubated at 30 μM final concentration with ADAM17C at 2.5 ng/μL (specific activity of 2430 U/μg enzyme) in 25 mM Tris–HCl buffer, pH 8.0 at 37 °C. Aliquots from each cleavage reaction were taken at 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, after over-night incubation, and stopped by the addition of 1% TFA. The aliquots were analyzed by RP-HPLC on a Jupiter C18 1×50 mm column (Phenomenex) heated at 50 °C, using a 20 min linear gradient from 0.1% formic acid in water to 0.1% formic acid in acetonitrile for the p15 and p15m peptides and a 30 min linear gradient from 0.1% TFA in water to 0.1% TFA in acetonitrile for J1X and J1Xm. The column effluent was diverted to an ESI ion trap mass spectrometer (Amazon SL, Bruker) and the mass spectra collected in positive ion mode. For each time point, ion chromatograms of selected ions characteristic of each molecule were extracted (Table S2).

3. Results

Deconvolution of the far-UV CD of J1X (Fig. 2A) suggests the presence of a mixed α+β secondary structure, in agreement with predictions (Fig. 1) and the presence of tertiary structure was confirmed by a negative band in the near-UV (data not shown). The two disulfides bonds were found to adopt a bead-like topology (C948-C958; C980-C1002) (Fig. 1; Figure S2), and limited proteolysis showed rapid cleavage of the N-terminal and C-terminal parts (Figure S3) and the presence of a resilient core region. The apparent MW of 18.0 kDa found by size-exclusion chromatography is ~30% larger than the calculated MW (13.9 kDa) and is consistent with a monomeric protein with a partially disordered state of the N-terminal and C-terminal segments (Figure S4).

Enzymatic cleavage of J1X by ADAM17C occurs between E1054 and V1055 (1046 SSLIAAVAE|VRVQRR 1060) (Fig. 3), as determined by RP-HPLC coupled to MS analysis (Table S2). The same site was found using a synthetic polypeptide, p15, bearing the cleavage sequence (Table S2). The processing rates for the J1X protein and the p15 peptide are, however, drastically different (Fig. 4); whereas p15 is completely cleaved already after 15 min, J1X is only partially cleaved after 4 h. To get more insight into the structural properties of the cleavage sequence, we acquired far-UV CD spectra of the p15 peptide in buffer and in the presence of increasing concentrations of TFE (Fig. 2B). The p15 peptide is mainly disordered in buffer but shows a significant helical propensity in the presence of TFE, in agreement with secondary structure predictions (Fig. 1). The coil–helix transition was estimated to occur at 20% TFE and a maximum helix content of 45%, reached at 40% TFE, was calculated from the MRE at 222 nm.

The VR→G mutation does not affect the structural properties of J1X in a significant way. The mutant protein, J1Xm, shares the same topology of disulfide bonds (Fig. 1; Figure S2) and hydrodynamic properties with the wild-type. The far-UV CD is only slightly different (Fig. 2A), the increase in the MRE at 222, 208, and 190 nm being

