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Trafficking of PrP^C to mitochondrial raft-like microdomains during cell apoptosis

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The cellular form of prion protein (PrP^C) is a highly conserved cell surface GPI-anchored glycoprotein that was identified in cholesterol-enriched, detergent-resistant microdomains named "rafts." The association with these specialized portions of the cell plasma membrane is required for conversion of PrP^C to the transmissible spongiform encephalopathy-associated protease-resistant isoform. Usually, PrP^C is reported to be a plasma membrane protein, however several studies have revealed PrP^C as an interacting protein mainly with the membrane/organelles, as well as with cytoskeleton network. Recent lines of evidence indicated its association with ER lipid raft-like microdomains for a correct folding of PrP^C, as well as for the export of the protein to the Golgi and proper glycosylation. During cell apoptosis, PrP^C can undergo intracellular re-localization, via ER-mitochondria associated membranes (MAM) and microtubular network, to mitochondrial raft-like microdomains, where it induced the loss of mitochondrial membrane potential and cytochrome *c* release, after a contained raise of calcium concentration. We suggest that PrP^C may play a role in the multimolecular signaling complex associated with cell apoptosis.

Lipid rafts and their components may, thus, be investigated as pharmacological targets of interest, introducing a novel and innovative task in modern pharmacology, i.e., the development of glycosphingolipid targeted drugs.

Keywords: rafts, microdomains, mitochondria, gangliosides, apoptosis, scrambling, prion protein

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The Role of Cellular Prion Protein in Cell Activation

The prion protein (PrP) was first detected in attempts to identify the infective agent of transmissible spongiform encephalopathies.[1] Later, several isoforms of this protein were described and named, in particular PrP^C,[2] either membrane-bound or soluble, and PrP^{Sc}.[3] These conformational isoforms have essentially the same amino acid sequence, but different biochemical properties. Physiological prion protein is a sialoglycoprotein with two possible glycosylation sites, leading to diglycosylated, monoglycosylated and nonglycosylated forms. PrP is present in body fluids and in the plasma membrane of neural and lymphocytic cells.[4] PrP has been also described as a component of plasma membrane-derived microvesicles, suggesting that microvesicles may contribute both to the intercellular mechanism(s) of PrP diffusion and signaling, as well as prion spread and neuroinvasion.[5]

The cellular form of PrP (PrP^C) is a highly conserved cell surface GPI-anchored glycoprotein that was first identified in vitro as a molecule able to bind Cu^{2+},[6] in neurons and other cells, including lymphocytes. While GPIs were originally regarded as simple anchors that tethered proteins to cell membranes, it is now recognized that GPIs are involved in protein sorting and cell signaling.

Like other GPI-anchored proteins, most of PrP^C, as well as PrP^{Sc}, were found in cholesterol-enriched, detergent-resistant microdomains ("rafts") of

the neural plasma membrane and lymphocytes,[4,7,8] which are also enriched in several cytoplasmic proteins, including tyrosine kinases.[9] The association with these specialized portions of the cell plasma membrane is required for conversion of PrP^C to the transmissible spongiform encephalopathy-associated protease-resistant isoform.[10]

An increasing number of GPI-anchored proteins, including CD59, Thy-1, CD14 and Fc-ε-R1 are associated with cell responses and the activation of multiple cell-signaling pathways. Cell activation commonly occurs following cross-linkage of GPI-anchored proteins by antibodies, suggestive of a density dependent effect.

Crosslink of PrP^C on the surface of T-cell lines has been associated with various cellular responses, such as intracellular calcium mobilization, Src and Erk kinase activation or capping of lipid microdomains[11] in a large membrane zone where PrP^C colocalize with Thy-1 or fyn kinase.[12] The contribution of PrP^C to the classical T-lymphocyte activation process has been characterized by clustering the T-cell receptor component CD3, as well as PrP^C, with soluble and surface immobilized antibodies, respectively. Thus, cross-linking of PrP^C with surface-immobilized antibodies is sufficient to initiate several different signaling cascades. A modulating function of PrP^C during the fine tuning of the lymphocyte activation process is therefore very conceivable.

