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Live-cell FRET imaging reveals clustering of the prion protein at the cell surface induced by infectious prions

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ABSTRACT

Prion diseases are associated to the conversion of the prion protein into a misfolded pathological isoform. The mechanism of propagation of protein misfolding by protein templating remains largely unknown. Neuroblastoma cells were transfected with constructs of the prion protein fused to both CFP-GPI-anchored and to YFP-GPI-anchored and directed to its cell membrane location. Live-cell FRET imaging between the prion protein fused to CFP or YFP was measured giving consistent values of 10 ± 2%. This result was confirmed by fluorescence lifetime imaging microscopy and indicates intermolecular interactions between neighbor prion proteins. In particular, considering that a maximum FRET efficiency of 17 ± 2% was determined from a positive control consisting of a fusion CFP-YFP-GPI-anchored. A stable cell clone expressing the two fusions containing the prion protein was also selected to minimize cell-to-cell variability. In both, stable and transiently transfected cells, the FRET efficiency consistently increased in the presence of infectious prions — from 4 ± 1% to 7 ± 1% in the stable clone and from 10 ± 2% to 16 ± 1% in transiently transfected cells. These results clearly reflect an increased clustering of the prion protein on the membrane in the presence of infectious prions, which was not observed in negative control using constructs without the prion protein and upon addition of non-infected brain. Our data corroborates the recent view that the primary site for prion conversion is the cell membrane. Since our fluorescent cell clone is not susceptible to propagate infectivity, we hypothesize that the initial event of prion infectivity might be the clustering of the GPI-anchored prion protein.

1. Introduction

Prions, a class of proteinaceous infectious self-reproducing agents discovered by S. Prusiner, cause fatal neurodegenerative diseases due to misfolding and aggregation of the prion protein. Also termed transmissible spongiform encephalopathies (TSEs), these diseases occur when an abnormal isoform of the prion protein named PrP^{Sc} acts as a transmissible agent being able to induce the conversion of endogenous cellular prion protein (PrP^{C}) into new PrP^{Sc} molecules which then form aggregates. The infectious characteristic of prion diseases imposes threat to public health as shown by the outbreak of mad cow disease (BSE) in the 80s. The appearance of this novel disease in cattle, its source of infection likely to be related to feed and especially its most probable transmissibility to humans by consumption of BSE-contaminated beef products [1] has stimulated investigation on prion diseases. Moreover, the mechanism of propagation of protein misfolding by corruptive protein templating thought to be exclusive of prion diseases has now been proven to occur in other proteophaties such as Alzheimer, Parkinson and Huntington diseases conferring renewed interest to the study of prion diseases [2–4]. Increasingly efforts to solve key aspects of prion diseases have been undertaken but several questions still require definite insight such as the cell biology of prion conversion and the role of the prion protein in other neurodegenerative diseases, namely as receptor for Aβ oligomers [5], or the proteolytic cleavage of PrP^{C} impact on prion diseases [6]. The infectivity mechanism of prion diseases requires protein interaction between PrP^{Sc} and the host PrP^{C}, which is a glycoprotein anchored to the cell membrane through a glycophosphatidylinositol (GPI) anchor. The nature of the PrP^{Sc} entity is still under scrutiny and recent reports point to a dynamic collection of two distinct populations of particles [7]. Enhancement of resistance to proteolysis of PrP^{Sc} reduces its infectivity by decreasing frangibility pointing to a key role of PrP^{Sc} oligomers on infectivity [8]. It seems that rates of transmissibility and disease progression are governed by the selection of progressively less stable, faster replicating PrP^{Sc} conformers. The membrane location of PrP^{C} and especially its GPI-anchor seem to be key for prion conversion and pathogenesis [9–12]. One of the aspects that need deeper insight is certainly the characterization of the initial event in prion conversion and infectivity: (i) How do cells respond to extracellular prion aggregates? (ii) What is the initial step and where is the primary site of prion conversion? Recently, it was shown that prion infectivity is extremely rapid and the plasma

