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Deep membrane insertion of prion protein upon reduction of disulfide bond

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ABSTRACT

The membrane may play a role in the pathogenesis of the prion protein (PrP). Cytoplasmic expression of PrP causes the conversion of PrP to a self-perpetuating PrP^{Sc}-like conformation and the interaction of polypeptide chain with the hydrophobic core of the membrane is believed to be closely correlated with neurodegeneration. However, it is still elusive what factors govern the membrane interaction of PrP. Here, we show that PrP penetrates deeply into the membrane when the single disulfide bond is reduced, which results in membrane disruption and leakage. The proteinase K treatment and the fluorescence quenching assays showed that a predicted transmembrane domain of PrP penetrates into the membrane when the disulfide bond was reduced. Therefore, the oxidation state of PrP might be an important factor that influences its neurotoxicity or pathogenesis.

Prion diseases, also called transmissible spongiform encephalopathies (TSEs), are infectious disorders affecting the brains of humans and animals. In the prion disease, a portion of the prion protein (PrP) converts from its normal conformation, PrP^{C}, to an insoluble and protease-resistant pathogenic conformation, PrP^{Sc} [1]. It is believed that PrP^{Sc} is the transmissible element in the prion disease [2–4].

There is evidence that the polypeptide chain of PrP interacts with the membrane. Binding of PrP to the membrane results in structural changes in PrP and the extent and nature of these structural changes are dependent upon the factors such as pH, lipid compositions, recombinant construct as well as initial conformation of PrP [5–8]. Furthermore, it has been shown that binding of PrP to the membrane can destroy the integrity of the lipid bilayer structure [7,8]. Recently, the protein misfolding cyclic amplification (PMCA) assay showed that PrP, lipids and a synthetic polyanions were the minimal set of components for scrapie [9]. Thus, it seems that the membrane is a crucial factor in pathogenesis of prion disease.

Cytosolic expression of PrP causes the conversion of PrP to a self-perpetuating PrP^{Sc}-like conformation [10]. It has also been shown that the interaction between cytoplasmic PrP and the hydrophobic core of the membrane is closely correlated with neurotoxicity [7]. It is possible that the interaction of cytosolic PrP with the hydrophobic core of the membrane leads to the disruption of the lipid bilayer structure. Aggregation of PrP-bound lipid membranes and the change in membrane permeability can ultimately cause neuronal cell death.

However, it is still elusive what factors determine the membrane-binding of PrP. It is not known what causes the lethal deep insertion of PrP into the membrane. Provided that PrP^{C} behaves normally on cell surface, and also provided that membrane-binding of PrP is correlated with the conformational change, there must be an internal structural factor that regulates the membrane-binding of PrP. In the present study, we found that the membrane-disrupting ability of PrP is closely correlated to the oxidation state of the single disulfide bond of PrP and deeper membrane insertion occurs when the disulfide bond is reduced.

Materials and methods

Materials. 1-Palmitoyl-2-oleoyl phosphatidylcholine (POPC) and 1,2-dioleoyl phosphatidylserine (DOPS) were purchased from Avanti Polar Lipids (Birmingham, AL).

Protein expression and purification. The C-terminal fragment spanning residues 90–231 of Syrian Hamster prion protein was expressed from pET28a-PrP(90–231) [11] in *E. coli* BL21(DE3). The inclusion body of PrP was refolded as described previously [11]. Refolded proteins were eluted with 20 mM Tris–HCl pH 7.4, 500 mM NaCl and 500 mM imidazole. The solution was centrifuged to remove precipitates. Finally, salts were removed from solution using PD-10 desalting column (GE Healthcare).

Preparation of LUV and Nanodisc. Large unilamellar vesicles (LUV, 100 nm diameter) were prepared by extrusion as described elsewhere [12,13]. Nanodiscs, a discoidal membrane structure, were assembled as described except that full-length apolipoprotein A-1 was used in this study [14]. The self-assembled Nanodiscs

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were collected using size exclusion chromatography. The mole fraction of POPC:DOPS was 65:35 for both liposome and Nanodisc.

Contents release. Large unilamellar vesicles (LUV) used in calcein release were prepared as described above. After incorporating 70 mM calcein into LUV, unencapsulated dye was removed with a Superdex 200 gel filtration column. Release of the fluorescent dye calcein from preloaded LUV was monitored using SpectraMaxM2 fluorescence spectrophotometer (Molecular devices, Sunnyvale, CA). Excitation and emission wavelengths were set at 430 and 520 nm, respectively. The addition of Triton X-100 to a final concentration of 0.1% (v/v) was used to determine maximal calcein release. The percentage of calcein release was defined as;

$$\% \text{ calcein release} = (I_t - I_o)/(I_f - I_o)^*100 \quad (1)$$

where, I_o is the initial fluorescence intensity at $t = 0$, I_f is the total fluorescence intensity observed after addition of Triton X-100, and I_t is the fluorescence intensities measured as a function of time. The BSA-encapsulated LUVs were prepared as in case of calcein-encapsulated LUVs. BSA-encapsulated LUV was separated from free BSA using Superdex 200 gel filtration column.