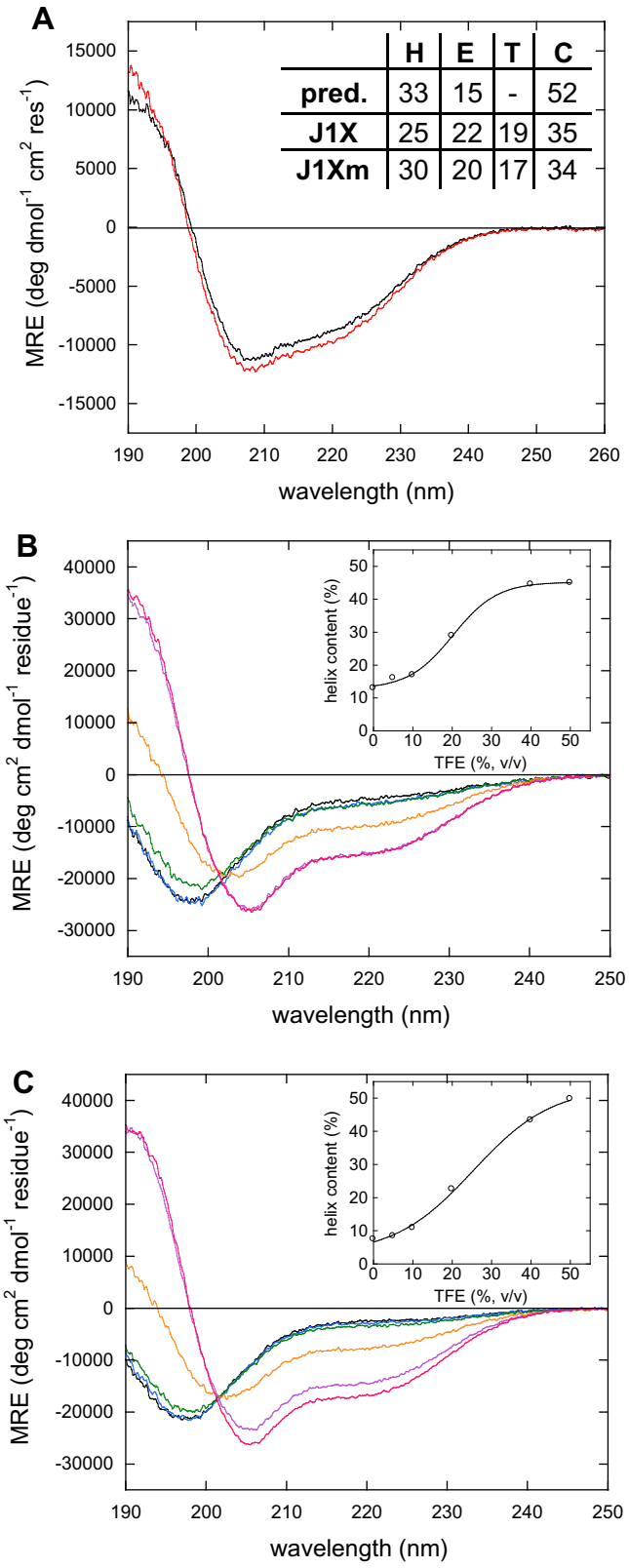

Fig. 2. Circular dichroism. Far-UV CD of (A) J1X (16 μM; black line) and J1Xm (16 μM; red line) in Tris buffer (5 mM, pH 7.4), (B) p15 (45 μM) and (C) of p15m (70.5 μM) in buffer (5 mM Tris, pH 7.5, black line) and at increasing TFE concentrations (v/v) (5%, blue; 10%, green; 20%, orange; 40%, magenta; 50%, red); inset in panel A: predicted and calculated secondary structure content (%) (H, helix; E, strand; T, turns; C, coil); insets in panels B and C: helix content (%) calculated from the MRE at 222 nm vs. TFE concentration (%, v/v). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

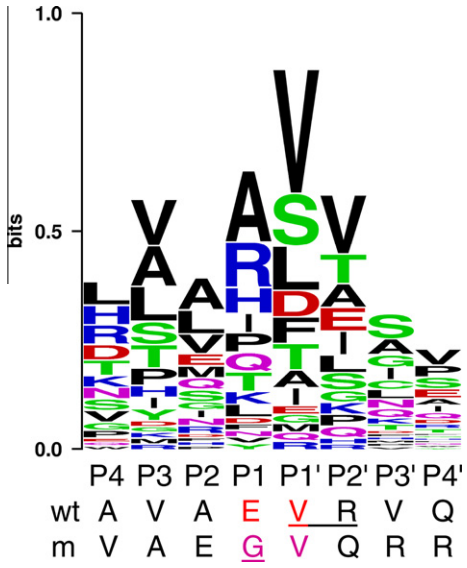

Fig. 3. Cleavage site. Sequence logo of the cleavage site in ADAM17 substrates and amino acid sequence of the experimental cleavage site found in the wild-type (J1X and p15, in red) and in the mutant (J1Xm and p15m, in magenta) Jagged-1 juxtamembrane region; residues involved in the VR→G mutation are underlined. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

consistent with a slight increase in the helical content (Fig. 2A). Although this difference has to be confirmed by more detailed structural studies, it is supported by limited proteolysis experiments, notably by a missed cleavage in the C-terminal region (Figure S3).

RP-HPLC coupled to MS analysis showed that J1Xm is also processed by ADAM17C, but with a shift in the position of the cleavage (G1055) to the following valine residue (Table S2) with respect to the wild-type sequence (Fig. 3). The same cleavage site was found in a synthetic polypeptide, p15m, bearing the mutant cleavage sequence (1046 SSLIAAVAEG|VQRR 1059) (Table S2). Comparable processing rates were observed for the p15m peptide and the recombinant J1Xm protein (Fig. 4). Far-UV CD spectra show that the p15m peptide is mainly disordered in buffer, much alike the p15 wild-type, and displays a similar, although not identical behavior in the presence of increasing concentrations of TFE (Fig. 2C). The maximum helix content was 50% at 50% TFE, but the lack of a plateau in the ellipticity at 222 nm in the range of the tested TFE concentrations hampered the reliable estimation of the transition midpoint.