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membrane is the primary site for prion conversion [13]. Imaging FRET (fluorescence resonance energy transfer) has been increasingly used to examine the membrane organization of GPI-anchored proteins [14,15]. FRET measurements have the high sensitivity of fluorescence measurements and are sensitive to near-Angstrom biological relevant distances thus being widely used to study biomolecular interactions in cells [16]. The cyan and yellow fluorescent protein (CFP and YFP, respectively) pair has been commonly used to measure FRET in cells due to their excitation and emission properties, suitable Förster distance and easy photobleaching of YFP [17]. Using a novel cell system, expressing fusions between the prion protein and the CFP and YFP attached to the cell membrane through a GPI anchor, we have carried out imaging FRET measurements to characterize the interaction of infectious prions with cells. Imaging FRET can be performed in several ways including donor lifetimes [18], acceptor photobleaching [19], acceptor photoactivation [20] and variations on sensitized acceptor fluorescence also called sensitized emission [21]. Acceptor photobleaching FRET on fixed cells was first measured to characterize intermolecular FRET on this novel cell system. Then, sensitized-emission FRET was chosen to perform live-cell measurements, as the alternative based on acceptor photobleaching causes phototoxicity and it is prone to errors resulting from mobility of the donor and acceptor during scan time [22]. Using live-cell sensitized emission FRET from CFP to YFP both fused to PrP, this work shows that exogenous infectious prions promote clustering of the prion protein attached to the cell membrane as the initial step of interaction with cells.

2. Material and methods

2.1. DNA constructs

2.1.1. Site-directed mutagenesis of the mouse ORF of PrP to generate the epitope to the mAb 3F4

The ORF of mouse PrP was mutagenized targeting the residues L108M and V111M to generate the specific epitope for the mAb 3F4. The L108M mutation was first obtained with the primers 5′-cca aaa acc atg aag cat gtg gca ggg-3′ (forward) and 5′-ccc tgc cac atg ctt cat gtt ggt ttt tgg-3′ (reverse). The V111M mutation was then generated using the template with the L108M mutation and the primers 5′-cca aca tga agc ata tgg cag ggg ctg cgg-3′ (forward) and 5′-ccg cag ccc ctg cca tat gct tca tgt tgg-3′ (reverse).

2.1.2. Generation of PrP-FP-GPI constructs targeted to the plasma membrane

The sequences of YFP and CFP were ligated to the PrP sequence that codes for the GPI-anchor preceded by 15 bp from mature PrP, to assure that cleavage and GPI-anchor are processed correctly. This ligation was performed first by amplification of the fluorescence protein sequences from the plasmids pEYFP and pECFP with the primers 5′-ggg aga aga tcc agc atg gtg agc aag ggc-3′ (forward) and 5′-gga tct ccc gtc ctt gta cag ctc gtc-3′ (reverse) and by amplification of the sequence that codes for the GPI-anchor with the primers 5′-gac gag ctg tac aag gac ggg aga aga tcc-3′ (forward) and 5′-agt gga tcc tca tcc cac gat cag gaa-3′ (reverse). SOEing of the two previous PCR products was carried out using the primers 5′-gac gag ctg tac aag gac ggg aga aga tcc-3′ (forward) and 5′-gga tct tct ccc gtc ctt gta cag ctc gtc-3′ (reverse) [23]. The sequence that codes for FP-GPI was then amplified with the primers 5′-ttt tga att cat ggt gag caa ggg cga gga g-3′ (forward) and 5′-agt gga tcc tca tcc cac gat cag gaa-3′ (reverse). The forward and reverse primers have recognition sites for EcoRI and BamHI restriction enzymes, respectively. The ORF of PrP with L108M and V111M mutations was amplified to generate a GPI-anchorless sequence with recognition sites for the restriction enzymes HindIII and EcoRI using the primers 5′-ctt agg ctt atg gcg aac ctt ggc tac-3′ (forward) and 5′-gcc ctt gct cac cat gct gga tct tct ccc-3′ (reverse). The sequences YFP-GPI and CFP-GPI generated by SOEing and the PrP-anchorless sequence were digested with EcoRI and ligated with T4 ligase (Promega, USA). The resulting PrP-YFP-GPI and PrP-CFP-GPI sequences were then digested with HindIII and BamHI and cloned into pcDNA 5.0/Hyg and pcDNA 3.1/Zeo plasmids (Invitrogen, USA), respectively. Restriction enzymes were acquired both from Promega (USA) and Nzytech (Portugal).

