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Cell membrane translocation of the N-terminal (1–28) part of the prion protein

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Abstract

The N-terminal (1–28) part of the mouse prion protein (PrP) is a cell penetrating peptide, capable of transporting large hydrophilic cargoes through a cell membrane. Confocal fluorescence microscopy shows that it transports the protein avidin (67 kDa) into several cell lines. The (1–28) peptide has a strong tendency for aggregation and β-structure formation, particularly in interaction with negatively charged phospholipid membranes. The findings have implications for how prion proteins with uncleaved signal peptides in the N-termini may enter into cells, which is important for infection. The secondary structure conversion into β-structure may be relevant as a seed for the conversion into the scrapie (PrP^{Sc}) form of the protein and its amyloidic transformation.

Keywords: Prion protein N-terminus; Cell penetrating peptide; Aggregation; β-Structure; Membrane interaction

The infection process involved in the prion diseases has been the subject of intensive research. We report here a novel aspect of the prion protein (PrP) sequences, which may play an important role for the cellular transport of PrPs, and for the structure conversion associated with the formation of the scrapie form of a PrP. The N-terminal sequences of the PrPs are very similar to constructed chimera of peptides, which have been shown to function as cell penetrating peptides (CPPs) [1]. A CPP is capable of mediating so-called energy-independent cellular uptake of linked hydrophilic macromolecules into a variety of cells and is seemingly independent of any chiral receptor. Based on the sequence similarities we decided to test the hypothesis that the N-terminal sequence of a PrP, with a non-cleaved signal sequence, is also active as a CPP. We have found that a PrP(1–28) peptide is indeed a CPP, with the ability to carry the 67 kDa protein avidin into several cell lines, as judged from confocal fluorescence microscopy. We have also undertaken a study by circular dichroism (CD) and NMR spectroscopy of the secondary structures of the peptide induced in various membrane mimetic solvents.

One type of the synthetic CPPs [2] is composed of a signal peptide, for membrane anchoring, continued by a nuclear localisation sequence (NLS), for intracellular addressing. The chimeric peptide (sequence **S1** below, with residues 1–17 from a signal peptide and 18–23 from an NLS) was easily internalised into fibroblasts and was found to localise mainly to the nucleus [2].

Although the mechanism by which a CPP enters a cell carrying a large cargo is still not well understood [1], the positive charges and the hydrophobic character of most CPPs suggest that a particular membrane interaction is required. Also other native sequences may have similar patterns and we found that this seems to hold for the N-terminal of PrPs when the N-terminal signal sequence remains. Compare the following peptide sequences:

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Name		Sequence
Chimeric CPP	**(S1)**	**MGLGLHLLVLAAALQG A *KKKRKVC*** [2]
Mouse Prp(1–28)	**(S2)**	**MANLGYWLLALFVTM WTDVGLC *KKRPKP***
Human Prp(1–28)	**(S3)**	**MANLGCWMLVLFVAT WSDLGLC *KKRPKP***
Bovine PrP(1–30)	**(S4)**	**MVKSKIGSWILVLFVAM WSDVGLC *KKRPKP***

The general feature of both the chimeric CPP (**S1**) [2] and the N-terminal sequences of the three examples of PrP is mainly a hydrophobic signal peptide part of 16–23 residues followed by a basic putative NLS part of about six residues.

This inspired us to investigate whether PrP(1–28) is also a CPP and chose to study the chemically synthesised mouse PrP(1–28) peptide (**S2**).

Materials and methods

Peptide synthesis

The peptides were synthesised in a stepwise manner in a 0.1 mmol scale on an automated peptide synthesiser (Applied Biosystems Model 431A) using the *t*-Boc solid-phase peptide synthesis strategy. *tert*-Butyloxycarbonyl amino acids (Neosystem, Strasbourg, France) were coupled as hydroxybenzotriazole (HOBt) esters to a *p*-methylbenzylhydrylamine (MBHA) resin (Neosystem, Strasbourg, France) to obtain amidated peptides at the C-terminus. Biotin and fluorescein were coupled as previously described [3]. The molecular weight was determined by MALDI–TOF mass spectrometer (Voyager STR).

