

# The intracellular region of the Notch ligand Jagged-1 gains partial structure upon binding to synthetic membranes

Matija Popovic, Alfredo De Biasio, Alessandro Pintar and Sándor Pongor

Protein Structure and Bioinformatics Group, International Centre for Genetic Engineering and Biotechnology (ICGEB), Padriciano, Trieste, Italy

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#### Correspondence

A. Pintar and S. Pongor, Protein Structure and Bioinformatics Group, International Centre for Genetic Engineering and Biotechnology (ICGEB), AREA Science Park, Padriciano 99, I-34012 Trieste, Italy Fax: +39 040226555 Tel: +39 04023757354 E-mail: pintar@icgeb.org, pongor@icgeb.org

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Notch ligands are membrane-spanning proteins made of a large extracellular region, a transmembrane segment, and a  $\sim$  100–200 residue cytoplasmic tail. The intracellular region of Jagged-1, one of the five ligands to Notch receptors in man, mediates protein-protein interactions through the C-terminal PDZ binding motif, is involved in receptor/ligand endocytosis triggered by mono-ubiquitination, and, as a consequence of regulated intramembrane proteolysis, can be released into the cytosol as a signaling fragment. The intracellular region of Jagged-1 may then exist in at least two forms: as a membrane-tethered protein located at the interface between the membrane and the cytoplasm, and as a soluble nucleocytoplasmic protein. Here, we report the characterization, in different environments, of a recombinant protein corresponding to the human Jagged-1 intracellular region (J1 tmic). In solution, J1 tmic behaves as an intrinsically disordered protein, but displays a significant helical propensity. In the presence of SDS micelles and phospholipid vesicles, used to mimick the interface between the plasma membrane and the cytosol, J1 tmic undergoes a substantial conformational change. We show that the interaction of J1\_tmic with SDS micelles drives partial helix formation, as measured by circular dichroism, and that the helical content depends on pH in a reversible manner. An increase in the helical content is observed also in the presence of vesicles made of negatively charged, but not zwitterionic, phospholipids. We propose that this partial folding may have implications in the interactions of J1 tmic with its binding partners, as well as in its post-translational modifications.

Ligands to Notch receptors [1,2] are type I membrane spanning proteins, all sharing a poorly characterized N-terminal region and a Delta/Serrate/Lag-2 domain, which are required for receptor binding, a series of tandem epidermal growth factor-like repeats, a transmembrane segment, and a unique cytoplasmic tail of  $\sim 100-200$  amino acids [3]. Five different ligands to Notch receptors have been identified in mammals, three orthologs (Delta-1, -3 and -4) of *Drosophila*  Delta and two orthologs (Jagged-1 and -2) of *Drosophila* Serrate. Although the molecular mechanisms of ligand specificity are still unclear, evidence from *in vivo* studies suggests that each ligand exerts nonredundant effects. Gene knock-out of Jagged-1 [4] or Delta-1 [5], heterozygous deletion of Delta-4 [6] or homozygous mutants in Jagged-2 [7] all lead to severe developmental defects and embryonic lethality in mice. There is no significant sequence similarity shared among the

#### Abbreviations

DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] sodium salt; DMPS,

<sup>1,2-</sup>dimyristoyl-sn-glycero-3-[phospho-L-serine] sodium salt; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate-d<sub>6</sub> sodium salt; HSQC, heteronuclear single quantum correlation; MRE, mean residue ellipticity; nrmsd, normalized root mean squared deviation of the fit; PDZ, domain present in PSD-95, Dlg, and ZO-1/2; RIP, regulated intramembrane proteolysis; TFE, 2,2,2-trifluoroethanol.

1086	$\mathbf{W}$ vtafywalrkrrkpgs $\underline{H}\underline{H}$ sasednttnnvreqlnqiknpiek $\underline{H}$ gantvpikdyenknskmskirt $\underline{H}$ nseveedd $\underline{M}$ dk $\underline{H}$ qqkarfakqpaytlvdreekppngtptk $\underline{H}$ pn $w$ tnkqdnrdl $\underline{P}$ saqslnr $w$ $\underline{P}$ IV	1218
psipred	chhhhhhhhhhcccccccccccchhhhhhhhhhhhhhhh	
jnet	chhhhhhhhhcccccccccccchhhhhhhhcccccccc	
sspro	cchhhhhhhhhcccccccccccchhhhhhhhhhhhhhcccc	
Cons	chhhhhhhhhhcccccccccccchhhhhhhhhhhhhh	
Prob	6688899988747888888888777656799999875475666477876654466887524344257888875647888877655779864554466789999988899988877776567877875543337	

**Fig. 1.** Secondary structure predictions. Amino acid sequence of J1\_tmic and secondary structure predictions (h, helix; e, β-strand; c, coil) obtained running PSIPRED, JNET and SSpro from the PHYRE web server (http://www.sbg.bio.ic.ac.uk/). The consensus secondary structure and the score are also shown; segments with high score are highlighted in gray. Histidine residues are underlined, tryptophans are in italics, and the C-terminal PDZ binding motif is in bold.

