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PrP^c capping in T cells promotes its association with the lipid raft proteins reggie-1 and reggie-2 and leads to signal transduction

Claudia A. O. Stuermer,* Matthias F. Langhorst,* Marianne F. Wiechers,* Daniel F. Legler,* Sylvia Hannbeck von Hanwehr,* Andreas H. Guse,[†] and Helmut Plattner*

*Department of Biology, University of Konstanz, Konstanz, Germany; [†]Institute of Biochemistry and Molecular Biology I: Cellular Signal Transduction, Center of Experimental Medicine, University Hospital Hamburg-Eppendorf, Hamburg, Germany

Corresponding author: Claudia A. O. Stuermer, Dept. of Biology, University of Konstanz 78457 Konstanz, Germany. E-mail: Claudia.Stuermer@uni-konstanz.de

ABSTRACT

The cellular prion protein (PrP^c) resides in lipid rafts, yet the type of raft and the physiological function of PrP^c are unclear. We show here that cross-linking of PrP^c with specific antibodies leads to 1) PrP^c capping in Jurkat and human peripheral blood T cells; 2) to cocapping with the intracellular lipid raft proteins reggie-1 and reggie-2; 3) to signal transduction as seen by MAP kinase phosphorylation and an elevation of the intracellular Ca²⁺ concentration; 4) to the recruitment of Thy-1, TCR/CD3, fyn, lck and LAT into the cap along with local tyrosine phosphorylation and F-actin polymerization, and later, internalization of PrP^c together with the reggies into limp-2 positive lysosomes. Thus, PrP^c association with reggie rafts triggers distinct transmembrane signal transduction events in T cells that promote the focal concentration of PrP^c itself by guiding activated PrP^c into preformed reggie caps and then to the recruitment of important interacting signaling molecules.

Key words: noncaveolar microdomains • reggie/flotillin • PrP^c cross-linking

The cellular prion protein (PrP^c) is a glycosylated glycosylphosphatidyl inositol (GPI-) anchored protein that is mostly expressed on the surface of neurons and immune cells (1–4). PrP^c has gained considerable attention due to the conversion of α helix to β sheet structures leading to the protease-resistant conformer designated PrP^{sc} (PrP^{sc}) and the spreading of prion disease (1, 4, 5). The physiological function of PrP^c is still under debate: PrP^c has been implicated in cell adhesion, differentiation, copper binding (6), neuroprotection against oxidative stress (7, 8), apoptosis (9), and transmembrane signaling via a lipid raft-based mechanism (10).

Clearly, PrP^c resides in plasma membrane lipid rafts/microdomains (2, 11, 12). Lipid rafts are discussed as platforms for proteins involved in signal transduction, allowing for example GPI-anchored proteins to signal across the plasma membrane (13, 14). Cross-linking of raft proteins

by natural ligands or antibodies (Abs) leads to so-called clustered rafts, ~100–200 nm in size (15, 16) that can be visualized at the light microscopic (LM) level. In fact, an activation of the nonreceptor Src kinase fyn was reported to occur in neurites of a neuroectodermal cell line in a caveolin-1-dependent manner using AB-induced PrP^c cross-linking (10).

Conflicting views exist, however, concerning the association of PrP^c with caveolin-1 and caveolae as opposed to its association with noncaveolar lipid rafts (16), particularly as neurons and lymphocytes lack caveolin-1 and caveolae (14, 17, 18).