Cell culture

Mouse neuroblastoma cells, N2A, were cultivated in Dulbecco's minimal essential medium with Glutamax-I, supplemented with 10% (vol/vol) fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Cell penetration experiments

The experiments were performed essentially as previously described [3,4].

The fluorescent probes used were the following: avidin-FITC from Sigma–Aldrich (No. A2050); Hoechst 33258 from Molecular Probes, Holland; concanavalin A conjugated to Alexa Fluor 594 from Molecular Probes, Holland. The images were obtained by a Leica DMIRE2 fluorescence microscope. Hoechst 33258 was used to visualise cell nuclei. Concanavalin A complexed to Alexa Fluor 594 was used to visualise cell membranes.

Peptide only experiments. The cells used for internalisation were seeded out on round glass coverslips in a 24-well plate. One day post-seeding, the cells were semi-confluent and the medium was changed to serum-free medium. The fluorescein-labelled peptides were added, with a final concentration of 5 μM. After 1 h of incubation at 37 °C, the cells were washed three times with 1 ml Hepes–Krebs–Ringer- (HKR) buffer and fixed with 3% paraformaldehyde solution in phosphate-buffered saline solution (PBS) for 10 min. The cells were then washed three times with 1 ml HKR-buffer and the coverslips were mounted and sealed for microscopy studies.

Peptide–cargo complex experiments. These experiments were performed as described above, with the difference that biotinylated peptide coupled to avidin-FITC (i.e., cargo protein with a fluorescein fluorescence probe) with a final peptide concentration of 5 μM was used.

Vesicle preparation

The phospholipids (1-palmitoyl-2-oleyl-phosphatidylcholine, POPC and 1-palmitoyl-2-oleyl-phosphatidylglycerol, POPG) were purchased from Avanti Polar Lipids, Alabaster, USA. Small unilamellar vesicles (diameter less than 100 nm) were prepared by initially dissolving the phospholipids at the desired concentration in chloroform, then removing solvent by vacuum for 3 h, and then dissolving the lipids in between 0 and 50 mM potassium phosphate buffer (pH 7.0). The ice-cooled dispersion was sonicated using a Heat System Model 350 A Sonifier under nitrogen gas until the sample became transparent (ca. 30 min, with the microtip at low output). Titanium particles from the microtip and lipid debris were removed by centrifugation at 25,000*g*.

CD and fluorescence spectroscopy

CD spectra were recorded on a Jasco J-720 spectropolarimeter, using 0.5 or 1 mm optical path quartz cuvettes. The temperature was regulated by a PTC-343 controller, set at 4 °C. Tryptophan fluorescence was measured on a Perkin–Elmer LS 50B Luminescence Spectrometer. Measurements were made at 20 °C, using 280 nm excitation wavelength.

NMR spectroscopy

Deuterated SDS was purchased from Cambridge Isotope Laboratories, Andover. NMR samples were prepared by dissolving the peptide powder to a concentration of 2 mM in 300 mM deuterated SDS, forming micelles, in 90% H_2O/10% D_2O. The pH was 3.4, uncorrected pH-meter reading.

The NMR spectra were acquired at 45 °C using a Varian Unity-600 spectrometer with a 600 MHz proton frequency. Two-dimensional NOESY and TOCSY spectra were recorded. The chemical shifts were referenced to TSPA.