In our work, confocal microscopy of T human lymphoid cells showed colocalization at the level of the plasma membrane of PrP^C with the Src-family non-receptor tyrosine kinase Fyn, but not Src itself. A polyclonal anti-PrP^C antibody coimmunoprecipitated Fyn, and conversely, anti-Fyn pulled down PrP^C in both resting and CD28/CD3 stimulated cells. Following stimulation, anti-PrP^C also coimmunoprecipitated the Syk family tyrosine kinase ZAP-70, which binds both CD3ζ chains and CD45-associated phosphatase upon TCR stimulation.[8] These data support the interpretation that PrP^C associates with the TCR complex and with at least some of the latter's intracellular downstream effectors, suggesting that PrP^C is a component of the multimolecular signaling complex within microdomains involved in T-cell activation.

Cross-linking of PrP^C in Jurkat T cells and peripheral blood T lymphocytes produced both cocapping of PrP^C with the raft-associated proteins reggie-1 and reggie-2 (flotillin-2 and flotillin-1, respectively), as well as a transient calcium-mediated signal and phosphorylation of Erk1/2, which were somewhat distinct from similar signals induced by TCR/CD3 stimulation. In addition, PrP^C crosslinking also dragged the Src-family kinases Fyn and Lck, as well as CD3 and LAT to the cap, consistent with recruitment of a functional TCR complex, followed by endocytosis of PrP^C together with the reggies.[12]

These results show that cross-linked PrP^C can promote the assembly of TCR complex components within rafts, and this dynamic behavior of a set of cell surface, transmembrane and juxtamembrane molecules may constitute the basis of several PrP^C-mediated T-cell responses.

Furthermore, PrP^C has in vivo consequences during the activation of the immune system by regulating the ability of APC to stimulate proliferative T-cell responses.[13]

Cellular Prion Protein Intracellular Trafficking

After translation and post-translational modifications, most of PrP^C derived from secreted form is believed to be exported to the cell surface, where it is connected to the plasma membrane via a glycosylphosphatidylinositol anchor. This localization is supported by several studies,[7,8] demonstrating an interaction of PrP^C with lipid rafts, small and highly dynamic cholesterol and sphingolipid-enriched domains present on the plasma membrane.[14]

Usually, PrP^C is reported to be a plasma membrane protein, however several studies have revealed the presence of endogenous PrP^C as an interacting protein mainly with the membrane/organelles,[15] as well as with cytoskeleton network. Indeed, lipid microdomains are similarly formed at subcellular organelles, which include endoplasmic reticulum, Golgi and mitochondria, named lipid raft-like microdomains, with relatively low concentrations of cholesterol and glycosphingolipids.[16,17] Increasing evidence suggests that events taking place in these compartments play a modulating role in the pathogenesis of several neurodegenerative disorders.[18] In line with these observations, recent studies[15,19] implicated the potential role of association with endoplasmic reticulum (ER) lipid raft-like microdomains for a correct folding of PrP^C, as well as for the export of the protein to the Golgi and proper glycosylation,[20] suggesting a quality control mechanism for ER raft-like microdomains in distribution of mature PrP^C among cellular compartments and distinct regions of the plasma membrane. Immature and misfolded proteins are normally retained in ER and subject to the ER-associated degradation pathway.[15,21] Alteration of ER homeostasis and proteasomal dysfunction in cells overexpressing PrP^C lead to accumulation of cytosolic PrP species with aberrant biochemical properties and to neurotoxicity in vivo.[22] Furthermore, alteration in the intracellular trafficking of the prion protein could also play a role in the pathology of the inherited disease, as evidenced by the observation that some pathological mutants have a different intracellular localization compared with the wild-type protein.[23] Campana et al.[15] demonstrated that some pathogenic PrP mutants, in particular mutant PrP T182A, inhibited a correct scrapie-like conversion of the protein, protecting cells from neurodegenerative disease. In addition, it has been shown that cholesterol and sphingolipid depletion decreased the amount of PrP^{Sc} production in infected cells and led to the non-polarized distribution of PrP^C at the surface of hippocampal cultured neurons.[24] Furthermore, the glycosylation patterns of PrP^C may also affect protein trafficking and biophysical features.[25] It demonstrates an accumulation of PrP^C in the Golgi compartment of neurons from transgenic mice expressing only nonglycosylated PrP^C, although neither neurological signs nor neurodegeneration were found in these mice.

