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Communication

The engineered peptide construct NCAM1-Aβ inhibits fibrillization of the human prion protein (PrP)

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In prion diseases, the prion protein (PrP) becomes misfolded and forms fibrillar aggregates that are responsible for prion infectivity and pathology. So far, no drug or treatment procedures have been approved for prion disease treatment. We have previously shown that engineered cell-penetrating peptide constructs can reduce the amount of prion aggregates in infected cells. However, the molecular mechanism underlying this effect is unknown. Here, we use atomic force microscopy (AFM) imaging to show that the amyloid aggregation and fibrillization of the human PrP protein can be inhibited by equimolar amounts of the 25 residues long engineered peptide construct NCAM1-Aβ.

Keywords: Creutzfeldt-Jakob disease, AFM imaging, amyloid, drug design, drug transport, protein-peptide interaction

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Abbreviations: AFM, Atomic force microscopy; PrP, prion protein; NCAM1, Neural cell adhesion molecule-1

INTRODUCTION

Prion and amyloid diseases are both characterized by aggregation of misfolded proteins or peptides (Miller, 2009; Verma *et al*., 2015; Sengupta & Udgaonkar, 2018; Jaunmuktane & Brandner, 2020), such as the prion (PrP) protein (Creutzfeldt-Jakob disease), α-synuclein (Parkinson's disease), and amyloid-β (Aβ) and tau (Alzheimer's disease). Many of these proteins and peptides may co-aggregate or at least influence each other's aggregation (Luo *et al*., 2016a; 2016b; Wallin *et al*., 2018; Ren *et al*., 2019). Factors that modulate the aggregation of one of these proteins, such as small molecules, potential drug compounds, lipids, and metal ions, can often also modulate aggregation processes of other proteins in this family (Robinson & Pinheiro, 2010; Richman *et al*., 2013; Wärmländer *et al*., 2013; Chemerovski-Glikman *et al*., 2016; Wallin *et al*., 2017; Ambadi Thody *et al*., 2018; Österlund *et al*., 2018; Gielnik *et al*., 2019; Owen *et al*., 2019; Wärmländer *et al*., 2019). This suggests that at least some of the underlying mechanisms may be the same in prion and amyloid diseases (Miller, 2009; Prusiner, 2012; Sabate, 2014; Collinge, 2016; Jucker & Walker, 2018; Jaunmuktane & Brandner, 2020). However, prion aggregates are particularly infectious, as they spread between the cells (Jucker & Walker, 2018; Jaunmuktane & Brandner, 2020), cross the blood-brain barrier and other membrane barriers (Banks *et al*., 2009; Urayama *et al*., 2016; Keller *et al*., 2018), and are not degraded by cellular processes, such as proteinase digestion (Löfgren *et al*., 2008; Söderberg *et al*., 2014).

The toxic species in amyloid and prion diseases are generally considered to be small toxic oligomeric aggregates (Verma *et al*., 2015; Sengupta & Udgaonkar, 2018; Sang *et al*., 2019), but so far no drugs or treatments that target such aggregates have been approved against prion diseases (Lee *et al*., 2019; Hyeon *et al*., 2020; Mashima *et al*., 2020). Potential drug molecules may interfere with oligomer formation in various ways: by reducing production of the protein, by inhibiting its aggregation, by diverting the aggregation pathway(s) towards non-toxic forms, or by reducing the lifetime of the toxic forms, for example by promoting rapid aggregation into larger non-toxic aggregates.

We have previously demonstrated anti-prion properties of short peptide constructs (up to 30 residues) with sequences derived from the unprocessed N-termini of mouse and bovine prion proteins: such PrP-derived peptides induced lower amounts of prion aggregates resistant to proteinase K in prion-infected cells (Löfgren *et al*., 2008; Söderberg *et al*., 2014).