Fluorescence quenching assay. Fluorescence quenching experiment was performed using acrylamide as a quencher. Fluorescence spectra were recorded for each quencher concentration, using an excitation wavelength at 280 nm. The protein and lipid concentrations were 2 μM and 2.5 mM, respectively. When the protein-to-lipids ratio was less than 1/1000, there was little membrane aggregation and the fluorescence could be measured without significance interference (data not shown). The degree of quenching was analyzed according to the following Stern–Volmer equation.

$$F_0/F = 1 + K_{SV}[Q] \quad (2)$$

where, F_o and F are the fluorescence intensities in the absence and presence of acrylamide, respectively, K_{SV} is the Stern–Volmer constant for collisional quenching, and [Q] is the concentration of the quencher. The insertion of PrP into membrane was also measured using two types of lipid quencher. Lipid quencher, 6,7-Br2-PC or 11,12-Br2-PC, was added in replacement of part of POPC while maintaining the DOPC mole fraction at 35%. The degree of quenching was determined as a function of the mole fraction of added bromated PC [13,15].

Results

Enhanced membrane-disrupting ability of reduced PrP and co-precipitation with membrane

There is evidence that the polypeptide chain of PrP_{90-231} interacts with the phospholipid membrane [6,16,17]. The calcein release assay employing negatively charged phosphatidyl glycerol (PG) vesicles showed that the integrity of the membrane was severely perturbed, resulting in the release of the vesicle-entrapped dye upon binding of PrP [18]. However, the reduced PrP has not been tested yet for its membrane disrupting ability. It is believed that the disulfide bond of PrP is reduced when PrP is retrograde transported to endoplasmic reticulum or cytosol, which seems to be closely related to prion pathogenesis [16,19].

In order to measure the effect of disulfide bond reduction on the leakage of internal contents, we prepared calcein-encapsulated large unilamellar vesicles (LUV) of 100 nm diameter. The disruption of membrane would release the internal calcein into bulk solution resulting in the decrease of self-quenching and the increase in the fluorescence intensity. In the presence of PrP, we could observe a gradual increase of the fluorescence intensity consistent with the previously reported results (Fig. 1A). Interestingly, however, we observed much severer membrane disruption when PrP was reduced by DTT. Increase of the fluorescence intensity was much steeper in the presence of DTT than in the absence of DTT, indicating that the membrane-disrupting ability of PrP was enhanced by the reduction of the disulfide bond. Next, an LUV containing bigger internal contents, BSA, was prepared. The release of entrapped BSA from LUV showed clear difference between the two forms of PrP. Reduced PrP efficiently disrupted LUV and released BSA into bulk solution while the intact oxidized PrP did not. The results showed that the reduction of the single disulfide bond enhanced the membrane-rupturing function of PrP.

Above results raise the possibility that PrP interacts with the membrane differently dependent upon its oxidation state. To check this possibility, we measured the ability of PrP to induce aggregation of two forms of membrane LUV (Fig. 1C) and Nanodisc [14] (Fig. 1D) in the presence and absence of the disulfide bond. The extent of membrane precipitation was assessed by measuring the residual fluorescence intensity of the rhodamine-incorporated membrane after removing the precipitate by centrifugation. PrP induced significant membrane aggregation only when the disulfide bond was reduced by DTT as expected from Fig. 1(Fig. 1C). Interestingly, the SDS–PAGE analysis of the membrane aggregates indicated that reduced PrP co-precipitated with LUVs. A discoidal membrane structure, so called Nanodisc, was also subjected to PrP-induced membrane precipitation assay (Fig. 1D). Because the Nanodisc contains apolipoprotein A-I (apoA-I) for membrane scaffolding, the membrane-associated co-precipitation of PrP can be easily visualized by the presence of apoA-I in pellet fraction. As in case of LUV, the discoidal membranes also precipitated only when the disulfide bond was reduced by DTT, indicating that PrP induces membrane aggregation and co-precipitates along with membranes when the disulfide bond is reduced.

The images taken by transmission electron microscopy showed that LUVs were aggregated by reduced PrP (Fig. 2). Little morphological changes were observed when LUVs (~100 nm diameter) were treated with the oxidized PrP (Fig. 2A and B). However, we could observe flocculation of LUVs of sizes over 200–300 nm when LUVs were treated with reduced PrP. More distinctively, the aggregated LUVs by the reduced PrP contained embossing-like rough surfaces while the surfaces of LUV treated by the oxidized PrP were smooth. These rough surfaces might be the scars of membrane rupture by PrP and are reminiscent of the flat high-rise domains observed with LUVs treated with PrP106–126 peptide in another study [20].

Change in the membrane-binding mode of PrP upon the reduction of the disulfide bond

Above results suggest that PrP affects the membranes differently dependent on the status of the disulfide bond. The content release and the membrane-aggregating ability are dramatically increased by the reduction of disulfide bond, raising the possibility that the membrane-binding mode of PrP is altered when the disulfide bond is reduced. In Fig. 3A, it is shown that λ_{max} of emission spectra of PrP measured in the presence of the membrane shifts depending on the state of disulfide bond. The blue shift occurred when DTT was added to the PrP/liposome mixture, an indicative of membrane insertion of PrP.

To characterize the interaction between PrPs and membrane lipids more systematically, we have used a fluorescence quenching assay employing a soluble quencher acrylamide [13]. There are two Trp residues in our construct PrP_{90-231} and a putative transmembrane domain resides between these two Trp residues [21]. This approach allowed us to assess the sequestering of the Trp residues from the solution phase due to the insertion into the membrane. Fig. 3B shows the Stern–Volmer plots for acrylamide quenching of Trp fluorescence of PrP when its disulfide bond is either oxidized