Results

Cell penetration of peptide and cargo

In order to investigate the efficiency of cell membrane translocation by the mouse PrP(1–28) peptide without cargo, N2a cells were incubated at 37 °C for 1 h (Fig. 1A) with a fluorescein-labelled peptide and then fixed. The peptide was prone to aggregation in aqueous solution but was found to be an efficient CPP. The fluorescence micrograph shows that the peptide is found in the cytosol and gives a marked perinuclear staining, possibly coinciding with the Golgi (Fig. 1A). Incubating the cells at 4 °C produced the same staining pattern (not shown), although the fluorescence was less intense, indicating that less peptide was internalised. Bowes melanoma and N1e neuroblastoma cells were treated according to the same protocol and yielded a similar staining pattern as the N2a cells.

Next, the ability of the peptide to carry a cargo was investigated. The protocol was similar to that used in

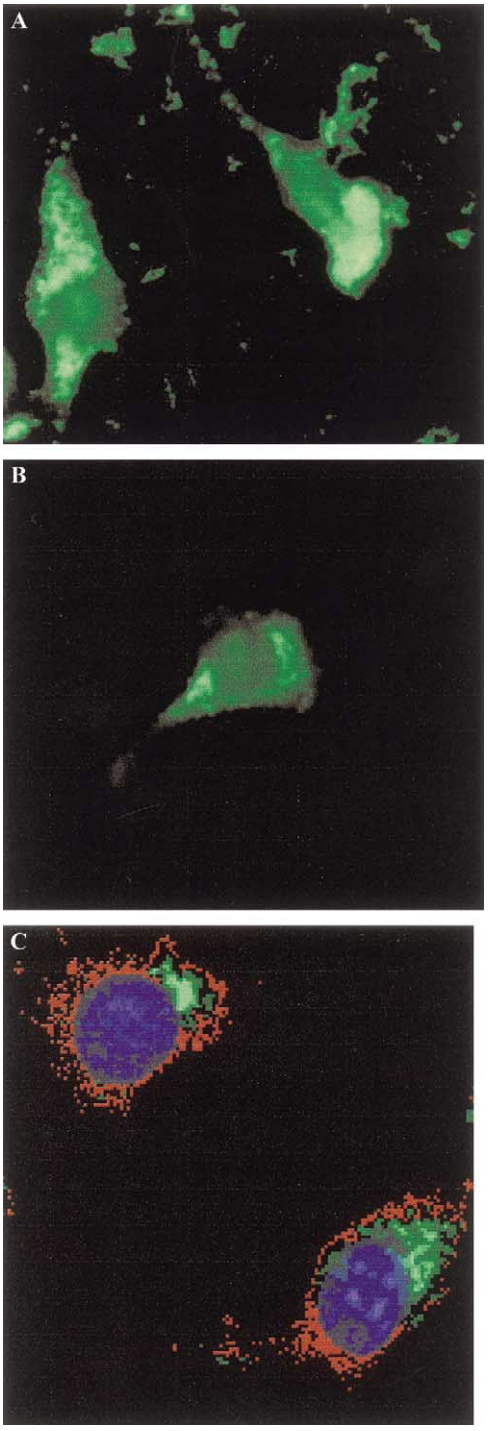

Fig. 1. Cellular internalisation of mouse PrP(1–28) in N2a cells. The cells were incubated for 1 h at 37 °C with a final peptide concentration of 5 μM. (A) Confocal microscope picture showing, in green, the localisation of PrP(1–28) coupled to fluorescein. The peptide is mainly localised perinuclearly and in the cytosol. (B) Confocal picture showing the localisation of PrP(1–28) conjugated to avidin-FITC, in green, yielding the same staining pattern as the fluorescein-coupled peptide alone. The protein concentration was 3.7 μM. (C) Confocal picture of the peptide–avidin-FITC complex, in green, with the same conditions as in (B). In addition, the nucleus is visualised in blue, using Hoechst 33258, and the plasma membrane is visualised in red, using concanavalin A conjugated to Alexa Fluor 594.

