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Targeting prion propagation using peptide constructs with signal sequence motifs

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ABSTRACT

Synthetic peptides with sequences derived from the cellular prion protein (PrP^C) unprocessed N-terminus are able to counteract the propagation of proteinase K resistant prions (PrP^{Res}, indicating the presence of the prion isoform of the prion protein) in cell cultures (Löfgren et al., 2008). The anti-prion peptides have characteristics like cell penetrating peptides (CPPs) and consist of the prion protein hydrophobic signal sequence followed by a polycationic motif (residues KKRPKP), in mouse PrP^C corresponding to residues 1–28. Here we analyze the sequence elements required for the anti-prion effect of KKRPKP-conjugates. Neuronal GT1-1 cells were infected with either prion strain RML or 22L. Variable peptide constructs originating from the $mPrP_{1-28}$ sequence were analyzed for anti-prion effects, measured as disappearance of proteinase K resistant prions (PrP^{Res}) in the infected cell cultures. We find that even a 5 amino acid N-terminal shortening of the signal peptide abolishes the anti-prion effect. We show that the signal peptide from PrP^C can be replaced with the signal peptide from the Neural cell adhesion molecule-1; $NCAM1_{1-19}$, with a retained capacity to reduce PrP^{Res} levels. The anti-prion effect is lost if the polycationic N-terminal PrP^C-motif is conjugated to any conventional CPP, such as TAT_{48-60}, transportan-10 or penetratin. We propose a mechanism by which a signal peptide from a secretory or cell surface protein acts to promote the transport of a prion-binding polycationic PrP^C-motif to a subcellular location where prion conversion occurs (most likely the Endosome Recycling Compartment), thereby targeting prion propagation.

Introduction

The physiological role(s) of the cellular prion protein (PrP^C) appear to be many and varied [1]. The prion protein is also involved in protein misfolding disorders such as Creutzfeldt-Jacob's disease in humans, Bovine Spongiform Encephalitis (BSE)[1] in cattle, or Scrapie in sheep, which belong to a family of important neurodegenerative amyloid diseases [2]. The so called Scrapie isoform of the prion protein (PrP^{Sc}, in infectious prions), probably in an oligomeric form [3], is a biological and medical concept associated with infection and disease. Here we will generally use the term PrP^{Res}, chemically defined as the isoform of PrP which is resistant to proteinase K, a characteristic closely but not entirely linked to prion infectivity [4,5].

It has recently become clear that other amyloid diseases share important traits with the prion diseases, such as transcellular spread of amyloid material. This makes prion studies relevant also for a wider range of amyloid diseases [6].

While the cellular localization of PrP^C varies between cell types, the protein generally follows the secretory pathway (as reviewed in [7]). Most PrP^C is found on the plasma membrane, from where it is internalized and travels via early endosomes and recycling endosomes back onto the cell surface [8]. A small fraction of PrP^C is internalized for lysosomal degradation [9]. PrP^C traffics different uptake pathways depending on membrane subdomain dynamics [10].

Endocytosis is an important key event for prion propagation [11] and the Endosome Recycling Compartment (ERC) has been proposed as a major site of the PrP^{Sc}-induced misfolding of PrP^C (prion conversion) [8]. Like amyloid conversion in general, the conversion process of PrP^C into PrP^{Sc} is not well defined chemically.

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[1] *Abbreviations used:* BSE, Bovine Spongiform Encephalitis; ERC, Endosome Recycling Compartment; HSPGs, heparan sulfate proteoglycans; CPPs, cell penetrating peptides; FBS, fetal bovine serum; PBS, phosphate buffered saline; OD, optical density; NCAM1, Neural cell adhesion molecule-1.

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It appears to depend on PrP^{C} and/or PrP^{Sc} membrane insertion [12], and oligomeric PrP^{Sc} nucleating misfolding of PrP^{C} [13–15]. The efficiency of prion replication is affected by several proposed environmental cofactors [16] such as pH and presence of ions, as well as the presence of cell surface heparan sulfate proteoglycans (HSPGs) [17,18].

The gradually lowered pH (pH in the range 4.7–5.8) of maturing endosomes is a potential driving force for prion conversion [11] [19,20]. Although the ERC has been proposed as a major site for prion replication [8,21,22], other conversion sites also may exist, such as the plasma membrane [23]. While membrane rafts control prion uptake [10], neuronal cell surfaces in BSE infected cattle exhibit abnormal coated pit formations associated with presence of misfolded PrP [24]. Membrane proteins interacting with PrP^{C}/PrP^{Sc} in both raft and non-raft membrane domains may promote their endocytosis [10,25] and possibly also their toxicity [26]. Pinocytosis of prions has also been reported [27,28]. The prion conversion site(s) may vary between different cells [22,29–33], and most likely depends on factors like prion strain, PrP^{Sc}-subpopulations [34–36] as well as uptake routes.

The cell penetrating peptides (CPPs) are a heterogeneous group of peptide constructs, which are related to but generally less cytotoxic than antimicrobial peptides. CPPs have the capacity to internalize into cells and also bring with them various attached cargoes. CPPs have two proposed mechanisms for cellular uptake: by triggering an active uptake and possibly by passive transduction directly across the cell membrane [37]. The active endocytotic uptake may follow different pathways, leading to endosomal escape into the cytoplasm [38].