Recently, we identified PrP^C as a new component of mitochondrial raft-like microdomains in lymphoblastoid T cells undergoing CD95/Fas-mediated apoptosis, indicating that PrP^C could undergo intracellular re-localization via

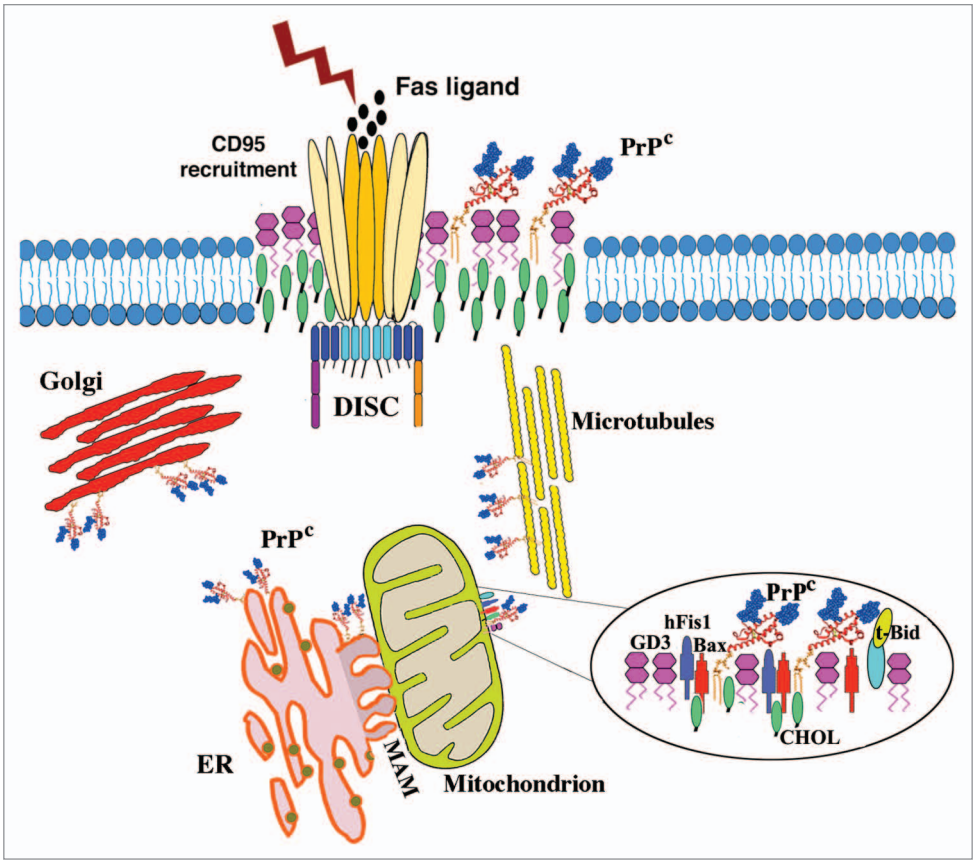

Figure 1. Schematic drawing depicting the trafficking of PrP^C to raft-like microdomains during cell apoptosis. In these microdomains, PrP^C may interact with different molecules; some of these are constitutively present, including ganglioside GD3, cholesterol and the fission protein hFis1, whereas Bcl-2 family proteins (t-Bid and Bax) are recruited, after CD95/Fas triggering. DISC, death-inducing signaling complex; ER, endoplasmic reticulum; MAM, endoplasmic reticulum-mitochondria associated membranes; t-Bid, truncated Bid; CHOL, cholesterol.