Cleavage rates estimated by RP-HPLC coupled to MS analysis at different time points also showed that p15m is processed more slowly than p15, whereas the opposite trend was observed for the recombinant proteins J1Xm and J1X (Fig. 4).

4. Discussion

Sequence analysis of Jagged-1 suggested that the ADAM17 putative cleavage site resides within a hydrophobic stretch of amino acids (1048 LIAAVAEV 1055) located ~15 residues N-terminal to the membrane-spanning segment. The cleavage site belongs to Jagged-1 juxtamembrane region, which is delimited by the membrane-spanning segment at the C-terminus and by the VWC-like domain at the N-terminus (Fig. 1). This so far uncharacterized region shows no sequence similarity with other unrelated proteins or with known protein domains but is predicted to be globular (data not shown) and to have both α and β secondary structure segments (Fig. 1). The region chosen for expression (JAG1_HUMAN, residues 945–1067, J1X) includes all the predicted secondary

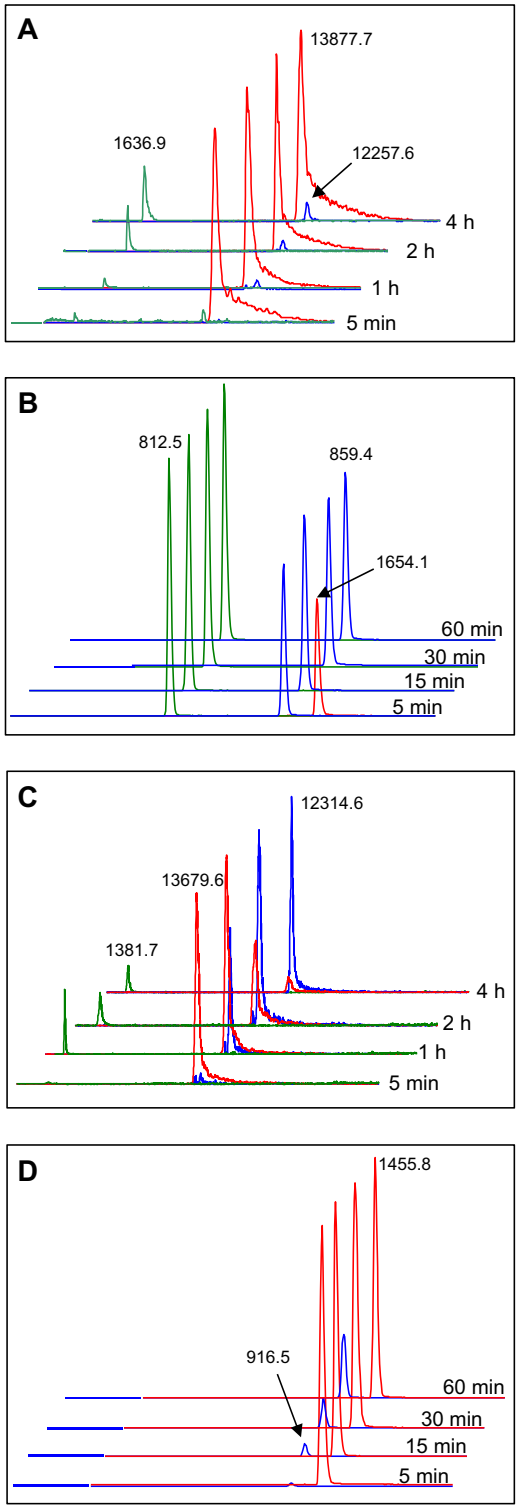

Fig. 4. Enzymatic cleavage. Ion chromatograms (EIC) extracted from the LC–MS analyses of (A) J1X, (B) p15, (C) J1Xm, and (D) p15m digested with ADAM17C; EIC traces at different digestion time points of the starting, uncleaved product in red, of the cleaved N-terminal fragment in blue, of the cleaved C-terminal fragment in green; the deconvoluted mass value (Da) of each species is also shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

