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Prion protein is a component of the multimolecular signaling complex involved in T cell activation

Vincenzo Mattei[a], Tina Garofalo[a], Roberta Misasi[a], Annapia Circella[a], Valeria Manganelli[a], Giuseppe Lucania[a], Antonio Pavan[b], Maurizio Sorice[a,*]

[a] *Dipartimento di Medicina Sperimentale e Patologia, Università 'La Sapienza', viale Regina Elena 324, 00161 Rome, Italy*
[b] *Dipartimento di Medicina Sperimentale, Università di L'Aquila, Via Vetoio Coppito 2, 67100 L'Aquila, Italy*

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Abstract In this study we analyzed the interaction of prion protein PrP^C with components of glycosphingolipid-enriched microdomains in lymphoblastoid T cells. PrP^C was distributed in small clusters on the plasma membrane, as revealed by immunoelectron microscopy. PrP^C is present in microdomains, since it coimmunoprecipitates with GM3 and the raft marker GM1. A strict association between PrP^C and Fyn was revealed by scanning confocal microscopy and coimmunoprecipitation experiments. The phosphorylation protein ZAP-70 was immunoprecipitated by anti-PrP after T cell activation. These results demonstrate that PrP^C interacts with ZAP-70, suggesting that PrP^C is a component of the multimolecular signaling complex within microdomains involved in T cell activation.

Key words: Prion protein; Lipid domain; Raft; Ganglioside; T cell activation

1. Introduction

Prions are unprecedented infectious pathogens that cause a group of invariably fatal neurodegenerative diseases mediated by an entirely novel mechanism. Prion diseases may present as genetic, infectious, or sporadic disorders, all of which involve modification of the prion protein PrP^C, as a constituent of mammalian cells [1].

PrP^C is a highly conserved glycoprotein bound to the cell surface by glycosylphosphatidylinositol (GPI)-anchored linkage in neurons and other cells [2], including lymphocytes [3].

Although there is much information available on the role of PrP^{Sc} in the disease process, relatively little is known about the physiological functions of PrP^C. 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 [4,5], which are also enriched in several cytoplasmic proteins, including tyrosine kinases [6]. 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 [5,7].

PrP^C has been also detected in human lymphocytes [8], where the level of PrP^C was up-regulated as a consequence of T cell activation [3]. The host immune system has been shown to play a role in the pathogenesis of prion disease in animal models [9,10], but the mechanism by which it participates in the propagation of PrP^{Sc} is not known.

In human lymphocytes glycosphingolipid-enriched microdomains (GEM) were observed [11]. In these microdomains GM3 represents the main ganglioside constituent [12] and is involved in modulating signal transduction by assembly with signal transducer molecules, including Fyn, Lck [13] and, after activation, the phosphorylation protein ZAP-70 [12]. In a preliminary study [14] we demonstrated a specific PrP^C–GM3 association within microdomains of human T cells.

The aim of this study was to evaluate a possible role of PrP^C in tyrosine signaling pathway triggered by GEM in T cells. In this concern, we demonstrated the interaction of PrP^C with the transducer protein ZAP-70, which plays a key role in the transduction signaling pathway ending to T cell activation.

2. Materials and methods

2.1. Immunoelectron microscopy

Human lymphoblastoid CEM cells were incubated with anti-PrP monoclonal antibody (MoAb) (6H4, Prion Diagnostica, Milan, Italy) for 1 h at 4°C. Cells were fixed with 2% glutaraldehyde (1 h at 4°C), washed and labeled with protein A-colloidal gold (Pharmacia Fine Chemicals, Uppsala, Sweden) for 3 h at 4°C. Cells were post-fixed in osmium tetroxide 1% in Veronal acetate buffer, pH 7.4, for 2 h at 4°C, stained with uranyl acetate (5 mg/ml), dehydrated in acetone and embedded in Epon 812. Samples were then sectioned and examined under an electron microscope (Philips CM10, Eindhoven, The Netherlands).