previous experiments [4], which demonstrated CPP properties of the pIsl peptide sequence, derived from the homeodomain of a rat transcription factor. Briefly, Nα-biotinyl PrP(1–28) coupled to avidin-FITC was added to the N2a cells and incubated at 37 °C for 1 h (Fig. 1B) or 3 h (not shown). Most of the protein–peptide complex precipitated and formed large aggregates on the cell surfaces. Nevertheless, the peptide–protein complex was internalised into the cells, where it could be found in the cytosol of N2a cells (Fig. 1B). The cytosolic staining for both peptide (Fig. 1A) and peptide–protein complex (Fig. 1B) was diffuse, indicating uptake by the cell penetrating process, but also punctate, indicating some uptake by endocytosis. The staining pattern was similar for N1e and Bowes melanoma cells, both after 1 and 3 h incubation.

The peptide-treated cells drastically changed morphology, indicating that the peptide stressed the cells. As amphipathic peptides can disturb and perforate the plasma membrane, a membrane leakage assay was performed, as previously described [5]. Surprisingly, the assay showed that the PrP(1–28) does not cause leakage of deoxyglucose from cells, in contrast to other known amphipathic CPPs [5]. In addition, an MTT cytoproliferation assay [6] was performed. The assay showed that the peptide was indeed toxic, with only 50% of the N2a cells surviving after 20 h in 5 μM PrP(1–28), as compared to control cells where no peptide was added.

Secondary structure induction

A second question concerned secondary structures of the PrP(1–28) peptide itself, this time without biotin attachment, both in aqueous solution and in the presence of various membrane mimetic solvents, as studied by CD and NMR spectroscopy. Fig. 2A shows CD spectra of 10 μM peptide in aqueous solution or interacting with small unilamellar phospholipid vesicles, prepared as previously described [7]. The induced secondary structures vary significantly with the environment. The CD spectra of the peptide show mostly random coil in pure water and more β-like structure in a buffered solution. We found that increasing peptide concentration, increasing the temperature, or addition of salt increased the β-structure contribution. This behaviour mirrors that of a previously studied (12–28) fragment of the amyloid (Aβ) peptide involved in Alzheimer's disease, which has a strong tendency to aggregate and turn into β-structure in aqueous solution, particularly at high concentration, high temperature, and with added salt [8]. In the presence of neutral POPC or negatively charged POPG vesicles the CD spectra of PrP(1–28) indicate a dominating α-helical or β-structure, respectively (Fig. 2A).

In 50 mM phosphate buffer, pH 7, the 10 μM PrP(1–28) peptide exhibited a tryptophan fluorescence shifted