The existence of noncaveolar lipid raft microdomains is clearly revealed by the pattern of the two proteins reggie-1 and reggie-2 (18, 19, 20), also known as flotillin-2 and flotillin-1 (22). In lymphocytes, the reggie proteins exhibit a strikingly polarized expression known as “capping” (20, 23, 24). AB-mediated sequestration of GPI-anchored proteins such as Thy-1 results in Thy-1 capping and cocapping with the reggies (20) and seems to involve transmission of signals into the cell (13, 25, 26, 27, reviewed in 14). Signaling leading to full T cell activation with effects on proliferation, cytokine secretion and formation of the immune synapse requires capping of the TCR/CD3, phosphorylation cascades, a long-lasting increase in the cytosolic and nucleoplasmic Ca²⁺ concentration ([Ca²⁺]_i) and cytoskeletal reorganization (28, 29). It is, however, less clear which aspects of the T cell response repertoire are induced by AB-mediated activation and aggregation of GPI-anchored proteins alone. Such analysis is highly relevant from the cell biological point of view to unravel the function of PrP^c. This, in turn, requires identification of its interaction partners and their dynamic association in lipid rafts (10, 11, 30, 31).

Here, we report that AB-mediated PrP^c cross-linking in Jurkat T cells and peripheral blood T lymphocytes leads to capping of PrP^c and its association with the reggies. We demonstrate further that cross-linked PrP^c coclusters with Thy-1, TCR/CD3 complex, fyn, lck, and LAT (linker of activated T cells) in the cap region where an increase of tyrosine phosphorylation and actin polymerization is indicative of signal transduction. This is substantiated by our results showing a brief elevation of [Ca²⁺]_i in response to PrP^c cross-linking, and increased phosphorylation of the MAP kinases ERK 1/2 (extracellular regulated kinases 1/2). Moreover, suppression of the increase in [Ca²⁺]_i by a membrane permeable Ca²⁺ chelator interferes with PrP^c capping. Thus, PrP^c capping triggers signal transduction cascades that involve reorganization of actin and the recruitment of raft-associated signaling molecules, as well as TCR/CD3 to the cap. Furthermore, cross-linked PrP^c and the reggies are incorporated together in globular intracellular structures, including limp-2 positive endosomes/lysosomes showing the close association of PrP^c and reggie at the plasma membrane and during internal trafficking.

METHODS

Cell culture

Jurkat T cells were grown in RPMI 1640 (Invitrogen, Karlsruhe, Germany) containing 10% FCS (Sigma, Deisenhofen, Germany). For immunohistochemistry and confocal laser scanning microscopy Jurkat T cells were centrifuged onto polylysine-coated cover slips. Human peripheral blood lymphocytes (PBL) were isolated from fresh blood of healthy donors by separation on Ficoll-Paque (Pharmacia, Uppsala, Sweden) followed by the depletion of monocytes using anti-CD14 Microbeads (Miltenyi, Bergisch Gladbach, Germany).

Antibodies

Primary antibodies were anti-ESA (Transduction Laboratories, Lexington, KY) recognizing reggie-1 (20, 32), affinity purified anti-reggie-1 pAB and anti-reggie-2 pAB (20), anti-flotillin-1 (reggie-2) mAB and anti-phosphotyrosine mAB (Transduction Laboratories), anti-PrP^c mAB (6H4, Prionics, Zurich, Switzerland), anti-PrP^c pAB (kindly provided by A. Aguzzi, Zurich), anti-Ick pAB and anti-LAT pAB (Biomol, Hamburg, Germany), anti-phospho ERK1/2 (Thr202, Tyr204) pAB and anti-ERK1/2 pAB (Cell Signaling Technology, Beverly, USA), anti-Thy-1 mAB (human AF9; BioTrend, Köln, Germany), anti-CD3 ϵ (M-20), anti-CD55 pAB and anti-fyn pAB (Santa Cruz Biotechnology, Santa Cruz, USA), anti-limp-2 mAB and pAB (33) (kind gift of S. Hoening, University of Göttingen, Germany), FITC-coupled phalloidin (Molecular Probes, Leiden, Netherlands), anti-HRP pABs (Sigma, Munich, Germany). Cross-linking ABs goat anti-mouse IgG and IgM, rat anti-mouse IgG (all Dianova, Hamburg, Germany) and goat anti-rabbit IgG (Southern Biotechnology, Birmingham, Alabama, USA) were “AffiniPure”. AB-gold conjugates for EM analyses were obtained and used as described previously (20).