structure segments and an even number of cysteines, to allow for the formation of disulfide bonds. Experimental results confirmed that J1X behaves as a structured, mainly globular domain, which is cleaved by ADAM17C between E1054 and V1055. This cleavage

site is somewhat unusual. While ADAM17 shows a strong preference for valine at position P1′ of the cleavage site [7], aliphatic residues such as alanine or leucine are most commonly found at positions P1, P2, P3, and P4 (Fig. 3; Table S1). We thus expected J1X to be cleaved between A1051 and V1052 (1046 SSLIAA|VAEVRVQRR 1060). As a positively charged residue (arginine or lysine) is often present at position P1, a second possible cleavage is before V1057 (1046 SSLIAAVAEVR|VQRR 1060). Instead, we identified the peptide bond between E1054 and V1055 as the target of ADAM17C and no other cleavage product could be detected. Although unusual, a negatively charged residue at position P1 was found also in another substrate, the surface glycoprotein of Ebola virus [21].

The VR→G mutation associated with Alagille syndrome [14] does not affect the biochemical and overall structural properties of J1X in a radical way, and both the p15 and p15m peptides display similar, although not identical, conformational properties in buffer as well as in the presence of TFE, as determined by far-UV CD. Both J1Xm and p15m are cleaved between G1055 and V1056 (1046 SSLIAAVAEG|VQRR 1059) and not at the expected position (1046 SSLIAA|VAEGVQRR 1059). These results show that, while the consensus sequence recognized by ADAM17 can indeed be quite variable, there is no promiscuity in the cleavage, probably because of the substantially different cleavage rates of the potential substrates.

The differences in cleavage rates observed for the same sequence within different structural contexts (the protein and the peptide) and for different sequences (the wild type and the mutant) in similar structural contexts show that the amino acid sequence of the cleavage site is not the only determinant in ADAM17 proteolytic activity. If sequence alone had a major impact on substrate recognition, the VR→G mutation would have the same effect on the peptide and on the protein, which is not consistent with experimental results. Furthermore, we observed very different cleavage rates for p15 and J1X. Although the length of the peptide is important, truncation studies showed that residues beyond position P3′ are not involved in substrate recognition [10]. The contributions of sequence, structure, and dynamics to substrate recognition and processing are linked and cannot be evaluated separately at this stage, but we propose that the structure and probably the dynamics of the cleavage site within the protein environment play a central role, as already put forward [13]. Differential cleavage rates may in turn represent an effective way to achieve substrate selectivity and provide a rationale to explain disease-associated mutations. Indeed, the p15m peptide is processed at a significantly lower rate than the wild-type. Because the two peptides display similar conformational properties in buffer, as determined by far-UV CD, the different cleavage rate may be due to a sub-optimal recognition of the mutant peptide by ADAM17C. J1Xm, on the contrary, is cleaved more rapidly than the wild-type protein in the experimental conditions used. This apparently contradictory result may be explained in terms of subtle structural changes and different dynamics of the cleavage region occurring upon the VR→G mutation [22]. If the difference in cleavage rates reported here for J1X and J1Xm is maintained also *in vivo*, it can be speculated that the VR→G mutation within the ADAM17 cleavage site leads to an unbalance in Notch signaling due to an increased level in Jagged-1 shedding.

On the grounds of secondary structure predictions, experimental secondary structure determined by far-UV CD, biochemical studies, and observed cleavage rates, it is likely that in the native protein the cleavage site adopts a well defined, helical conformation, which is in equilibrium with the extended conformation required for cleavage. In the cell, the transition from the structured form, more resilient to proteolysis, to the mainly disordered form, which would be recognized and processed by ADAM17, could be triggered by a mechanical pulling force such that generated by receptor/ligand recognition. Probably, additional factors play a role in substrate recognition and cleavage kinetics. It cannot be ruled out that long range interactions involving additional regions, other than the juxtamembrane of Jagged-1 and the catalytic domain of ADAM17, are involved in substrate recognition. Moreover, a recent report showed that desialylation of the N-linked oligosaccharides of the substrate is associated with ADAM17 activation, suggesting that post-translational modifications may play a regulatory role in sheddase activity [23]. Finally, the effects of the cell membrane outer layer on the structure of both the substrate and ADAM17 are yet to be investigated. More in-depth studies addressing the structure and dynamics of the juxtamembrane region will clarify the principles of substrate recognition by ADAM17, and possibly lead to the design of inhibitors that can target the interaction of ADAM17 with a selected substrate.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.02.022.

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