2.2. Immunoprecipitation experiments

Briefly, CEM cells were lysed in lysis buffer (20 mM HEPES, pH 7.2, 1% Nonidet P-40, 10% glycerol, 50 mM NaF, including protease inhibitors). Parallel experiments were performed using Triton X-100 as detergent and the results were virtually the same. After preclearing, the supernatant was immunoprecipitated with the polyclonal anti-PrP (C-20) or anti-Fyn (FYN3) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) plus protein A-acrylic beads. The immunoprecipitates were checked by Western blot, using the anti-PrP MoAb (6H4) or anti-Fyn MoAb (FYN15, Santa Cruz).

The PrP^C immunoprecipitate was subjected to ganglioside extraction according to the method of Svennerholm and Fredman [15]. The eluted glycosphingolipids were dried and separated by high-performance thin-layer chromatography (HPTLC) aluminum-backed silica gel 60 (20×20) plates (Merck, Darmstadt, Germany). Chromatography was performed in chloroform:methanol:0.25% aqueous KCl (5:4:1, v:v:v). Plates were immunostained for 1 h at room temper-

*Corresponding author. Fax: (39)-6-4454820.
E-mail address: maurizio.sorice@uniroma1.it (M. Sorice).

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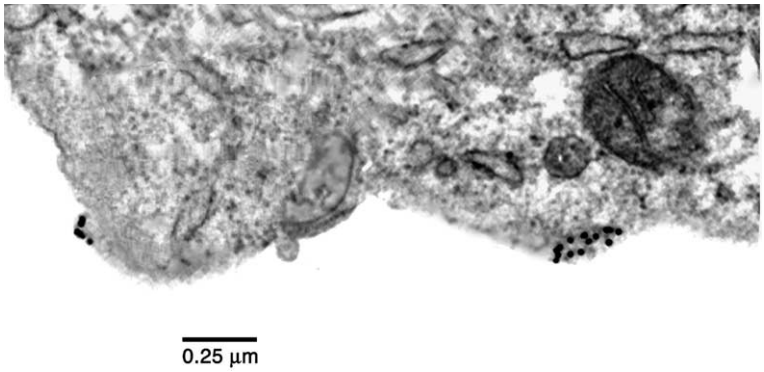

Fig. 1. Immunolabeling of PrP^C on CEM plasma membrane. Small clusters are localized over the microvilli and over the non-villous portion of the plasma membranes.

ature with horseradish peroxidase-conjugated cholera toxin, B subunit (CTxB) (Sigma, St Louis, MO, USA), or, alternatively, with GMR6 anti-GM3 MoAb (Seikagaku, Chuo-ku, Tokyo, Japan) and then horseradish peroxidase-conjugated anti-mouse IgM (Sigma). Immunoreactivity was assessed by chemiluminescence reaction using the ECL Western blocking detection system (Amersham, Buckinghamshire, UK).

The Fyn immunoprecipitate was analyzed by Western blot with the anti-PrP (C-20).

2.3. Analysis of PrP^C–Fyn colocalization on the cell surface by scanning confocal microscopy

CEM cells were labeled with anti-PrP 6H4, for 1 h at 4°C, followed by addition (45 min at 4°C) of fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Sigma). The cells were fixed in acetone/methanol (1:1, v:v) in phosphate-buffered saline for 5 min at 4°C and then labeled with anti-Fyn (FYN3) or anti-Src (pp60src, Biomol, Plymouth Meeting, PA, USA) polyclonal antibodies for 1 h at 4°C, followed by addition (45 min at 4°C) of Texas red-conjugated anti-rabbit IgG (Calbiochem, La Jolla, CA, USA). The images were acquired with a Leica TCS SP2 confocal laser scanning microscope. Images were collected at 512×512 pixels and processed with the confocal software 4.7.