Determination of intracellular Ca²⁺ concentration

Fura2/AM, BAPTA/AM and Ionomycin were purchased from Calbiochem (Bad Soden, Germany), Concanavalin A from Sigma. OKT3 was purified from hybridoma supernatant on protein G-Sepharose (Amersham Pharmacia, Freiburg, Germany).

Jurkat T lymphocytes were loaded with Fura2/AM as described previously (34) and kept in the dark at room temperature (RT) until use. [Ca²⁺]_i was measured in aliquots of 1×10⁶ Fura2-loaded cells using a Hitachi F-2000 spectrofluorimeter operated in the ratio mode (alternating excitation wavelengths 340 and 380 nm, emission wavelength 495 nm) with gentle stirring. At the end of each experiment the maximal ratio was obtained by addition of Triton-X-100 (0.1%), the minimal ratio by addition of Tris/EGTA (4mM/40mM) and Ca²⁺ concentrations were calculated as described (35).

Isolation of lipid rafts

Lipid rafts were isolated exactly as described previously (36).

Immunoprecipitation experiments and cell lysates

Pelleted cells (10⁷) were homogenized on ice in lysis buffer (1% NP-40, 50 mM Tris, 150 mM NaCl, 2 mM EDTA, pH 7.4 + protease inhibitor cocktail (Complete Mini; Roche, Mannheim, Germany)). The homogenates were cleared by centrifugation.

ABs were coupled to magnetic protein G-beads (15 μ l; Dynal, Hamburg, Germany). Mouse IgG₁ ABs and rabbit preimmune serum served as control. Cell homogenate was added and incubated overnight. Beads were pelleted on a magnetic rack and washed in TBS/1% NP-40 and TBS/0.1% NP-40. Pelleted beads were boiled in SDS-sample buffer.

Alternatively cells were washed in HBSS, starved, and stimulated as indicated. Cells were lysed by addition of 10× lysis buffer (50 mM Hepes, 20 mM EDTA, 10% Triton-X-100, 50 mM MgCl₂ + protease and phosphatase inhibitors (phosphatase inhibitor cocktail, Calbiochem)) on

ice and lysates were cleared by centrifugation. Protein concentration was determined using the Bradford assay.

Gel electrophoresis and immunoblotting

Gel electrophoresis and immunoblotting were performed according to standard procedures using Hybond-C Super nitrocellulose (Amersham Buchler, Braunschweig, Germany) or Immobilon-P (Millipore, Bedford, USA) membranes, enhanced chemoluminescence substrate SuperSignal (Pierce Chemical, Rockford, IL) and ECL hyperfilm (Amersham Buchler).

Immunocytochemistry

mAB and pAB against the surface protein PrP^c were applied to live cells at 4°C for 1 h. Cross-linking of surface-bound PrP^c mAB was achieved by incubating the cells with goat anti-mouse, goat anti-rabbit, or rat anti-mouse ABs, respectively, at 37°C for 5–10 min. After cross-linking of PrP^c, cells were washed and fixed in 4% formaldehyde and permeabilized by immersion in methanol. After washing, nonspecific binding sites were blocked with BSA. The cells were then incubated with primary antibodies in blocking solution (overnight) as detailed below, washed with PBS, and incubated with the appropriate combination of secondary ABs: either donkey anti-rabbit (or anti-mouse, or anti-rat) Cy-3 (Dianova), donkey anti-goat (or goat anti-mouse or goat anti-rabbit) Alexa 488. The ganglioside GM1 was stained using Cholera toxin B subunit (Sigma). FITC-coupled phalloidin was used to stain actin filaments in fixed cells. The cells were mounted using Mowiol containing *n*-propylgallate as an antifading agent. Immunolabeled cells were analyzed by confocal laser scanning microscopy (LSM 510; Zeiss, Oberkochen, Germany) equipped with a Plan-Apochromat ×63 oil immersion objective (n.a. = 1.4). Images were acquired with the LSM 510 software and processed using Photoshop (Adobe Systems, San Jose, CA).