intracellular region of the different ligands, apart from the identical PDZ binding motif (ATEV) found at the C-terminus of Delta-1 and Delta-4. The cytoplasmic tail of Jagged-1 (Fig. 1) contains a different C-terminal PDZ interacting motif (EYIV), whereas neither Delta-3 nor Jagged-2 present a PDZ recognition motif. Jagged-1 has indeed been shown to interact in a PDZdependent manner [8] with afadin, a protein located at cell-cell adherens junctions. The cytoplasmic tail of Notch ligands is also required for endocytosis [9]. Mind bomb 1 (Mib1) has been recently suggested to be the E3 ubiquitin ligase responsible for mono-ubiquitinvlation of Jagged-1 in mice [10]. Finally, there is compelling evidence that Notch ligands, much like Notch receptors, undergo a proteolytic processing that is mediated by ADAM proteases and by the preseni $lin/\gamma$ -secretase complex [11]. A membrane-tethered C-terminal fragment of Jagged-1 comprising part of the transmembrane segment and the intracellular region expressed in COS cells was shown to localize mainly in the nucleus, and to activate gene expression through the transcription factor activator protein 1 (AP1/p39/jun) enhancer element [12].

The intracellular region of Jagged-1 can then exist in at least two distinct forms that experience two different environments. The first is a membrane-tethered protein located at the interface between the membrane and the cytoplasm, and the second is a soluble nucleocytoplasmic protein. We expressed and purified a recombinant protein starting at the putative intramembrane cleavage site and comprising part of the transmembrane segment and the entire intracellular region of human Jagged-1 (J1\_tmic) (Fig. 1), and studied its conformational properties in aqueous solution in the presence of a secondary structure promoting cosolvent like TFE and, to mimick the interface with the cell membrane, in the presence of SDS micelles or phospholipid vesicles. We show that J1\_tmic is mainly disordered in solution, but partially gains structure upon binding to the negatively charged surface of SDS micelles or to negatively charged phospholipid vesicles, with an increase in its  $\alpha$ -helical content. The transition between different environments, the membrane-cytosol interface and the cytoplasm, may affect the conformational properties of many receptor cytoplasmic tails that undergo regulated intramembrane proteolysis (RIP) mediated by presenilin/ $\gamma$ -secretase.

#### Results

#### J1\_tmic is mainly unstructured in solution

The presence of secondary structure in J1\_tmic was investigated by CD spectroscopy. The far-UV CD spectrum of J1\_tmic (Fig. 2) in Tris buffer shows a strong minimum at 198 nm, which is typical of disordered proteins. The content of secondary structure was estimated through deconvolution of the CD spectrum in the range 190–240 nm using several methods [13]. The best fit between the experimental and calculated spectra was obtained with CDSSTR (nrmsd = 0.013), including spectra of unfolded proteins [14] in the reference set. The results show a high content of unordered structure (65%) and a poor residual presence of secondary structure (4% helix, 19% strand and 12%



**Fig. 2.** Circular dichroism. Far-UV CD spectra of J1\_tmic (7.5  $\mu$ M) in 5 mM Tris/HCl buffer, pH 7.4, in the presence of different concentrations of 2,2,2-trifluoroethanol (TFE) (%, v/v).

turns) (supplementary Table S1). Very similar results were obtained from the CD spectrum of J1\_tmic purified in native conditions, confirming that the purification process did not affect the intrinsic conformation of J1\_tmic (data not shown).

NMR results support these findings (Fig. 3A,B). In the <sup>1</sup>H-<sup>15</sup>N HSQC spectrum of the <sup>15</sup>N-labeled protein, only  $\sim 90$  backbone HN cross-peaks of the expected 125 are detectable ( $\sim 70\%$ ), most of them clustered in a narrow region of between 7.7 and 8.4 p.p.m. (Fig. 3B) and many resonances suffer from extensive line broadening. The average value of HN chemical shifts is 8.08 p.p.m. with a dispersion ( $\sigma$ ) of 0.22 p.p.m. For comparison, the random coil values for a protein of the same amino acid composition would have an average of 8.18 p.p.m and a dispersion of 0.16 p.p.m. (supplementary Figure S1). The lack of chemical shift dispersion in the HN region as well as in the methyl region (data not shown) is an indicator of the lack of globular structure, and of little, if any, secondary structure [15]. The presence of strong and sharp resonances accompanied by much weaker peaks in the <sup>1</sup>H-<sup>15</sup>N HSOC spectrum, and the few peaks that could be identified in the HN-H $\alpha$  region of the <sup>1</sup>H-<sup>15</sup>N heteronuclear single quantum correlation/total correlated spectroscopy (HSQC-TOCSY) spectrum (data not shown) also point to the presence of conformational exchange processes. The lack of chemical shift dispersion in the HSQC spectrum obtained from in-cell NMR experiments (Fig. 3A and supplementary Figure S2) is a further confirmation of the lack of globular structure, even in the molecular crowding conditions of a cell-like environment [16].

To better characterize the conformation of J1\_tmic in solution, we studied its hydrodynamic properties through size exclusion chromatography. J1\_tmic (15.5 kDa) is eluted from the size-exclusion column as a peak corresponding to a 25.6 kDa globular protein (Fig. 4). The sharpness and symmetry of the peak (Supplementary Figure S3) indicates the presence of a single, well-defined species. The calculated Stokes radius,  $R_{\rm S}$ , for an apparent mass (*m*) of 25.6 kDa is 23.57  $\pm$  0.35 Å. This is slightly larger than the calculated value ( $R_{\rm S}N = 19.6 \pm 0.3$  Å) for a globular protein with the same number of residues as J1\_tmic but considerably smaller than the expected value for a completely extended chain ( $R_{\rm S}U = 36.4 \pm 0.7$  Å) as can be measured in denaturing conditions [17].