2.4. Coimmunoprecipitation of PrP^C and ZAP-70

Immunoprecipitation of PrP^C was performed as reported above from untreated or treated cells (anti-CD3, Ortho-Clinical Diagnostics, Ratinan, NJ, USA, plus anti-CD28, Pharmingen, La Jolla, CA, USA, 10 µg/ml) for 1 h at 37°C. The immunoprecipitates were analyzed by Western blot with polyclonal anti-ZAP-70 (LR, Santa Cruz).

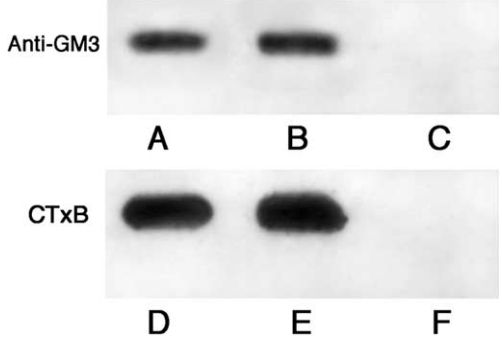

Fig. 2. Coimmunoprecipitation of PrP^C and gangliosides. CEM cells were immunoprecipitated with anti-PrP. The immunoprecipitates were subjected to ganglioside extraction and analyzed by HPTLC. A: Reactivity of anti-GM3 MoAb with standard GM3. B: Reactivity of anti-GM3 MoAb with PrP^C immunoprecipitate. C: Reactivity of anti-GM3 MoAb with immunoprecipitate with IgG with irrelevant specificity. D: Reactivity of CTxB with standard GM1. E: Reactivity of CTxB with PrP^C immunoprecipitate. F: Reactivity of CTxB with immunoprecipitate with IgG with irrelevant specificity.

3. Results

3.1. PrP^C distribution on CEM cell plasma membrane

To investigate the cell surface distribution of PrP^C, immunoelectron microscopy was performed. It showed an uneven distribution of PrP^C on the cell plasma membrane. The gold immunolabeling was distributed in small clusters (Fig. 1).

3.2. PrP^C–ganglioside interaction

Since gangliosides GM3 [12] and GM1 [16] have been shown to be components of the signaling complex within microdomains involved in T cell activation, we investigated the interaction of PrP^C with these gangliosides. Acidic glycosphingolipids, extracted from the PrP^C immunoprecipitates, were immunostained by a high selective anti-GM3 MoAb [17] and by CTxB, indicating that PrP^C strictly interacts with GM3 (Fig. 2B) and GM1 in CEM cell microdomains (Fig. 2E). The immunoprecipitate was revealed as PrP^C, as detected by Western blot, using the anti-PrP MoAb (not shown). In control samples the immunoprecipitation with IgG with irrelevant specificity, under the same condition, did not result in detectable levels of gangliosides (Fig. 2C–F).

3.3. PrP^C–Fyn association on cell plasma membrane

We analyzed the possible association of PrP^C with Fyn, a protein involved in the signal transduction pathway triggered by PrP^C in neural cells [18].

The results obtained by scanning confocal microscopy confirmed that most of the cells showed an uneven signal distribution of PrP^C molecules over the CEM cell plasma membrane (Fig. 3A,D). Similar staining was obtained with the anti-Fyn antibody (Fig. 3B). In order to determine the possible association between PrP^C and Fyn, we superimposed the double immunostaining of PrP^C and Fyn. The merged image of anti-PrP^C and anti-Fyn staining revealed yellow areas, resulting from the overlap of green and red fluorescence, which correspond to nearly complete colocalization areas (Fig. 3C). As a control, we analyzed the possible association of PrP^C with the monoacylated kinase Src (Fig. 3E). The merged image of anti-PrP^C and anti-Src revealed minimal colocalization areas, indicating a weak association between the two proteins (Fig. 3F).