For inhibition experiments, cells were preincubated with BAPTA/AM (50μM final) for 30 min at 37°C.

Electron microscopy

For EM analysis, stimulated and nonstimulated Jurkat T cells, were fixed in 8% of freshly depolymerized formaldehyde + 0.1% glutaraldehyde in PBS pH 7.4, 1 h, 0°C, then overnight in the same fixative at 4°C. They were washed first, 3 × 10 min in PBS on ice, then 3 × 10 min in PBS with 50 mM glycine added, then 2 × 10 min in PBS without glycine. Cells were carefully pelleted by centrifugation and the pellet stirred in 8% gelatin (dissolved in water), followed by centrifugation (2 h, 0°C). Pellets were transferred into PBS supplemented with 2.3 M sucrose and 20% polyvinyl pyrrolidone (PVP10) for impregnation overnight at 4°C. Pellets were mounted on cryo-stabs for freezing in liquid nitrogen and ultrathin cryo-sectioning with a Leica Ultracut E equipped with a FC4 unit. Sections were exposed to mABs against PrP^c followed by goat anti-mouse F(ab)₂ coupled to gold particles; subsequently, sections were exposed to reggie-1 pAB, followed by protein A gold conjugate. Gold conjugates used were either of 6 and 10 nm, respectively (Au₆, Au₁₀), as described previously (20).

Alternatively, a combined pre- and postembedding labeling procedure on plastic section was used. Live cells were exposed to anti-PrP^c mAB (1 h, 4°C) and then exposed to goat anti-mouse F(ab)₂ coupled with Au₁₀ gold (1 h, 37°C) and then fixed in 8% formaldehyde and 0.1% glutaraldehyde in 0.1 M Pipes buffer (with 1 mM CaCl₂, pH 7.2, 0°C, 1 h). Dehydration in ethanol was performed by progressively lowering the temperature, followed by embedding in LR Gold for UV polymerization at -35°C. Labeling on sections was performed with protein A (pA)-Au₅ conjugates after exposure of the sections to anti-reggie Abs and anti-lck pAB.

After washing with double-distilled water, cryosections were stained for 3 min with 2% aqueous uranyl acetate supplemented with methylcellulose, whereas plastic sections were stained with uranylacetate only. Sections were washed again, and analyzed in a Zeiss EM10 (80 kV, 30 µm objective aperture).

RESULTS

Coexistence of PrP^c, reggie-1 and reggie-2, fyn and lck in lipid rafts

To confirm lipid raft-association of PrP^c, the reggie proteins, fyn and lck in Jurkat T cells, we isolated lipid rafts according to standard procedures. Western blot analysis of collected fractions using specific ABs against reggie-1 ([Fig. 1A](#)), reggie-2 ([Fig. 1B](#)), PrP^c ([Fig. 1C](#)), fyn ([Fig. 1D](#)) and lck ([Fig. 1E](#)) revealed localization of all proteins in detergent-insoluble fractions and confirms raft localization of PrP^c and the reggies in lymphocytic cell lines (11, 12).

Application of specific ABs against PrP^c on living Jurkat T cells and confocal microscopy revealed cell surface labeling in small-sized patches with intervening regions with weak or no labeling ([Fig. 2A](#)), which resembled the distribution of Thy-1 ([Fig. 2B, C](#)) (2). Likewise, lck, fyn, LAT, TCR/CD3, and phosphotyrosine were distributed more or less evenly along the plasma membrane and so was F-actin ([Fig. 2D-I](#)). In contrast, reggie-1 and reggie-2 staining was observed at one aspect of the cell (reminiscent of a cap) in about half of the cell population ([Fig. 2J-O](#); and (24)), whereas the other half of the cell population showed a more widespread patchy distribution (20). Furthermore, reggie-1 and reggie-2 proteins were also present both in intracellular small vesicles and larger organelles (see below).