The PrP-derived peptides consisted of an N-terminal signal peptide segment (different for mouse and bovine PrP), together with a conserved positively charged and hydrophobic hexapeptide (KKRPKP) corresponding to the first six residues of the processed PrP protein. Our earlier studies had shown that peptides with such sequences displayed a cell-penetrating activity (Derakhshankhah & Jafari, 2018), and thus were able to interact with and penetrate cell membranes (Lundberg *et al*., 2002; Magzoub *et al*., 2005; Magzoub *et al*., 2006; Oglecka *et al*., 2008). The anti-prion effects of the PrP-derived peptides were lost when the KKRPKP hexapeptide was added alone or coupled to various other peptides with cell-penetrating properties (Söderberg *et al*., 2014). However, the anti-prion effects were retained when KKRPKP was coupled to the signal sequence of the neural cell adhesion molecule-1 (i.e., $NCAM1_{1-19}$) (Söderberg *et al*., 2014).

The mouse PrP_{1-28} segment and the $NCAM1_{1-19}$-KKRPKP construct are both amyloidogenic in themselves, as they form amyloid fibrils by self-aggregation (Mukundan *et al*., 2017; Pansieri *et al*., 2019). The $NCAM1_{1-19}$-KKRPKP construct was recently shown to also inhibit aggregation of the amyloid-β peptide involved in the Alzheimer's

Table 1. Primary sequences and molecular properties of the human PrP protein, the synthetically produced NCAM1-Aβ peptide construct (with both termini amidated), and its parts.

Protein	Sequence	Isoelectric point (pI)	Molecular weight [g mol^{-1}]	Net charge at pH 7	Theoretical hydrophobicity [kcal mol^{-1}]
huPrP$_{23-231}$	UniProt ID: P04156 (209 aa)	9.39	22747	+7	–
NCAM1$_{1-19}$-K-Aβ$_{16-20}$ (NCAM1-Aβ)	**NH$_2$**-MLRTKDLIWTL FFLGTAVSKKLVFF-**NH$_2$**	11.67	2974.7	+4	−3.83
NCAM1$_{1-19}$ (NCAM1)	**NH$_2$**-MLRTKDLIWTL FFLGTAVS-**NH$_2$**	11.39	2211.7	+2	−3.06
KKLVFF	**NH$_2$**-KKLVFF-**COOH**	10.69	781	+2	−0.77

disease (Henning-Knechtel *et al.*, 2020), and to promote *in vitro* aggregation of the amyloid protein S100A9 (Pansieri *et al.*, 2019), which is involved in amyloid-related and other inflammatory processes (Wang *et al.*, 2014; Horvath *et al.*, 2018; Wang *et al.*, 2019). Almost identical results were obtained for a similar amyloidogenic 25 residue-construct, i.e. NCAM1$_{1-19}$-KKLVFF (from here onwards: NCAM1-Aβ) (Pansieri *et al.*, 2019). The KLVFF sequence originates from the hydrophobic core (residues 16-20) of the Aβ peptide: this pentapeptide is known to inhibit aggregation of the full-length Aβ peptide (Tjernberg *et al.*, 1996). In the NCAM1-Aβ construct, an additional lysine residue was added to the KLVFF sequence for increased solubility (Pansieri *et al.*, 2019). Molecular properties of the NCAM1-Aβ sequence and its segments are shown in Table 1, including hydrophobicity values calculated according to the Wimley-White whole residue hydrophobicity scale (Wimley & White, 1996; Wang *et al.*, 2016).

As the NCAM1-Aβ construct inhibits fibrillization of the Aβ peptide (Henning-Knechtel *et al.*, 2020), but promotes (co-)aggregation of the S100A9 protein (Pansieri *et al.*, 2019), it is unclear how the construct may affect aggregation of the PrP protein – if at all. Here, we use Atomic Force Microscopy (AFM) imaging to investigate if there is a direct effect of the NCAM1-Aβ construct on *in vitro* fibrillization of the full-length human PrP protein. Answering this question might help clarify the mechanisms underlying the previously observed beneficial effects of such peptide constructs on PrP infectivity (Löfgren *et al.*, 2008; Söderberg *et al.*, 2014).