**Fig. 3.** NMR spectroscopy.  ${}^{1}H{}^{-15}N$  HSQC spectra of J1\_tmic (A) from in-cell experiments, (B) of the purified protein (0.5 mM) in H<sub>2</sub>O/D<sub>2</sub>O (90/10, v/v), pH 7.0, (C) in the presence of SDS (50 mM), pH 7.0, and (D) in the presence of SDS (50 mM), pH 5.6.





**Fig. 4.** Size-exclusion chromatography. Calibration standards are shown as open circles (1, horse myoglobin (17 kDa); 2, carbonic anhydrase (29 kDa); 3, bovine serum albumin (67 kDa); 4, lactate dehydrogenase (147 kDa)), J1\_tmic as a filled square (apparent m = 25.6 kDa); the calibration curve is also shown.

Our structural data on J1\_tmic collected by CD, size exclusion chromatography and NMR are consistent with a mainly disordered, but rather compact, state of the protein in solution, and the presence of very little or no secondary structure.

#### J1\_tmic exhibits intrinsic helical propensity

J1 tmic is predicted to adopt some secondary structure, as determined by subjecting the protein sequence to the analysis of different secondary structure predictors (PSIPRED [18], JNet [19], SSpro [20]) run from the PHYRE web server (http://www.sbg.bio.ic.ac.uk). From the consensus secondary structure prediction, four stretches of helix displaying a relatively high confidence can be identified (Fig. 1). These predictions led us to speculate that the J1\_tmic secondary structure might be stabilized in specific conditions. To test this possibility, we first analyzed the secondary structure of J1\_tmic in the presence of different concentrations of trifluoroethanol (TFE). Starting from a random-coil conformation in aqueous solution, a significant change in the secondary structure was observed upon addition of increasing amounts of TFE. The CD spectra developed a strong ellipticity at 206 nm and a shoulder at 222 nm, characteristic of an α-helical structure, at the expense of the minimum at 198 nm, showing that TFE induces an *a*-helical conformational in J1 tmic (Fig. 2). The J1\_tmic helical content increases from 4% to 50% upon TFE addition (0–50%, v/v), with a drastic change in ellipticity between 10 and 25% TFE. These results confirm that J1\_tmic possesses intrinsic helical propensity, and the measured  $\alpha$ -helical content is consistent with the predicted one (23–35% for the consensus prediction, depending on the threshold set for the probability score).

# J1\_tmic binds to SDS micelles and phospholipid vesicles

Binding of J1 tmic to SDS micelles and phospholipid vesicles was monitored by tryptophan emission fluorescence spectroscopy and fluorescence anisotropy, taking advantage of the two tryptophans present in the sequence. At increasing SDS concentrations, an increase from 0.07 to 0.12 in anisotropy was observed (Fig. 5A). At submillimolar concentrations (50-100 µM SDS) abnormally high anisotropy values were observed (data not shown), probably due to scattering associated with solution turbidity, which, however, disappeared at higher SDS concentrations. Tryptophan fluorescence emission spectra showed an increase in intensity and a blue-shift of the maximum from 355 to 350 nm in the presence of SDS (Fig. 5). In the presence of 1,2-dimyristoyl-sn-glycero-3-[phospho-rac-(1glycerol)] sodium salt (DMPG) phospholipid vesicles, changes were even more evident, with a marked increase in the emission intensity and a blue-shift from 355 to 345 nm (Fig. 5). Altogether, fluorescence data confirm binding of J1 tmic to SDS micelles and DMPG phospholipid vesicles, with at least partial embedding of one or both the tryptophan residues in a more hydrophobic environment [21,22].

As J1 tmic contains two tryptophans, W1091 in the N-terminal transmembrane region and W1196 in the C-terminal region, similar experiments were repeated on a recombinant protein, J1 ic [23], that lacks the transmembrane segment and thus contains only W1196. In this case as well, we could observe an increase in the anisotropy and a shift in the maximum from 356 to 346 nm upon addition of SDS (final concentration: 3 mm) but the shift was accompanied by a decrease, rather than an increase, in the fluorescence intensity (supplementary Figure S4). In the presence of DMPG phospholipid vesicles, the blue-shift was accompanied by an increase in the emission intensity, as measured with J1 tmic, and a blue-shift from 356 to 345 nm. Although these results are not conclusive with respect to the determination of the precise environment of the two tryptophans, they show that both J1\_tmic and J1\_ic bind to SDS micelles and DMPG



**Fig. 5.** Fluorescence spectroscopy. (A) Tryptophan fluorescence anisotropy and emission intensity of J1\_tmic (7.5  $\mu$ M) in 5 mM Tris/HCl buffer, pH 7.4, in the presence of increasing concentrations of SDS. (B) Tryptophan fluorescence emission spectra of J1\_tmic (7.5  $\mu$ M) in 5 mM Tris/HCl buffer, pH 7.4, in the presence of SDS (3 mM), and in the presence of DMPG (1 mM) phospholipid vesicles; excitation wavelength was set to 295 nm.

phospholipid vesicles, and thus that the transmembrane region of J1\_tmic is not absolutely required for binding. The reduced W1196 fluorescence emission in the presence of SDS micelles can be explained by the quenching effect of the negatively charged sulfate groups of SDS.