2.1.3. Generation of FP-GPI constructs targeted to the plasma membrane

To generate constructs of YFP-GPI and CFP-GPI targeted to the plasma membrane (negative control) the sequences coding for PrP-YFP-GPI and PrP-CFP-GPI, in the respective plasmids, were used as template for the primers 5′-ctt aag ctt atc atg gcg aac ctt ggc tac tgg ctg ctg gcc ctc ttt gtg act atg tgg act gat gtc ggc ctc tgc aaa aag cgg cca aag cct atg gtg agc aag ggc gag-3′ (forward) and 5′-agt gga tcc tca tcc cac gat cag gaa-3′ (reverse). The forward primer contains the coding region for the peptide signal that directs the protein to the ER and anneals with the FP coding sequence. The resulting constructs YFP-GPI and CFP-GPI were cloned into the plasmids pcDNA 5.0/Hyg and pcDNA 3.1/Zeo, respectively.

2.1.4. Generation of CFP-YFP-GPI construct targeted to the plasma membrane

To generate the construct CFP-YFP-GPI targeted to the plasma membrane (positive control) the construct CFP-GPI was used as template to replace the GPI-anchor sequence by the recognition site for the restriction enzyme EcoRI using the primers 5′-ctt aag ctt atc atg gcg aac ctt ggc tac-3′ (forward) and 5′-tat aat gaa ttc ctt gta cag ctc gtc cat-3′ (reverse). The construct PrP-YFP-GPI amplified by PCR was subcloned into the pGEM plasmid (Promega, USA) and then restricted with HindIII and EcoRI to remove the PrP coding sequence. The CFP sequence, including the peptide signal targeting to the ER, was then ligated to the pGEM plasmid between HindIII and EcoRI to generate the construct CFP-YFP-GPI. This construct was then subcloned into pcDNA5.0/Hyg between HindIII and BamHI restrictions sites.

2.1.5. Generation of PrP-mtq2-GPI construct targeted to the plasma membrane

The sequence of m-turquoise2 (mtq2) from the pmtq2 vector was amplified using the primers 5′-ggg aga aga tcc agc atg gtg agc aag ggc-3′ (forward) and 5′-gga tct ccc gtc ctt gta cag ctc gtc-3′ (reverse) and digested with EcoRI and Bsp1407I (Thermo, Germany). After digestion, the resulting sequence was ligated to the pGem vector containing PrP-YFP-GPI to remove the YFP coding region. The newly formed PrP-mtq2-GPI sequence in pGem was digested with HindIII and BamHI and cloned into pcDNA 3.1/Zeo for mammalian expression.

2.1.6. Generation of mtq2-GPI constructs targeted to the plasma membrane

To generate the construct of mtq2-GPI targeted to the plasma membrane (negative control) the sequence coding for mtq2 from the pmtq2 plasmid was used as template for the primers 5′-ctt aag ctt atc atg gcg aac ctt ggc tac tgg ctg ctg gcc ctc ttt gtg act atg tgg act gat gtc ggc ctc tgc aaa aag cgg cca aag cct atg gtg agc aag ggc gag-3′ (forward) and 5′-gga tct tct ccc gtc ctt gta cag ctc gtc-3′ (reverse). The amplified sequence was digested with HindIII and Bsp1407I. The forward primer contains the coding region for the peptide signal that directs the protein to the ER and anneals with the FP coding sequence. The resulting construct was cloned into CFP-GPI-pcDNA 3.1/Zeo plasmid, digested with HindIII and Bsp1407I, to replace the coding sequence of CFP with that of mtq2.

2.1.7. Generation of mtq2-YFP-GPI construct targeted to the plasma membrane

To generate the construct mtq2-YFP-GPI targeted to the plasma membrane (positive control), the construct mtq2-GPI was used as template to replace the GPI-anchor sequence by the recognition site for the restriction enzyme EcoRI using the primers 5′-ctt aag ctt atc atg gcg aac ctt ggc tac-3′ (forward) and 5′-tat aat gaa ttc ctt gta cag ctc gtc cat-3′