MATERIALS AND METHODS

Sample preparation

Full-length recombinant human prion protein (huPrP) was prepared according to a previously published protocol (Zahn *et al.*, 1997; Morillas *et al.*, 1999), albeit with some modifications. The plasmid contained the full-length (23-231)huPrP protein in fusion with an N-terminal HisTag, and the thrombin cleavage site was cloned into the pRSETB vector (Invitrogen, USA). The construct was expressed in *E. coli* (BL21-DE3) grown in LB growth medium with 100 μg/mL ampicillin. Expression was induced by isopropyl β-D-galactopyranoside (IPTG) at OD_{600}=0.8. Sonication of the lysates was performed in a buffer containing 100 mM Tris at pH 8, 10 mM K_2HPO_4, 10 mM glutathione (GSH), 6 M GuHCl, and 0.5 mM phenylmethane sulfonyl fluoride (PMSF). The solution was centrifuged, and the supernatant loaded onto Ni-NTA resin (GE Healthcare) and eluted with buffer E (100 mM Tris buffer at pH 5.8, 10 mM K_2HPO_4, and 500 mM imidazole). After washing the resin, the protein was purified with two-step dialysis, initially against 10 mM phosphate buffer with 0.1 mM PMSF at pH 5.8, and then against Milli-Q H_2O with 0.1 mM PMSF. After thrombin cleavage, the pure huPrP protein (i.e., with the HisTag removed) was concentrated using an Amicon Ultra 0.5 mL centrifugal filter (Merck & Co., USA) with an NMWL cut-off of 3 kDa. The final protein concentration was determined by spectrophotometry using an extinction coefficient of ε_{280} = 57995 M^{-1}cm^{-1} (Gasteiger *et al.*, 2005). The quality of the final protein was controlled by mass spectrometry (molecular mass 22747 Da – Table 1).

The NCAM1-Aβ peptide (Table 1) was purchased as a custom order from the PolyPeptide Group (France) in lyophilized form. It was synthetically produced with both the N- and C-termini amidated. The peptide was stored frozen and dissolved in Milli-Q water prior to the experiments. Its concentration was determined via triplicate UV absorption measurements at 280 nm, using a DS-11 spectrophotometer (DeNovix, USA) and an extinction coefficient of ε_{280}=5500 M^{-1}cm^{-1} (Gasteiger *et al.*, 2005).

Sample incubation

The initial buffer in the huPrP solution was exchanged to ultrapure water by triplicate diafiltration using an Amicon Ultra 0.5 mL centrifugal filter (Merck & Co., USA) with an NMWL cut-off of 3 kDa. Samples of 0.5 μM NCAM1-Aβ, 2.5 μM NCAM1-Aβ, 0.5 μM huPrP, and 0.5 μM NCAM1-Aβ+0.5 μM huPrP were then prepared in 10 mM sodium phosphate buffer, pH 7.5, with 100 mM NaCl and 2 M urea. The urea was added as it has been previously shown to promote unfolding of the native PrP structure and rearrangement of the C-terminal domain, which is needed for fibril formation (Swietnicki *et al.*, 2000; Julien *et al.*, 2009; Glynn *et al.*, 2020; Wang *et al.*, 2020). To reduce the time needed for aggregation, the samples were incubated for up to 72 hours at 50°C with magnetic stirring at 400 rpm (Sneideris *et al.*, 2020). Subsamples were taken out for AFM imaging (below) after 8 and 72 hours, respectively.

Atomic force microscopy (AFM) imaging

Incubated samples, 5 μL, were transferred to freshly cleaved mica plates and left to absorb for 1 min, rinsed three times with 300 μL of pure water, and then dried under a gentle flow of nitrogen. AFM imaging was performed on a JPK Nanowizard 4 (Bruker, Germany) AFM unit using Tap150Al-G cantilevers (Ted Pella Inc., USA) in air intermittent contact mode. The scan rate was 0.3–0.7 Hz, and the scanned area size was either 5 μm × 5 μm or 10 μm × 10 μm, with pixel resolutions of respectively 512 × 512 or 1024 × 1024. The AFM images were analyzed using the Gwyddion 2.54 software (Necas & Klapetek, 2012).