ER-mitochondria associated membranes (MAM) and microtubular network (**Fig. 1**). Since MAMs represent a subcompartment of the ER connected to the mitochondria and since they display the characteristics of lipid rafts,[26] we investigated whether PrP^C may be present in this compartment. Our immunoelectron microscopy observations revealed that PrP^C was present in both MAM and mitochondrial membranes. Moreover, when we analyzed the PrP^C intracytoplasmic trafficking in CD95/Fas treated cells, we found that microtubular network-perturbing agent demecolcine impaired either mitochondrial re-localization of PrP^C or apoptosis induction. Hence, we hypothesized that microtubules could play key roles in the intracellular directional re-distribution of PrP^C, as well as in the recruitment of this small polypeptide to the mitochondrial compartment following CD95/Fas triggering.[27]

Mitochondrial Raft-Like Microdomains

Raft-like microdomains represent preferential sites on the mitochondrial membrane where some key reactions can be catalyzed, contributing to cell apoptosis execution.[16,28] In these dynamic structures some molecules are constitutively present, including gangliosides (GD3 and GM3), cholesterol, the voltage-dependent anion channel-1 (VDAC-1) and the fission protein hFis1, whereas PrP^C [27] and Bcl-2 family proteins (truncated Bid, t-Bid and Bax) are recruited, after CD95/Fas triggering.[16] Although phospholipid content is relatively low, cardiolipin is also a constituent of these specialized platforms on mitochondrial membrane, at the contact sites formed between the mitochondrial inner and outer membranes.[29]

Raft-like microdomains can exert a role in the trafficking pathways associated with cell death and actively participate to the structural and biochemical remodeling leading to injury and apoptotic cell death program execution. In particular, these microdomains appear to be involved in a series of intracellular functions, such as: (1) the membrane "scrambling" that participates in cell death execution pathways,[27] (2) the remodeling of organelles, e.g., changes of curvature in mitochondria, (3) the recruitment of proteins to the mitochondria, including molecules associated with mitochondrial fission,[30]

and (4) the mitochondrial oxidative phosphorylation and ATP production.[31] Both mitochondria depolarization and cytochrome *c* release are dependent on raft-like microdomain integrity, since the disruption of raft-like microdomains by methyl-β-cyclodextrin prevented mitochondria depolarization or cytochrome *c* release.

The Role of Cellular Prion Protein in Cell Apoptosis

Several studies suggested that PrP^C is involved in apoptotic signaling pathways. Preliminary studies by Paitel et al. showed that PrP^C overexpression sensitizes cells to staurosporine-induced caspase-3 activation. They further established that this PrP^C-related pro-apoptotic phenotype can be enhanced by proteasome inhibitors and prevented by PrP sequestration by anti-PrP antibodies.[32]

In addition, the PrP^{Sc} protein or PrP (106–126) induces neuronal cell death by increasing p53, p-ERK and p-p38 protein levels and decreasing bcl-2 protein levels.[33]

Hachiya et al. showed that transgenic mice harboring a high copy number of wild-type mouse PrP^C developed a spontaneous neurological dysfunction, probably due to mitochondria-mediated neuronal apoptosis in aged transgenic mice overexpressing wild-type PrP^C. The aged mice exhibited an aberrant mitochondrial localization of PrP^C concomitant with decreased manganese superoxide dismutase activity, cytochrome *c* release, caspase-3 activation and DNA fragmentation, most predominantly in hippocampal neuronal cells.