Peptides derived from the unprocessed prion protein N-terminus can cross biological membranes also when complexed with cargoes [39–41]. These CPP-like PrP-peptides contain the prion protein hydrophobic signal peptide (mouse PrP residues 1–22), normally removed from PrP^{C} during biosynthesis, followed by a polycationic nuclear localization-like sequence, the NLS-like sequence (mouse PrP residues 23–28; KKRPKP). The membrane translocation of such a peptide with a sequence derived from the mouse or bovine PrP ($mPrP_{1-28}$ or $bPrP_{1-30}$, see Table 1) was suggested to occur through raft-dependent macropinocytosis, initiated/mediated by cell surface HSPGs and/or negatively charged phospholipids, followed by endosomal escape [41].

We have previously reported that externally added $mPrP_{1-28}$ and $bPrP_{1-30}$ significantly down-regulate PrP^{Res} (proteinase K resistant PrP) levels in prion-infected cells [42]. To further analyze the biochemical background for this anti-prion effect, our present work explores the effects of varying peptide constructs added to prion infected cell cultures. The peptide constructs contain the polycationic segment KKRPKP, expected to bind to PrP^{Res} [43,44]. The polycationic sequence is coupled to different CPPs, or to an alternative signal peptide targeting the ER for secretary proteins (from the Neural cell adhesion molecule-1; NCAM1-19) (Table 1). While the constructs based on CPP sequences had no effect on PrP^{Res}, the construct based on the NCAM-1 was even more efficient than the original $mPrP_{1-28}$ to reduce the PrP^{Res} levels.

Materials and methods

Cell cultures and RML infection

The GT1-1 cell line [45] is derived from immortalized murine gonadotropin-releasing hypothalamic neuronal cells. Cells were cultivated in Dulbecco's modified Eagle's medium 4.5 g/L glucose with Glutamax I (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 5% heat-inactivated horse serum (HS), and 50 U/ml penicillin–streptomycin (PEST). Confluent cell cultures were split at a ratio of 1:5 once a week using 1X-trypsin–EDTA (Gibco BRL). Infection of GT1-1 cells with RML/Chandler prion isolate to generate ScGT1-RML cells was performed as previously [46], and of of GT1-1 cells with 22L prion isolate to generate ScGT1-22L cells [46], by using 0.1% homogenate of mouse brains infected with the prion strain. The RML/Chandler isolate homogenate was a generous gift from Prof. Stanley B. Prusiner (Institute for Neurodegenerative Diseases and Department of Neurology, University of California, San Francisco, CA, USA). The 22L isolate homogenate was obtained from The Roslin Institute, University of Edinburgh, Scotland. Both ScGT1cell lines were regularly tested for presence of PrP^{Res}.

Peptide synthesis

Peptide synthesis was performed on a SYRO multiple peptide synthesizer (MultiSyn Tech Gmbl) using a polystyrene-based Rink amide resin (0.4–0.6 mmol/g). Standars Fmoc (9 H-fluoren-9-ylmethoxycarbonyl)-AA-OH were coupled using HBTU (O-benzotriazole-N, N, N′, N′-tetramethyl-uroniumhexafluorophosphate) as activating reagent and DIEA (diisopropylethyl amine) as base. Peptides were purified by RP-HPLC, C18 preparative column (5 μm), acetonitrile–water [0.1% TFA] and analyzed by MALDI MS (Perkin-Elmer prOTOF™ 2000 O-TOF, positive mode).

Peptide treatments of cell cultures

The peptide constructs, as listed in Table 1, were tested for effects on PrP^{C} levels in GT1 cells and effects on PrP^{Res} in cells infected with either of prion strains RML/Chandler or 22L. Before treatment of cells with the respective peptide, the GT1, ScGT1-RML, or ScGT1-22L cells were seeded on 12-well Petri cell culture plates in HS/FBS/PEST-supplemented DMEM. $0.5 * 10^{6}$ cells /well were seeded out 7 days before harvest and analysis of prion protein levels. Peptides were added at a final concentration of 5 μM if not stated otherwise, in HS/FBS/PEST-supplemented DMEM. Equivalent addition of phosphate buffered saline (PBS) was used for negative controls (UT C). All treatments were conducted over 5 days. Cells were subjected to two additions of indicated peptide, at 5 and 3 days prior to cell harvest, and medium was changed prior to each peptide addition.

Immunodetection of prion protein

Following peptide treatment, cells were washed thrice in ice cold PBS, trypsinated and washed in PBS by centrifugation at 200×*g* for 5 min. Cell pellets were dissolved in ice cold extraction buffer (0.5% Triton X-100, 0.5% NaDoc, 150 mM NaCl, 10 mM EDTA, 50 mM Tris pH 7.5 at 0 °C). Debris was removed after centrifugation for 2 min at 5000×*g* at 4 °C. Protein content was measured by Bradford assay and samples diluted to achieve equal protein concentration in samples of a given experiment. Each sample was split into two parts; one part cell extract and one part to be subjected to proteinase K (PK)-digestion. For cell extracts, samples were boiled for 5 min in 1/4 final volume of 4xLaemmli sample buffer. For PK-digestion, samples were prepared as previously described [46]. Samples were applied on 12% SDS–PAGE or 4–12% bis-MES NuPAGE® gels followed by transfer onto PVDF membrane. Following transfer, each membrane was stained with ponceau-S solution. Analysis by Western blot was performed as described previously [47]. For immunodetection, enhanced chemiluminescense (ECL) was used and images recovered in a LAS-1000 Luminescent Image Analyzer V2.6 (Fuji Photo Film Co., Ltd) using the Fujifilm software MultiGauge V3.2.