# J1\_tmic gains helical structure upon binding to SDS micelles

The secondary structure of J1\_tmic in the presence of SDS micelles, which provide a model for the hydro-phobic/hydrophilic interface found in lipid membranes, was analyzed by CD. As already seen with



Fig. 6. Circular dichroism in the presence of SDS. Far-UV CD spectra of J1\_tmic (7.5  $\mu$ M) in 5 mM Tris/HCl buffer, at different pH values (7.4 and 6.0) in buffer alone and in the presence of SDS (3 mM).

TFE, at increasing concentrations of SDS J1\_tmic undergoes a significant conformational change towards an  $\alpha$ -helical structure, reaching a maximum of ~ 17% of  $\alpha$ -helix at saturation (3 mM SDS), as estimated from CDSSTR (Fig. 6). At the same SDS concentration (3 mM), the  $\alpha$ -helical content reversibly increases as the pH decreases (17% of  $\alpha$ -helix at pH 7.4 versus 33% at pH 6) (Supplementary Figure S5, Supplementary Table S1), whereas the same pH change does not induce any significant change in the  $\alpha$ -helical content in the protein in the absence of SDS (Fig. 6, Supplementary Table S1).

The conformation of J1 tmic in the presence of SDS was further analyzed by NMR spectroscopy. The <sup>1</sup>H-<sup>15</sup>N HSOC spectrum of J1 tmic obtained in the presence of SDS micelles is somewhat different from that of the protein alone (Fig. 3C-D). Although several resonances are still missing, probably due to overlap, HN cross-peaks appear to be of similar intensity and slightly better dispersed. Most HN backbone resonances are still clustered in a relatively narrow region (7.5-8.5 p.p.m.), but the average value of HN chemical shifts (7.97 p.p.m.) is smaller and the dispersion slightly larger ( $\sigma = 0.25$ ) compared to the values obtained for the protein alone (Supplementary Figure S1). Most of the expected cross-peaks in the  $H\alpha$ region of the <sup>1</sup>H-<sup>15</sup>N HSQC-TOCSY spectrum are still missing (data not shown). The lack of significant chemical shift dispersion in the HN and  $H\alpha$  chemical shifts is an evidence of lack of tertiary structure. On the other hand, NMR spectra suggest that the conformation of J1 tmic is at least partially restrained in the



Fig. 7. Circular dichroism in the presence of DMPG. Far-UV CD spectra of J1\_tmic (7.5  $\mu$ M) in 5 mM Tris/HCl and in the presence of DMPG (1 mM) phospholipid vesicles at pH 7.4 and pH 6.0.

presence of SDS micelles. At a lower pH, the appearance of both the HSQC and the <sup>1</sup>H-<sup>15</sup>N HSQC-TOCSY spectrum is markedly different. Most of the expected HN cross-peaks (93%) and of the H $\alpha$  peaks could be detected, and lines are much narrower than at pH 7. The average chemical shift of backbone amides is 8.04 p.p.m. and the dispersion 0.23 p.p.m. Also in these conditions, however, the lack of chemical shift dispersion points to the absence of tertiary structure.

# J1\_tmic gains helical structure upon binding to negatively charged phospholipid vesicles

As a model of biological membranes we also used vesicles prepared from various phospholipids that are typical components of eukaryotic cell membranes. The far-UV CD of J1 tmic in the presence of vesicles prepared from the negatively charged phospholipids DMPG (Fig. 7) or dimyristoylphosphatidylserine (DMPS) (Fig. 8) showed spectral variations similar to those obtained in the presence of SDS micelles (Fig. 6). The estimated α-helical content was 19% and 17% in the presence of DMPG and DMPS vesicles, respectively (lipid concentration: 1 mM; protein/lipid molar ratio = 1 : 130). On the contrary, no change could be detected in the presence of vesicles made of the zwitterionic phospholipid dimyristoylphosphatidylcholine (DMPC) (Fig. 8). In the presence of DMPG phospholipid vesicles, a decrease in pH from 7.4 to 6.0 led to a reversible increase in the helical content of J1 tmic from 19 to 36% (Fig. 7, supplementary Figure S8, supplementary Table S1).

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Fig. 8. Circular dichroism in the presence of phospholipid vesicles. Far-UV CD spectra of J1\_tmic (7.5  $\mu$ M) in 5 mM Tris/HCl buffer, pH 7.4, in the presence of DMPS, DMPG, or DMPC phospholipid vesicles.

# Discussion

The rationale of this work is based on recent evidence suggesting that the intracellular region of Jagged-1 exists in at least two distinct forms [12]. The first is a membrane-tethered protein experiencing the interface between the membrane and the cytoplasm, the second is a soluble nucleocytoplasmic protein, and is produced by intramembrane proteolytic cleavage by the preseni $lin/\gamma$ -secretase complex [12]. Although the precise cleavage site in Jagged-1 is not known, experimental evidence from the cleavage of Notch receptors suggests that it is placed at the first valine close to the inner side of the cytoplasm [24]. We thus expressed and purified a recombinant protein starting at the putative intramembrane cleavage site and comprising part of the transmembrane segment and the entire intracellular region of human Jagged-1 (J1 tmic), and studied its conformational properties in different conditions. SDS micelles and phospholipid vesicles were used to mimick the membrane/cytoplasm interface, whereas standard buffers were used to simulate the conditions experienced by the cleaved form. Additionally, we used incell NMR to reproduce the molecular crowding effects of a cell-like environment. Finally, TFE was used to investigate the intrinsic secondary structure propensity in conditions of reduced solvation.