PrP^c cross-linking induces capping and colocalization with reggie-1 and reggie-2

For cross-linking of PrP^c, we exposed live Jurkat T cells to anti-PrP^c mAB and to the polyvalent cross-linking goat anti-mouse secondary AB (20, 24). This induced the concentration of PrP^c in one aspect of the cell, the cap ([Fig. 3Aa-c](#); compare with [Fig. 2](#)), where PrP^c appeared as aggregates in a rather strict accumulation or more widely dispersed (see [Fig. 3A: k, h; 3Bb](#)). Double labeling of cells subjected to PrP^c cross-linking with anti-reggie-1 pAB and anti-reggie-2 pAB, respectively, led to a clear preferential concentration of the PrP^c aggregates in the cap region occupied by the reggies ([Fig. 3Aa-f](#)).

Cross-linked PrP^c colocalizes with Thy-1, CD3, and F-actin in the cap

PrP^c cross-linking and double labeling experiments with ABs against T cell components involved in signal transduction, showed that Thy-1 and CD3 colocalized with PrP^c in the cap

([Fig. 3Ag-l](#)). CD55, another GPI-anchored protein, however, did not accumulate in the cap region ([Fig. 3Bj-l](#)), showing specific aggregation processes within the PrP^c-induced cap

In the PrP^c cap region the Src kinases fyn and lck, and the adaptor protein LAT colocalized with PrP^c ([Fig. 3Am-o](#); [3Ba-c](#) and [g-i](#)) where increased staining for tyrosine-phosphorylated proteins ([Fig. 3Bd-f](#)), and, moreover, an increase of F-actin is apparent ([Fig. 3Ap-r](#)).

Thus, cross-linking of PrP^c triggers signaling steps leading to actin reorganization and to the dynamic rearrangement and close spatial association of various signaling molecules, which enhances signaling efficiency (37, 38).

Coaggregation of PrP^c and reggie in peripheral blood T lymphocytes

When human peripheral blood T lymphocytes were exposed to the same cross-linking and immunostaining procedure employed for the Jurkat T cell line, we observed that roughly 40% of cells showed anti-PrP^c immunolabeling. As demonstrated in [Fig. 4](#), PrP^c undergoes the same dynamic rearrangement and capping in primary T cells and coclusters with reggie-1 and reggie-2 ([Fig. 4a-f](#)), as well as with lck ([Fig. 4g-i](#)) in one aspect of the cell, implying that the same PrP^c-mediated stimulation events also occur in primary T cells.

Colocalization of PrP^c, reggie-1, reggie-2, and lck at the EM level

We examined whether these coclusters of PrP^c and reggies are also apparent at the higher resolution of the electron microscope (EM) using two double immunogold labeling methods on cryosections and plastic sections, each with specific advantages. The distribution of PrP^c and reggie-1 was analyzed in ultrathin sections of Jurkat cells before and after PrP^c cross-linking with anti-PrP^c mAB and anti-reggie-1, anti-reggie-2, and anti-lck pABs ([Fig. 5](#)). [Figure 5B](#) shows coclusters of PrP^c (Au₁₀ gold grains) and reggie-1 (Au₆) in a PrP^c cross-linked cell in a cross section of the plasma membrane. Such coclusters of PrP^c (Au₆) and reggie-1 (Au₁₀) are also apparent in a grazing section along the plasma membrane of PrP^c cross-linked cells ([Fig. 5C](#)). These gold clusters were larger and more closely spaced than those observed for PrP^c in untreated cells ([Fig. 5A](#)) and reggie-1 (not shown), consistent with the dynamic contraction of PrP^c and reggies upon PrP^c cross-linking. In unstimulated cells, both reggie-1 clusters (not shown) and PrP^c clusters ([Fig. 5A](#)) were <0.1 µm but the coclusters in PrP^c cross-linked cells also encompassed regions of >0.1 µm.