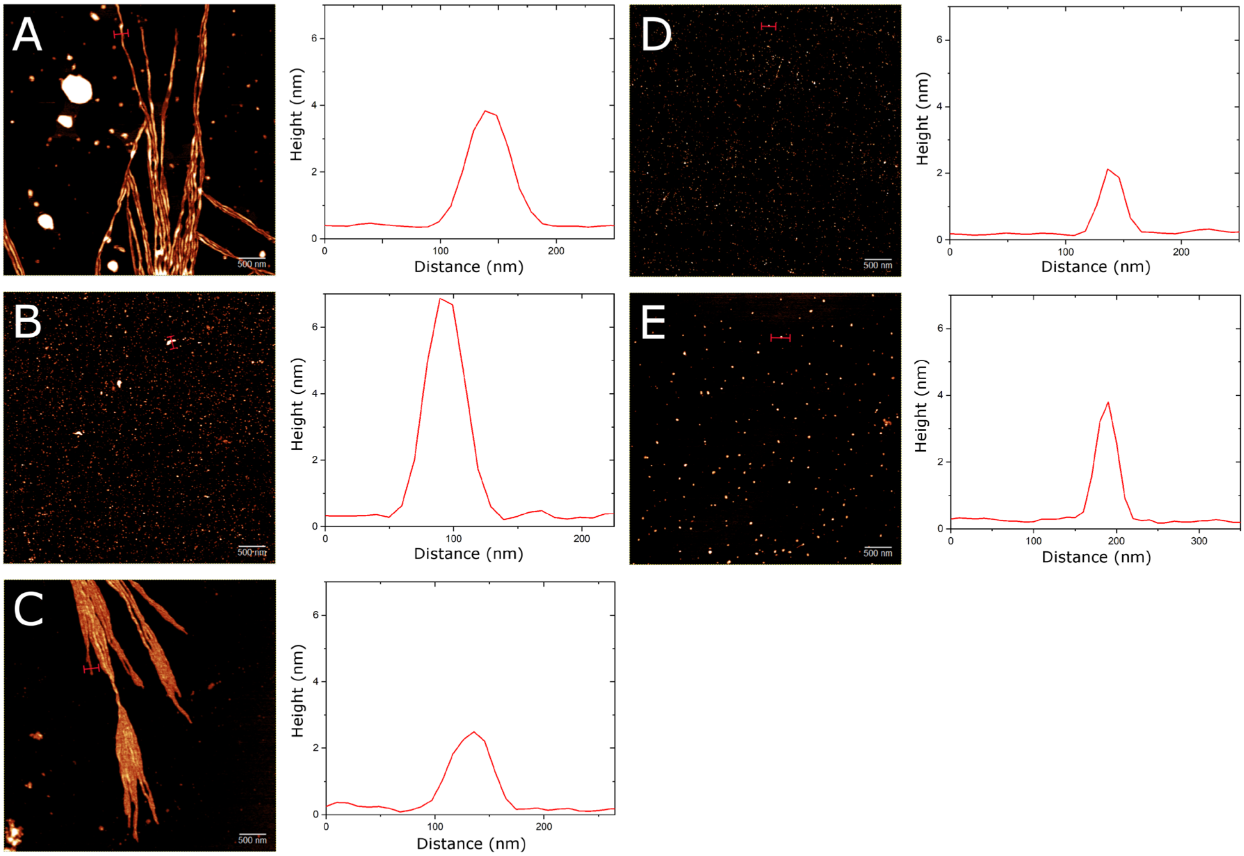

Figure 1. AFM images of:
(**A**) 0.5 µM huPrP protein; (**B**) 0.5 µM NCAM1-Aβ peptide; (**C**) 2.5 µM NCAM1-Aβ peptide; and (**D**) 0.5 µM huPrP protein+0.5 µM NCAM1-Aβ peptide. All samples in A–D were incubated for 8 hours. (**E**) 0.5 µM huPrP protein+0.5 µM NCAM1-Aβ peptide, incubated for 72 hours. All studied samples were incubated at 50°C in 10 mM sodium phosphate buffer, pH 7.5, with 100 mM NaCl and 2 M urea, and with magnetic stirring at 400 rpm. The white scale bars are 500 nm.