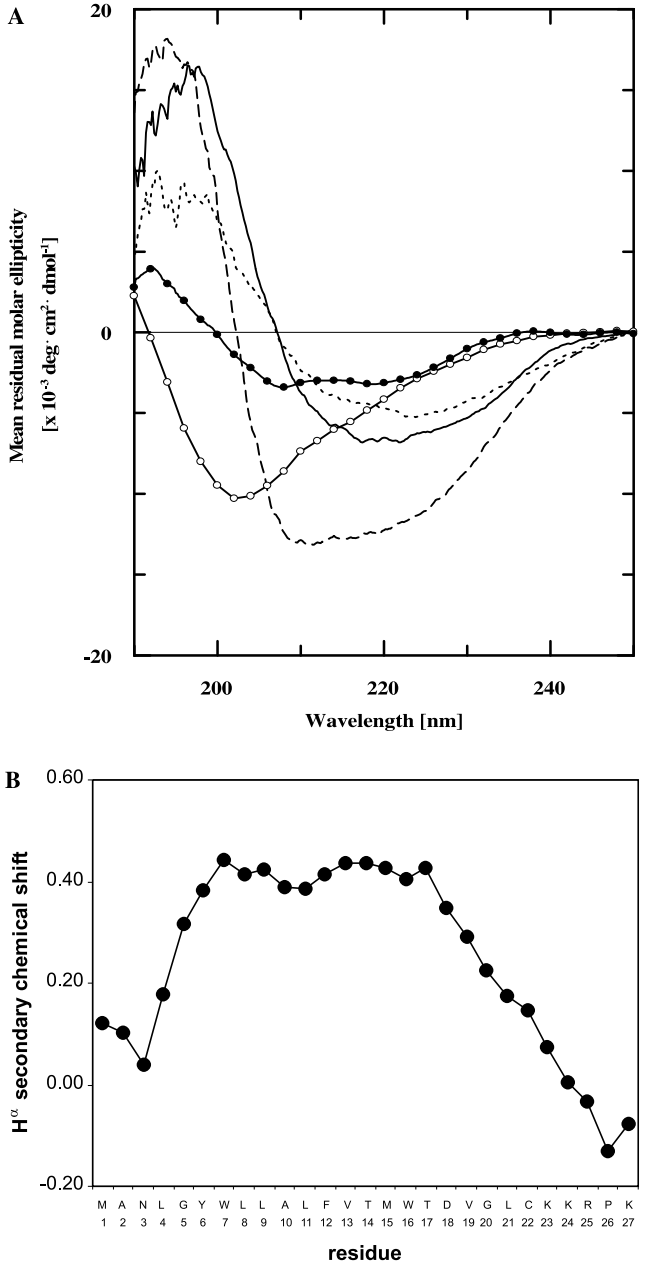

Fig. 2. (A) Circular dichroism spectra of mouse PrP(1–28) in various solvents, recorded at 4 °C. Ten micromolar PrP(1–28) in water (○); in 1 mM potassium phosphate buffer, pH 7.0 (●); in small unilamellar vesicles composed of 2 mM phospholipids in 1 mM potassium phosphate buffer, pH 7.0: neutral 1-palmitoyl-2-oleyl-phosphatidylcholine, POPC (———), negatively charged 1-palmitoyl-2-oleyl-phosphatidylglycerol, POPG (– – –), or a POPG/POPC mixture at a molar ratio of 30/70 (—). The low temperature was chosen as to minimise the aggregation of the peptide (cf. [8]). Contributions from background signals from the solvent were subtracted from the CD spectra acquired for the peptide. (B) Secondary chemical shifts in NMR spectra for the H^{α}-protons of mouse PrP(1–28) in deuterated SDS-d_{25} micelles, pH 3.4. The secondary shifts in (ppm) were calculated as a mean value over three residues $[\text{shift}(\text{res}^{i-1}) + \text{shift}(\text{res}^{i}) + \text{shift}(\text{res}^{i+1})]/3$. For the assignment, 2D-TOCSY and 2D-NOESY ^{1}H NMR spectra were recorded at 45 °C. The PrP(1–28) concentration was 2 mM and the concentration of SDS-d_{25} was 300 mM in 90% H_2O/10% D_2O.

towards shorter wavelengths compared to the fluorescence in pure water (data not shown). This indicates that the tryptophan fluorophore of the peptide is located in a hydrophobic environment and suggests an oligomeric aggregated state of the peptide. With samples stored for about 1 week no evidence for an amyloidic state was revealed by the thioflavin T fluorescence assay [9].

Parallel ^{1}H NMR experiments did not yield a resolved spectrum of the peptide around 1 mM concentration in pure water. Attempts to investigate diffusion showed that aggregates were formed that were larger (>100 kDa) than could be measured by the NMR technique. In a solvent with SDS micelles a well-resolved ^{1}H NMR spectrum of the peptide could be obtained and the resonances assigned according to standard procedures [10]. The secondary chemical shifts [11] of the H^{α}s along the peptide chain give clear evidence of induced α-helical structure (Fig. 2B) in this solvent.