Hovewer, conflicting results have been reported on the role of PrP^C on cell apoptosis. Indeed, a protective function of PrP^C has been hypothesized in T lymphocytes under oxidative stress.[35] Moreover, PrP^C may also activate neuroprotective signaling pathways. The neuroprotective functions of PrP^C have been attributed to its BCL-2-like properties.[36] PrP^C protects against cell death by preventing the conformational change of BAX that occurs during BAX activation.[37] A direct interaction of PrP^C with BAX is, however, not likely the mechanism by which PrP^C prevents Bax-mediated apoptosis, since cellular compartmentalization would prevent a direct interaction between PrP^C and the BCL-2 family of proteins.[38] A neuroprotective activity of PrP^C was also reported by Mitteregger et al. who revealed that both the C-terminal GPI anchor and the unstructured N-terminal domain are required for this physiological activity.[39] Thus, PrP^C might act as a signaling molecule at the cell surface to promote stress-protective signaling under physiological conditions, which can be switched to toxic signaling through an interaction with β-sheet-rich conformers. Similarly to other GPI-anchored proteins involved in signal transduction, PrP^C may act as a co-receptor in concert with a transmembrane protein to transduce the signal into the cell.

A new and interesting point of view suggests that the role of PrP^C on cell fate depends on its (intra)cellular localization. In particular, recent evidence showed that cholesterol-mediated PrP^C translocation from lipid rafts to non-lipid rafts prevents PrP(106–126)-mediated apoptosis and p-38 activation and caspase-3 activation.[40] The key role of intracellular localization of PrP^C is confirmed and extended by our study,[27] which revealed that PrP^C was redistributed to raft-like microdomains at mitochondrial membrane, as well as at endoplasmic reticulum (ER)-mitochondria associated membranes (MAM), following apoptotic stimuli. Our in vitro experiments also demonstrated that, although PrP^C had such an effect on mitochondria, it induced the loss of mitochondrial membrane potential and cytochrome *c* release only after a contained raise of calcium concentration. Thus, the functional role of PrP^C is strictly dependent on its association with lipid raft domains, in the presence of a raise of calcium concentration.

In conclusion, although contradictory evidence on the role of PrP^C in the apoptotic process has been published, our recent results support the view of a key role for PrP^C in the regulation of cell fate. Finally, since PrP^C raft-mediated trafficking appears to strictly depend upon apoptotic triggering, we can also suggest a reappraisal of the previously hypothesized formation of PrP^{Sc} within acidic compartments. Indeed, since raft-embedded PrP^C is part of the complex framework normally contributing to the death of the cell, a defective trafficking of PrP^C from and toward lipid rafts could also represent a sort of "risk factor" or favor an alteration of normal PrP^C catabolism, also leading to the formation of the 17-kD polypeptide hydrolysis to form the PrP 27–30 scrapie isoform.

Perspectives

These observations suggest that PrP^C may participate in the prion neurodegenerative cascade through the mitochondria-mediated events, at least in part. At the same time, the segregation of the infectious and neurotoxic properties of PrP suggests a new therapeutic strategy, since prevention of mitochondrial mislocalization of PrP^C can be regarded as putative therapeutic targets aimed at protecting cells from mitochondria-mediated apoptosis, even though the prion infection is not fully preventable. More detail can be developed into new therapeutic targets stages of regulation of the apoptotic process to prevent nerve degeneration in prion diseases.

These findings may also have a clinical relevance in terms of drug-delivery. In fact, cyclodextrins are being used in an ever-increasing way to camouflage undesirable pharmaceutical characteristics or to improve therapeutic indices and site-targeted delivery of different drugs, including some nonsteroidal anti-inflammatory drugs. Moreover, statins, which inhibit the enzyme HMG-CoA reductase, involved in the mevalonate pathway of cholesterol synthesis, are able to act on lipid rafts. On these bases, lipid rafts and their components may be investigated as pharmacological targets of interest in the therapeutic strategy of several human diseases, introducing a novel and innovative task in modern pharmacology, i.e., the development of glycosphingolipid targeted drugs.

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