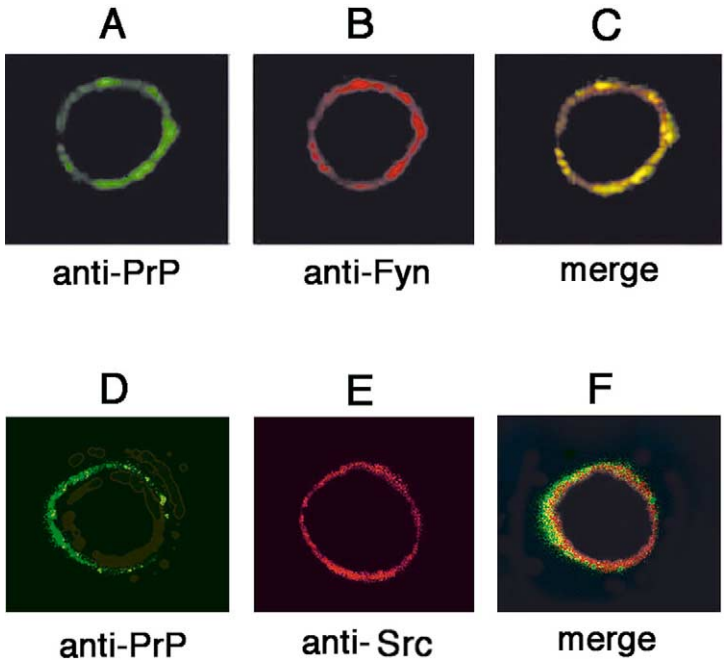

Fig. 3. Scanning confocal microscopic analysis of PrP^C association with Fyn and Src on cell plasma membrane. CEM cells were labeled with anti-PrP 6H4, followed by FITC-conjugated anti-mouse IgG. Cells were incubated with anti-Fyn or anti-Src, followed by Texas red-conjugated anti-rabbit IgG.

These findings indicate that PrP^C associates with Fyn on the plasma membrane of CEM cells.

3.4. PrP^C–Fyn coimmunoprecipitation

To verify whether PrP^C interacts with Fyn, cell-free lysates from CEM cells, either untreated or stimulated with anti-CD3 (10 μg/ml) plus anti-CD28 (10 μg/ml), were immunoprecipitated with the polyclonal anti-Fyn, followed by protein A-acrylic beads. Western blot analysis of the immunoprecipitates showed an about 35 kDa band, recognized by anti-PrP C-20, which was present in untreated cells (Fig. 4A), as well as after CD28/CD3 triggering (Fig. 4C). The immunoprecipitate was revealed as Fyn, as detected by Western blot using the anti-Fyn MoAb (not shown). No band was detected in the immunoprecipitates with IgG with irrelevant specificity, under the same condition (Fig. 4B).

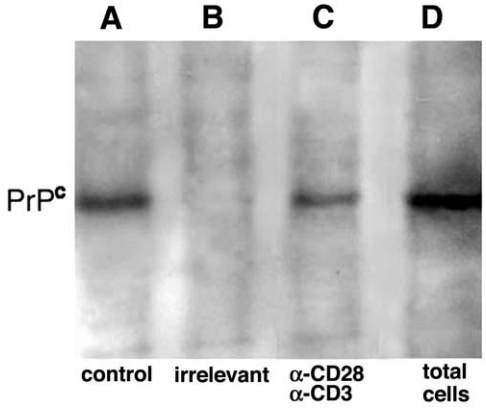

Fig. 4. Coimmunoprecipitation of Fyn and PrP^C. CEM cells were immunoprecipitated with anti-Fyn FYN3. The immunoprecipitates were analyzed by Western blotting with anti-PrP C-20. A: Control cells immunoprecipitated with anti-Fyn. B: Control cells immunoprecipitated with IgG with irrelevant specificity. C: Cells stimulated with anti-CD3 (10 μg/ml) plus anti-CD28 (10 μg/ml), immunoprecipitated with anti-Fyn. D: Total cell lysate.