In the presence of SDS micelles (Fig. 6) or vesicles made of negatively charged phospholipids (DMPG, DMPS) (Figs 7 and 8), which are prevalent components of the inner layer of the plasma membrane in eukaryotes, J1\_tmic gains secondary structure. The helical content measured by CD is consistent with secondary structure predictions (Fig. 1). No changes in CD spectra were observed in the presence of vesicles formed by a zwitterionic phospholipid like DMPC (Fig. 8), suggesting that the negative charge density at the surface of SDS micelles or phospholipid vesicles is required to promote binding and secondary structure formation. Interestingly, in the presence of SDS micelles, the formation of secondary structure is strongly pH-dependent, with a sharp increase in the helical content from pH  $\sim$  7 to  $\sim$  6. As J1 tmic contains six endogenous histidines, it is possible that protonation of one or more of the histidines is promoting helix formation or extension. A similar behavior was observed also in the presence of DMPG phospholipid vesicles (Fig. 7). The possible biological relevance of this observation is not clear. The biophysical properties of the interface between the cytoplasm and the plasma membrane are not very well known [25], and it is plausible that the negatively charged head groups of phospholipids present in the membrane of eukaryotic cells can generate a pH gradient [26]. From pH mapping by fluorescence, it has been actually reported in an early study that the effective pH in proximity of the membrane in yeast cells is  $\sim 6.0$  [27], which supports the physiological relevance of the pH-dependent secondary structure formation in J1 tmic.

The titration with SDS revealed that SDS triggers binding below its critical micellar concentration (2–8 mM, depending on ionic strength), with a saturated binding around 1 mM for 7.5  $\mu$ M J1\_tmic, suggesting that J1\_tmic can drive the formation of SDS micelles while binding on their surface. This effect is not unusual, as it has already been observed with  $\alpha$ -synuclein, another membrane-interacting protein [28].

It may be argued that the conformational changes observed are induced by the hydrophobic interaction of SDS or phospholipid vesicles with J1 tmic, rather than by the charged surface of micelles or vesicles. It can be remarked, however, that the fatty acid chains in SDS and in the phospholipids used are similar, if not identical. If the conformational change is induced by hydrophobic interactions, similar effects should be observed. On the contrary, CD spectra display distinct features, depending on the conditions. Most significantly, different types of phospholipids, depending on the charge of the polar head, have different effects. Moreover, hydrophobic interactions are expected to be rather insensitive to pH changes. On the contrary, in the presence of SDS micelles and DMPG phospholipid vesicles, the helical content of J1 tmic is markedly dependent on pH, suggesting that the conformational change is driven by polar, rather than hydrophobic interactions.

The partial folding of the cytoplasmic domain of Jagged-1 accompanied by its association with the inner side of the cell membrane may have relevant effects on the function of Jagged-1 in Notch signaling [8,10,12]. For instance, it may selectively mask certain residues that are potential targets for post-translational modifications such as phosphorylation, ubiquitination, or O-glycosylation by  $\beta$ -N-acetylglucosamine [29,30] while leaving others exposed for the same modifications. In a similar way, it may mask or expose selected binding motifs with respect to binding partners. The partial folding and association of the intracellular region of Jagged-1 with the membrane is also expected to reduce its 'capture radius' [31] towards protein targets like PDZ-containing proteins. Despite the high number of single pass membrane proteins involved in signaling, little is known about the structure and function of their cytoplasmic tails and, to our knowledge, only few examples have been reported [32,33]. The cytoplasmic tail of the T-cell receptor  $\zeta$ -chain [34,35] binds to lipid membranes through a lipid-induced coil-helix transition dependent on phosphorylation [33]. Other cytoplasmic domains related to multichain immune recognition receptors were found to be intrinsically disordered even when bound to lipids [36]. A role of the cytoplasmic tail of membrane-spanning proteins in protein-protein interactions has also been proved, e.g. the case of the association between the N-terminal region of the membrane-bound tyrosine kinase Lck with the cytoplasmic tail of the T-cell coreceptors CD4 or CD8 [37].

In solution, on the contrary, J1 tmic is mainly disordered (Figs 2 and 3). The strongly hydrophobic segment (VTAFYWAL) that is expected to be embedded in the membrane and to become exposed to the solvent upon cleavage of Jagged-1 is not sufficient to promote folding of J1 tmic in solution. Intrinsic disorder in the cytoplasmic region of type I membrane proteins that undergo regulated intramembrane proteolysis mediated by the presentlin/ $\gamma$ -secretase complex is probably not unique to Jagged-1. Intrinsic disorder propensity based on the amino acid composition only can be estimated from a plot of the protein mean net charge versus mean hydrophobicity [38]. Such a charge/hydrophobicity plot (Fig. 9) calculated for the intracellular region of a series of human membrane proteins that are cleaved by presenilin shows that most of the RIP substrates, including Jagged-1, actually fall in the left-hand side of the plot (natively unfolded proteins). All the proteins that clearly fall in the right-hand side of the plot contain, along with disordered stretches, structured domains (Supplementary Figure S9).