Improved ultrastructure is obtained with labeling on plastic sections which, however, is of lower sensitivity with regard to AB labeling. Still, as illustrated in [Fig. 5](#), mixed clusters of Au₁₀ and Au₅ gold grains for the detection of PrP^c and reggie-1 ([Fig. 5D](#)), PrP^c and reggie-2 ([Fig. 5E](#)) and PrP^c and lck ([Fig. 5F](#)) are clearly apparent at the plasma membrane and are in the range of 0.05 – 0.15 µm.

These results substantiate the coaggregation of cross-linked PrP^c with reggie-1 and reggie-2 in the Jurkat T cell cap and exemplify the close association of the intracellular nonreceptor kinase lck with the PrP^c and reggie coclusters.

Coimmunoprecipitation of PrP^c with reggie-1, fyn, and lck

Coimmunoprecipitation experiments and Western blot analyses were carried out to confirm colocalization and biochemical association of the proteins of interest. PrP^c mAB and pAB were used as the precipitating ABs and mouse IgG₁ and rabbit serum, respectively, as controls. As shown in [Fig. 6D](#), PrP^c was precipitated and detected by anti-PrP^c mAB. Moreover, reggie-1 was reliably detected in the PrP^c mAB precipitate (and not in the mouse IgG₁ control, [Fig. 6A](#)). Detection of reggie-1 in the precipitate with PrP^c pAB ([Fig. 6A](#)) was successful after PrP^c cross-linking (performed as described above). The control with rabbit serum ([Fig. 6A](#)) was carried out with the same cross-linking procedure. Furthermore, fyn as well as lck were detected in the precipitate using PrP^c mAB (and not in the control; [Fig. 6B, C](#)). In addition, reggie-1 was detected using lck pAB for precipitation ([Fig. 6A](#)) and is together with reggie-1 and reggie-2 associated with fyn (20). Thus, PrP^c, reggie, fyn, and lck are spatially organized in a complex allowing interactions and signaling across the plasma membrane.

PrP^c cross-linking induces transmembrane signaling

Ligation of the CD3 complex using mAB OKT3 (10 µg/ml), induced a biphasic Ca²⁺ signal consisting of a transient peak with a maximal amplitude of 450 nM ± 140 nM ($n = 6$, ± SDM) and a sustained plateau phase ([Fig. 7A](#)), which is essential for interleukin-2 production and proliferation in vivo (28). Unspecific cross-linking of cell surface glycoproteins by the lectin ConA activated a sustained Ca²⁺ signal similar to that induced by OKT3 but with a less pronounced peak (data not shown). No Ca²⁺ signal was observed after the addition of mAB 6H4 against PrP^c alone (up to a concentration of 10 µg/ml). Interestingly, however, cross-linking of PrP^c by the secondary AB induced a transient increase in [Ca²⁺]_i with a maximal amplitude of 143 nM ± 56 nM ($n = 9$, ± SDM) ([Fig. 7B](#)). [Ca²⁺]_i returned to baseline levels within 250 s ([Fig. 7D](#)), so cross-linking of PrP^c produced a distinct signal which differed from the sustained Ca²⁺ signaling after TCR/CD3 cross-linking. Increasing the concentration of mAB 6H4 (range tested 0.1–10 µg/ml) and/or the cross-linking antibody (range tested 1–20 µg/ml) neither changed the amplitude nor the duration of the Ca²⁺ signal. Addition of the cross-linking AB alone induced no Ca²⁺ signal ([Fig. 7C](#)).

In parallel experiments the phosphorylation status of the MAP kinases ERK1/2 was analyzed in Jurkat T cell lysates. We found an increase in ERK1/2 phosphorylation starting at 5 min after PrP^c cross-linking and lasting for over 20 min ([Fig. 7E](#)) which, however, was modest in comparison to cross-linking of the TCR/CD3 complex by mAB OKT3.