Discussion

The findings reported here have several important potential implications, both for infectivity aspects and the secondary structure conversion of the PrP. The evidence that PrP(1–28) is a CPP ('Trojan horse') and may carry a cargo into cells suggests that this cargo may also be the rest of the PrP protein or even the scrapie form, PrP^{Sc}. The whole PrP protein may be like a homeodomain transcription factor [12] in its overall facile passage through cell membranes without the need for active transport. Hence, it may be a member of the 'messenger protein' family.

Earlier studies [13,14] have shown that the PrP is synthesised in three topological forms in the ER. ^{Sec}PrP is fully translocated into the lumen, whereas ^{Ntm}PrP and ^{Ctm}PrP are single-spanning proteins of opposite transmembrane orientations. Increased generation of ^{Ctm}PrP is reported to be associated with development of neurodegenerative disease [15]. Interestingly, a recent study has shown that ^{Ctm}PrP contains an uncleaved N-terminal signal peptide [16]. In another study, a mutant PrP associated with the inherited Gerstmann–Sträussler–Scheinkler syndrome has also been reported to retain the N-terminal signal peptide in a significant proportion [17]. By itself the putative NLS sequence alone associated with the PrP was found not to be efficient in targeting the nuclear compartment with a cargo of green fluorescent protein [18]. In view of our present findings we would argue that the signal sequence and the putative NLS have to be coupled together as in the native sequence, to yield an efficient CPP with a potential nuclear targeting ability.

It should be added that none of the other common amyloid forming proteins associated with human disease [19,20] has a similar pattern of hydrophobic signal

sequence followed by a short stretch of mainly positively charged residues, as can be observed by visual inspection of the sequences. Although the present study concerns the mouse PrP sequence (**S2**), the similarity of this pattern to the corresponding bovine and human sequences (**S3** and **S4**) leaves little doubt that similar membrane translocation and structure conversion properties will be seen also in those cases. It therefore seems that there is a significant correlation between the literature reports on an uncleaved N-terminus of the PrP and prion related disease on the one hand, and the same N-terminal peptide and cell membrane translocation and membrane facilitated β-structure induction on the other hand.

We have earlier shown that the structural properties of the well-established CPP penetratin depend on the environment: the peptide is unstructured in water and undergoes an α-to-β transition 'catalysed' by a negative lipid surface, particularly at high peptide concentrations [7]. The PrP(1–28) peptide shows similar secondary structure variations and undergoes an α-to-β transition when the interacting medium is changed from neutral POPC to negatively charged POPG phospholipid vesicles. Another example of this behaviour is shown by the Alzheimer Aβ peptide which has been reported to undergo a lipid-catalysed α-to-β transition [21]. Similarly, the chimeric CPP peptide (**S1**) exhibits β-structure when it interacts with negatively charged phospholipid membranes [2].

The N-terminus (residues 23–121) of the human or bovine PrP(23–230) is flexibly disordered, as shown by NMR studies of the solution structure [22,23]. If a sequence with a strong β-structure propensity as well as a strong aggregation tendency is coupled to this flexibly disordered part, the (1–28) part may function as a seed to propagate β-structure further along the unstructured sequence. Activity as a seed for aggregation of the PrP has been ascribed also to the (106–126) region of the sequence [24]. Earlier studies on PrP(23–231) have shown that association of the N-terminal domain to a lipid bilayer induces a more ordered conformation within the so-called octa-repeat region in the flexibly disordered part of the protein [25], in agreement with observations that peptides with sequences corresponding to the octa-repeat region can adopt non-random conformations in non-aqueous solvents or in the presence of phospholipid vesicles [26].

The present results are highly interesting from a biological point of view. The detailed mechanisms of the infectivity of prion material are still not well known [27]. The CPP property of the PrP(1–28) part may play a role in the cellular transport of prion proteins and thus in infectivity. For the PrPs, the potent β-structure induction of the (1–28) region promoted by interaction with a negative membrane surface, together with a seeding role, might operate even between sequences from different species. This observation may be relevant for the origin of species barriers or lack thereof when PrP is transformed into the scrapie form.

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