Fig. 9. Intrinsic disorder. Mean net charge versus mean hydrophobicity calculated for the intracellular region of 37 human preseni $lin/\gamma$ -secretase substrates that undergo regulated intramembrane proteolysis. Proteins that contain globular domains in the cytoplasmic tail are shown as filled circles. The line ideally separates natively unfolded proteins (left-hand side of the plot) from natively folded ones (right-hand side of the plot). A4, amyloid β A4 precursor; APLP1/2, amyloid-like proteins 1/2; CADH1, E-cadherin; CADH2, N-cadherin; CD44, CD44 antigen; CSF1R, colony stimulating factor 1 receptor; DCC, netrin receptor; DLL1/4, delta-like 1/4; EFNB1/2, ephrin-B1/2; ERBB4, receptor protein tyrosine kinase erbB4; GHR, growth hormone receptor; IGF1R, insulin-like growth factor 1 receptor; IL1R2, interleukin 1 receptor II; JAG1, jagged-1; JAG1TMIC, J1\_tmic; JAG2, jagged-2; LEUK, leukosialin (CD43); LRP, low-density lipoprotein receptor related proteins; NTC1-4, Notch receptors 1-4; PCDG1, proto cadherin γ A1; PVRL1, nectin-1; SCN2B, sodium channel ß2 subunit; SDC3, syndecan-3; SORL, sortilin-related receptor; TNR16, tumor necrosis factor superfamily member 16; TYRP, tyrosinase-related proteins; VGFR1, vascular endothelial growth factor receptor 1.

Nevertheless, TFE can induce helix formation in J1\_tmic (Fig. 2) in even a more effective way than SDS micelles or phospholipid vesicles. The interaction of TFE with hydrophobic moieties of the polypeptide chain is supposed to be rather weak. Instead, TFE promotes secondary structure formation by reducing the protein backbone exposure to the aqueous solvent and favoring the formation of intramolecular hydrogen bonds [39]. Therefore, TFE stabilizes specific secondary structure elements in accordance with the intrinsic conformational propensities of the polypeptide chain. This is of particular significance in view of the fact that most of the presenilin/ $\gamma$ -secretase substrates considered in Fig. 9 release fragments that are translocated to the nucleus and are involved in transcriptional

regulation. This is the case also for Jagged-1, which has been shown to activate gene expression through the AP1 element [12]. Control of transcription by the released signaling fragments probably does not occur in a straightforward manner, but through the interaction with transcription factors and transcriptional complexes that have not been identified yet. In this scenario, the intrinsic propensity to adopt a particular type of secondary structure may facilitate folding when binding to target proteins occurs.

The identification of post-translational modifications that can play a role in the function and structure of Jagged-1 cytoplasmic tail, as well as the identification of binding partners at the membrane/cytoplasm interface, in the cytosol, and in the nucleus, represent issues that are worth further investigation.

### **Experimental procedures**

#### **Expression and purification**

The DNA encoding J1 tmic (corresponding to residues 1086-1218 of JAG1 HUMAN) was amplified by PCR from a template plasmid containing the codon-optimized synthetic gene encoding the intracellular region of human Jagged-1 (residues 1094-1218) [23]. The following forward and reverse primers [Sigma-Genosys (Cambridge, UK), purified by polyacrylamide gel electrophoresis] were used: 5'-TAA TAT TAG CAT ATG GTG ACC GCT TTC TAT TGG GCG CTG CGT AAA CGT CGT AAA CCG GGT AGC-3' and 5'-TAG TAG GGA TCC TCA TTA AAC GAT GTA TTC CAT ACG GTT CAG GCT-3'. The forward primer contains a NdeI restriction site (underlined) encoding the start methionine and 8 residues belonging to the putative transmembrane region (in italics). To avoid possible cross-linking, C1092 was mutated to alanine. The reverse primer contains a BamHI restriction site (underlined) and a double stop codon (in bold). The PCR product was purified, digested with NdeI and BamHI and directionally cloned into a pET-11a vector (Novagen, Darmstadt, Germany). DH5a E. coli cells were transformed, selected on Luria-Bertani plates containing 100 µg·mL<sup>-1</sup> ampicillin, and the positive clones subjected to automatic DNA sequencing. Correct clones were used to transform BL21(DE3) E. coli (Novagen) cells for expression. Bacteria were grown at 37 °C in Luria-Bertani medium containing 100  $\mu$ g·mL<sup>-1</sup> ampicillin to an optical density of ~1 and protein expression induced with isopropyl thio-B-D-galactoside (1 mm) for 3 h. Cells were harvested by centrifugation, resuspended in the lysis buffer [20 mM sodium phosphate buffer, 0.5 M NaCl, 50 mM CHAPS, 2% Tween 20, 1 mM DL-dithiothreitol, 10 mM imidazole, 0.5 mM EDTA, pH 7.4, containing one protease inhibitor cocktail tablet (Roche, Mannheim, Germany)] and sonicated. After centrifugation, the supernatant was loaded on a Ni<sup>2+</sup> Sepharose HisTrap HP column (1 mL, GE Healthcare, Piscataway, NJ, USA), the column washed with 20 mM sodium phosphate buffer, 0.5 M NaCl, 1 mM DL-dithiothreitol, 10 mM imidazole, pH 7.4 and the protein eluted with a 10-500 mM imidazole gradient. The crude material was purified by RP-HPLC on a Zorbax 300SB-CN column (9.4 × 250 mm, 5 µm, Agilent Technologies, Palo Alto, CA, USA) using a 0-50% gradient of 0.1% trifluoroacetic acid in H2O and 0.1% trifluoroacetic acid in acetonitrile, and freeze-dried. For preparation of the <sup>15</sup>N-labeled protein, cells were grown in M9 minimal medium (6 g·L<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>, 3 g·L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 0.5 g·L<sup>-1</sup> NaCl,  $0.12 \text{ g} \cdot \text{L}^{-1}$  MgSO<sub>4</sub>,  $0.01 \text{ g} \cdot \text{L}^{-1}$  CaCl<sub>2</sub>,  $0.5 \text{ g} \cdot \text{L}^{-1}$  $^{15}$ NH<sub>4</sub>Cl, 5 g·L<sup>-1</sup> D-glucose) supplemented with 1.7 g·L<sup>-1</sup> Yeast Nitrogen Base without amino acids and ammonium sulfate (Difco, Sparks, MD, USA) and containing  $100 \ \mu g \cdot m L^{-1}$  ampicillin. Expression and purification of the labeled protein were carried out as described above. The purified proteins were analyzed by liquid chromatographymass spectrometry to confirm their identity. The recombinant protein lacking the transmembrane region, J1 ic, was expressed and purified as described [23].