3.5. PrP^C–ZAP-70 coimmunoprecipitation after CD28/CD3 triggering

To verify the possible PrP^C interaction with ZAP-70, cell-free lysates from anti-CD3- and anti-CD28-treated and untreated cells were immunoprecipitated with the polyclonal anti-PrP, followed by protein A-acrylic beads. Western blot analysis of the immunoprecipitates revealed the presence of a 70 kDa band, recognized by anti-ZAP-70, which was present in the immunoprecipitates from activated cells (Fig. 5C). To confirm the positive band as PrP^C, the anti-ZAP-70 was

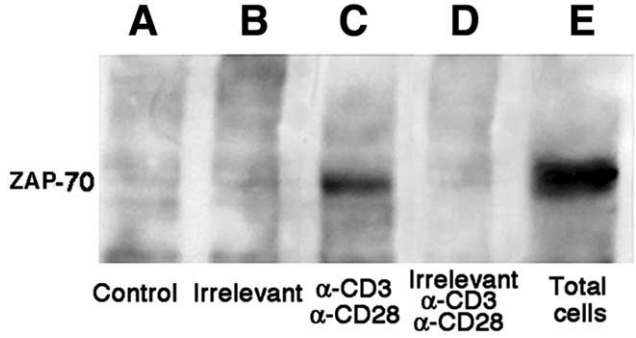

Fig. 5. Coimmunoprecipitation of PrP^C and ZAP-70. CEM cells were treated with anti-CD3 (10 μg/ml) plus anti-CD28 (10 μg/ml) and immunoprecipitated with anti-PrP C-20. The immunoprecipitates were analyzed by Western blotting with anti-ZAP-70. A: Control cells immunoprecipitated with anti-PrP. B: Control cells immunoprecipitated with IgG with irrelevant specificity. C: Cells stimulated with anti-CD3/CD28, immunoprecipitated with anti-PrP. D: Cells stimulated with anti-CD3/CD28, immunoprecipitated with IgG with irrelevant specificity. E: Total cell lysate.

stripped from the nitrocellulose and the membrane was then reprobed with anti-PrP 6H4 MoAb. The results showed the positive band as PrP^C (not shown). In contrast, no bands were detectable in the immunoprecipitates with IgG with irrelevant specificity (Fig. 5B–D) or from untreated cells (Fig. 5A).

4. Discussion

In this study we demonstrated the interaction of PrP^C with the transducer protein ZAP-70 after CD28 and CD3 engagement, strongly suggesting a role for PrP^C as a signaling molecule in lymphocytic cells. Recent insights, obtained in a neuronal differentiation model, indicated that PrP^C may be a signal transduction protein, since it couples to the tyrosine kinase Fyn and is able to increase the phosphorylation levels of the protein [18]. This binding was shown to be caveolin-1-dependent. Thus, following the demonstration that PrP^C is associated with ganglioside molecules within microdomains of human lymphocytic and neuronal cells [14], we investigated PrP^C–Fyn interaction in a T lymphocyte cell model. It could be interesting, since these cells, which might play a potential role in the propagation of the disease, lack caveolin-1 [19]. Until now, only indirect evidence suggested a role for PrP^C in signal transduction in human T lymphocytes [3,8]. On the basis of these findings, we primarily analyzed the distribution of PrP^C in T cells, revealing that the gold immunolabeling was distributed in small clusters. This finding is supported by recent papers, which have shown that prion protein is tightly clustered on neurons by using either monovalent antibody reagents [20] or a strong fixation procedure prior to labeling [21,22]. Moreover, we demonstrated that PrP^C coimmunoprecipitates with monosialoganglioside GM3 and with GM1, a well known raft marker [23]. It indicates that these specialized portions of the cell plasma membrane implicated in the signal transduction pathway of T cells [13,24], already identified in our previous papers [11,12], correspond to lipid rafts even if these cells lack caveolin-1 [19]. These findings confirm and extend, using TLC immunostaining, our preliminary observation on PrP^C–GM3 association [14]. They are also in agreement with the observations revealing that PrP^C, like many GPI-anchored proteins, resides within Triton X-100-insoluble, cholesterol- and sphingolipid-rich rafts in neuronal cells [25].