RESULTS AND DISCUSSION

AFM images of the aggregation products present in the samples after 8 hours of incubation are shown in Figs. 1A–D. The sample of 0.5 µM huPrP readily self-aggregated into long fibrils (Fig. 1A) that are approximately 3–4 nm thick (judged by their measured height, as width is not accurately represented in AFM images). This is somewhat thinner but still in line with the results of previous studies on PrP fibrils (Vazquez-Fernandez *et al.*, 2017; Yamaguchi & Kuwata, 2018; Terry & Wadsworth, 2019). A few very large aggregate clumps, over 10 nm high, can also be seen (Fig. 1A). For NCAM1-Aβ, the 0.5 µM sample shows small aggregate clumps (Fig 1B). Some of them are relatively large, with heights over 6 nm, and may or may not be early stages of fibrillar aggregates (Luo *et al.*, 2014). The 2.5 µM NCAM1-Aβ sample shows numerous mature fibrils, about 2–3 nm high, together with aggregate clumps (Fig. 1C). The more abundant amount of fibrils for 2.5 µM of NCAM1-Aβ confirms earlier results showing that NCAM1-Aβ self-aggregates faster at higher concentrations (Pansieri *et al.*, 2019).

Interestingly, the sample containing both 0.5 µM NCAM1-Aβ and 0.5 µM huPrP displays no fibrils, but only numerous small aggregate clumps, about 2 nm high (Fig. 1D). Even after 72 hours no fibrils can be seen, but the aggregate clumps are then fewer and larger, around 3–4 nm high (Fig 1E). It cannot be ruled out that these small aggregate clumps will eventually form fibrils. It is therefore not possible to tell if fibrillization is completely inhibited, or if the fibrillization rate is merely significantly reduced. Nonetheless, the absence of fibrillar aggregates of huPrP in the presence of equimolar concentrations of NCAM1-Aβ clearly shows that the peptide construct directly interacts with the huPrP protein and interferes with its aggregation and fibrillization. As both molecules are positively charged (Table 1), it stands to reason that they mainly interact via hydrophobic forces.

The aggregation-inhibiting effect of NCAM1-Aβ (Fig. 1) appears to provide an explanation, at a molecular level, for our earlier observations that such peptide constructs significantly reduce the levels of prion aggregates in prion-infected cells (Löfgren *et al.*, 2008; Söderberg *et al.*, 2014). As both the NCAM1-Aβ peptide and the huPrP protein can form amyloid fibrils by themselves (Figs. 1A and 1C), the two molecules may interact via cross-aggregation, to form smaller non-fibrillar co-aggregates (Fig. 1E) that could be less toxic than pure huPrP aggregates (Luo *et al.*, 2016a; 2016b). If so, the huPrP/NCAM1-Aβ interactions would be similar to the interactions between Aβ and NCAM1-Aβ (Henning-Knechtel *et al.*, 2020). In any case, the huPrP/NCAM1-Aβ interactions are very different from the interactions between NCAM1-Aβ and S100A9 protein, where amyloid aggregation is promoted (Pansieri *et al.*, 2019). Because the NCAM1-Aβ construct has different effects on different aggregating proteins, it would be interesting to study how this construct might affect the aggregation of other disease-related prion proteins, such as those involved in animal diseases like bovine spongiform encephalopathy (BSE), chronic wasting disease in cervids, and sheep scrapie (Vazquez-Fernandez *et al.*, 2017; Osterholm *et al.*, 2019). It would be also interesting to clarify the individual effects on PrP aggregation provided by the two parts of the peptide construct, i.e. the NCAM1 sequence and the KKLVFF hexapeptide (Table 1), or the minimal requirements for such a construct to be able to inhibit fibrillization. However, such studies should preferably be done with aggregation-prone PrP fragments, rather than with the very toxic full-length prion protein.

CONCLUSIONS

Our atomic force microscopy images show that the *in vitro* amyloid aggregation of the full-length human PrP protein is inhibited by equimolar amounts of the 25 residues long engineered peptide NCAM1-Aβ. Thus, a very likely molecular-level explanation for our previous observation that such cell-penetrating peptide constructs can reduce the amount of toxic prion aggregates in infected cells, is that these peptide constructs directly interact with the PrP protein and prevent its fibrillization.

Conflict of Interest

The authors declare no conflict of interest.

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