#### Size exclusion chromatography

The freeze-dried protein was dissolved in the elution buffer (Tris/HCl 50 mM, 100 mM KCl, pH 7.4), loaded onto a Sephacryl S-200 column (GE Healthcare) and eluted in the same elution buffer. The apparent molecular mass of J1\_tmic was deduced from a calibration carried out with the following molecular standards: lactate dehydrogenase (147 kDa), bovine serum albumin (67 kDa), carbonic anhydrase (29 kDa) and horse myoglobin (17 kDa). Stokes radii of native (R<sub>s</sub>N) and fully unfolded (R<sub>s</sub>U) proteins of known molecular mass (*m*) were determined according to the equations:  $log(R_sN) =$  $-(0.254 \pm 0.002) + (0.369 \pm 0.001) log($ *m*), and log(R<sub>s</sub>U) = $<math>-(0.543 \pm 0.004) + (0.502 \pm 0.001) log($ *m*) [17].

#### Preparation of phospholipid vesicles

The synthetic phospholipids DMPG, DMPS or DMPC (Avanti, Alabaster, AL, USA) were dissolved in CHCl<sub>3</sub>/CH<sub>3</sub>OH (2 : 1, v/v) in round-bottomed flasks and the solvent evaporated to obtain a thin lipid film. After drying after vacuum to remove residual solvent, lipids were hydrated in 5 mM Tris/HCl buffer, pH 7.4, to get a 10 mM lipid suspension which was sonicated to clarity at 37 °C in a high intensity bath sonicator (Branson 3200, Branson Sonic Power Co., Danbury, CT, USA).

#### **Circular dichroism**

Samples for CD spectroscopy were prepared dissolving the freeze-dried protein in 5 mM Tris/HCl buffer, pH 7.4.

Protein concentration (7.5 µM) was determined by UV absorbance at 280 nm using the calculated *\varepsilon*-value of 16 500 M<sup>-1</sup>cm<sup>-1</sup>. CD spectra were recorded at 25 °C or 37 °C on a Jasco J-810 spectropolarimeter (JASCO International Co., Tokyo, Japan) using jacketed quartz cuvettes of 1 mm pathlength. Five scans were acquired for each spectrum in the range 190-250 nm at a scan rate  $20 \text{ nm} \cdot \text{min}^{-1}$ . Mean residue ellipticity of (deg cm<sup>2</sup>·dmol<sup>-1</sup>· residue<sup>-1</sup>) was calculated from the baseline-corrected spectrum. A quantitative estimation of secondary structure content was carried out using SELCON3, CON-TINLL, and CDSSTR, all run from the DichroWeb server (www.cryst.bbk.ac.uk/cdweb/html/home/html) [40]. Helical content was also estimated from the mean residue ellipticity at 222 nm according to the formula  $[\alpha] = -100$  mean residue ellipticity<sub>222</sub>/40000 (1-2.57/N), where N is the number of peptide bonds.

#### Fluorescence spectroscopy

Samples prepared for CD were also used for fluorescence spectroscopy. Spectra were recorded at 25 °C or 37 °C on a Jobin-Yvon FluoroMax-3 spectrofluorimeter (Jobin Yvon-Horiba, Paris, France) equipped with a Peltier temperature control apparatus using  $1 \times 0.2$  cm pathlength quartz cuvettes. Excitation was set at 295 nm and spectra were recorded between 300 and 450 nm. Fluorescence anisotropy was measured at the maximum of emission using the same excitation wavelength. All anisotropy measurements were carried out at least five times. Measurements were corrected for the background and averaged.