Moreover, this investigation showed a strict association between PrP^C and Fyn in lymphocytic cells, as revealed by scanning confocal microscopy. In contrast, PrP^C showed a weak colocalization with the monoacylated kinase Src. This finding is in agreement with the observation that acylation, especially multiple acylation, regulates raft localization [26]. Furthermore, a strong PrP^C–Fyn interaction was indicated by the observation that PrP^C was coimmunoprecipitated by anti-Fyn both in untreated cells and after CD28/CD3 triggering. This finding is consistent with the already reported enrichment of Fyn in microdomains of human lymphocytes [24]. However, because Fyn is an intracellular protein but PrP^C is anchored to the outer membrane, the protein interaction is likely to involve intermediate factor(s). In neuronal cells microsequence analysis of Fyn immunoprecipitates prompted the identification of caveolin-1α and caveolin-1β [18]. In our cell system, in the absence of caveolin-1, this structural function is likely to involve ganglioside molecules, as suggested by the results of the present study. Interestingly, in a recent paper, Fyn was not found in coimmunoprecipitates with PrP^C, and does not colocalize in cerebellar granule cells lacking caveolin, as revealed by confocal immunofluorescence microscopy [27]. This discrepancy may be due to the different cell type and in particular to the different ganglioside pattern between the two cell types. Indeed, GM3 is the main ganglioside constituent in T cells, whereas it is present at a very low level in cerebellar granule cells, where it is virtually absent in the 'prion domain'. These findings, together with our observation of PrP^C–Fyn association in CEM cells, strongly supports a possible role for gangliosides (mainly GM3) as components involved in the linking of the extracellular PrP^C to the intracellular Fyn in lymphocytic cells.

From a functional point of view, in cerebellar granule cells the 'prion domain' contains a specific set of signaling components and this restriction would limit access of the components of other signaling pathways (e.g. Fyn) [27]. In lymphocytic cells, where PrP^C is involved in the signaling pathway ending in T cell activation, Fyn and PrP^C may be components of the same transduction pathway.

One of the main novelties of the present study is the demonstration that ZAP-70 was immunoprecipitated by anti-PrP antibody after triggering through CD28 and CD3. ZAP-70 is a Syk family kinase, activated via both a self- and an lck-dependent phosphorylation mechanism [28], which phosphorylates substrates which in turn lead to the subsequent docking and activation of other Src homology 2-containing molecules involved in the amplification and diversification of T cell receptor-initiated signaling. During T cell activation, phosphorylated ZAP-70 translocates from the cytoplasmic compartment to the cell plasma membrane microdomains [29], where it interacts with GM3 [12]. The observation of PrP^C–ZAP-70 coimmunoprecipitation is suggestive of a role for PrP^C in the signaling transduction pathway involved in T cell activation. It is already known that glial cells are stimulated by prion protein to activate tyrosine kinase (Lyn and Syk)-dependent signal transduction cascades [30]. This leads to a transient release of Ca^{2+} that results in activation of protein kinase C and Ca^{2+}-sensitive tyrosine kinase [31]. Additional evidence derives from the strict interaction of PrP^C with the neuronal phosphoprotein synapsin Ib and the adapter protein Grb-2 [32]. Although the physiological role of PrP^C in the signal transduction pathway ending in T cell activation is not understood, PrP^C may act as a membrane receptor transducer and is specifically regulated by cell activation [8]. In addition, PrP^C in human lymphocytes can modulate the cellular response to proliferative signals [3].

Taken together, the results of the present study provide the first evidence that PrP^C interacts with ZAP-70, indicating that it can be considered a component of the multimolecular signaling complex within GEM involved in T cell activation.

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