#### NMR spectroscopy

Protein samples for NMR spectroscopy were prepared dissolving the freeze-dried material in  $H_2O/D_2O$  (90 : 10, v/v) and adjusting the pH to 7.0 with small aliquots of 0.1 N NaOH, for a final protein concentration of  $\sim 0.5$  mM. The sample containing SDS was prepared by dissolving solid SDS sodium salt in the NMR sample, for a final SDS concentration of 50 mm. Additional spectra were recorded at pH 5.5. Spectra were recorded at 303 K on a Bruker spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at a <sup>1</sup>H frequency of 600.13 MHz and equipped with a  $^{1}\text{H}/^{13}\text{C}/^{15}\text{N}$  triple resonance Z-axis gradient probe. Transmitter frequencies in the <sup>1</sup>H and <sup>15</sup>N dimensions were set on the water line and at 118.0 p.p.m., respectively. HSQC and HSQC-TOCSY experiments were carried out in phasesensitive mode using echo/anti-echo-TPPI gradient selection and <sup>15</sup>N decoupling during acquisition. HSOC spectra were acquired with 1 K complex points, 256 t<sub>1</sub> experiments, 32 scans per increment, over a spectral width of 13 and 28 p.p.m. in the <sup>1</sup>H and <sup>15</sup>N dimensions, respectively. HSQC-TOCSY spectra were acquired with the same

For in-cell NMR experiments [41,42], 200 mL of E. coli culture was grown in M9 medium containing <sup>15</sup>NH<sub>4</sub>Cl as the only nitrogen source, as described above. The culture was split; in one sample expression was induced with isopropyl thio- $\beta$ -D-galactoside, and the other was used as control. Cells were centrifuged at  $\sim 500 g$  in a Sorvall RC5B centrifuge (Sorvall Instruments Inc., Newton, CT, USA) using a GSA rotor. The supernatant was discarded, and the pellet was gently resuspended in 50 mL of cold  $NaCl/P_i$  (10 g·L<sup>-1</sup> NaCl, 0.25 g·L<sup>-1</sup> KCl, 0.25 g·L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 3.6 g·L<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O, pH 7.2). After an additional centrifugation step, the pellet was gently resuspended in 500 µL NaCl/Pi, D2O (55 µL) was added, and a standard NMR tube was filled with the E. coli slurry. After NMR analysis, the slurry was recovered from the NMR tube, centrifuged for 2 min at  $\sim 14\ 000\ g$  in a Millipore MC-13 microcentrifuge (Amicon Bioseparations Inc., Beverly, MA, USA) and the clear supernatant subjected to further NMR analysis. HSQC spectra on the induced sample, on the control sample, and on the supernatant were acquired in identical conditions at 303 K with 1 K complex points, 128 t<sub>1</sub> experiments, 32 scans per increment, over a spectral width of 13 and 26 p.p.m. in the <sup>1</sup>H and <sup>15</sup>N dimensions, respectively, for a total experiment time of  $\sim 1$  h for each HSQC. A sample of freeze-dried, purified protein dissolved in NaCl/Pi was used to acquire a reference spectrum.

#### Intrinsic disorder

Disorder propensity was estimated from a plot of the mean net charge (absolute value) versus the mean hydrophobicity calculated using the normalized values of the Kyte & Doo-little scale [38]. Presenilin/ $\gamma$ -secretase substrates were taken from the literature [43].

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# **Supplementary material**

The following supplementary material is available online:

**Table S1.** CD. Helical content calculated from the ellipticity at 222 nm ( $H_{222}$ ), secondary structure content (H, helix, E, strand, C, disordered) calculated by CDSSTR through deconvolution of CD spectra, and normalized root mean squared deviation of the fit (nrmsd).

**Fig. S1.** NMR. Proton chemical shift distribution of detectable <sup>1</sup>HNs for J1\_tmic (gray bars), J1\_tmic in the presence of SDS, and of random coil values for a protein of the same sequence.

Fig. S2. In-cell NMR.  ${}^{1}H{}^{-15}N$  HSQC spectra of the *E. coli* slurry [(A), not induced; (B), induced and of the supernatant (C) of the induced culture].

Fig. S3. Size exclusion chromatography.

Fig. S4. Fluorescence spectroscopy.

Fig. S5. Effect of pH. Far-UV CD spectra of  $J1_{\text{tmic}}$  (7.5  $\mu$ M) in the presence of SDS (3 mM) at

pH 7.4, after acidification to pH 6, and after return to pH 7.4.

**Fig. S6.** Effect of pH. Far-UV CD spectra of J1\_tmic (7.5  $\mu$ M) in the presence of DMPG phospholipid vesicles (1 mM) at pH 7.4, after acidification to pH 6, and after return to pH 7.4.

**Fig. S7.** CD of J1\_ic. Far-UV CD spectra of J1\_ic (7.0  $\mu$ M) in buffer alone and in the presence of SDS (3 mM) at pH 7.4 and at pH 6.

**Fig. S8.** CD of J1\_ic. Far-UV CD spectra of J1\_ic (7.0  $\mu$ M) in buffer alone and in the presence of DMPG phospholipid vesicles (1 mM) at pH 7.4 and at pH 6.

**Fig. S9.** Intrinsic disorder. Domain architecture, as calculated by SMART, of human RIP substrates analyzed in this work.

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