To examine if the emerging Ca²⁺ signal and PrP^c capping are causally linked, cells were exposed to the membrane-permeable Ca²⁺ chelator 2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA/AM) and subjected to PrP^c cross-linking as before. In BAPTA/AM-treated Jurkat T cells, PrP^c accumulation in caps was seen in 27% (±3%) of the cells, whereas 63% (±6%) of the DMSO-treated control cells showed a cap ([Fig. 7F](#)). In BAPTA/AM-treated cells, PrP^c aggregates were distributed along the cell's circumference rather than capped in roughly 60% of the cells ([Fig. 7F](#)). The preformed cap revealed by reggie-1 staining was not affected by BAPTA/AM treatment; it could be observed in about half of the cell population exactly as under control conditions. PrP^c capping was impeded in cells without as well as in cells with a preformed reggie cap ([Fig. 7F](#)). To determine the effects of BAPTA/AM

on the molecules, which were recruited to the cap along with PrP^c after PrP^c cross-linking, we analyzed the localization of Thy-1, lck, and LAT in BAPTA/AM-treated cells after PrP^c cross-linking. All of these molecules failed to concentrate in the cap after PrP^c cross-linking in BAPTA/AM pretreated cells. For example, Thy-1 was distributed in small patches along the cell's circumference under these conditions, surprisingly these patches only partially colocalized with the PrP^c patches ([Fig. 7F](#)).

Therefore, condensation of PrP^c aggregates and other signaling molecules into caps seems to be dependent on the Ca²⁺ signal and its downstream effects, most likely on the actin cytoskeleton as incubation with cytochalasin D prior to PrP^c cross-linking also inhibits PrP^c capping (A. Reuter and C. A. O. Stuermer, unpublished data).

Internalization of PrP^c and reggie-1 and reggie-2

Upon exposure of Jurkat T cells to PrP^c cross-linking procedures for ≥10 min (37°C), a significant proportion of the cells had internalized almost all of the protein into globular intracellular organelles, that is, endosomes. Coimmunostaining on (permeabilized) cells revealed that PrP^c and reggie-1, as well as PrP^c and reggie-2, were to a large extent contained in the same endosomes ([Fig. 8A–F](#)). Optical sections and viewing the labeled structures in the three-dimensional reconstruction confirmed the impression reflected by [Fig. 8C, F](#) that many of the endosomal structures contain all stainings, i.e., reggie-1, reggie-2, and PrP^c. Internalized PrP^c after PrP^c cross-linking colocalized with anti-limp-2 ABs ([Fig. 8G–I](#)) indicating that internalized PrP^c is delivered to late endosomes/lysosomes. Localization to endosomes seems to be an additional characteristic feature of reggie-1 and reggie-2 in Jurkat T cells, since both reggies were colocalized with limp-2 in endosomes/lysosomes ([Fig. 8J–Q](#); compare [Fig. 2](#)), prior to cell activation by AB-cross-linking of GPI-proteins.

DISCUSSION

Our present results reveal that PrP^c activation by AB cross-linking promotes the dynamic assembly of PrP^c and interacting proteins in reggie raft microdomains in T lymphocyte caps. This association and signal transduction by PrP^c in T cells is shown by our microscopic (LM and EM) and biochemical analysis. PrP^c cocapping with the reggies recruits intracellular components involved in T cell signal transduction, that is, fyn and lck, to these sites and promotes an increase of tyrosine phosphorylation of proteins, as well as actin polymerization within the cap, and leads to an increased phosphorylation of the MAP kinases ERK1/2. The accumulation of PrP^c and other signaling molecules in the reggie cap seems to be directed by a distinct brief Ca²⁺ signal, since blockage of this Ca²⁺ signal by BAPTA/AM prevented PrP^c capping but not the formation of aggregates. Thus, PrP^c capping and its cocapping with reggies results in the transmission of instructive signals across the T cell plasma membrane, which is needed to guide PrP^c and its interaction partners to the cap and likely involves the cap-directed actin polymerization. It also effects the redistribution of specific raft components of the cell surface, that is, Thy-1 (but not CD55), and recruits the transmembrane TCR/CD3 complex along with LAT, fyn, and lck to the cap. Thereafter, the internalization of PrP^c and its transfer into reggie-positive endosomes/lysosomes is likely to regulate signaling by degradation or recycling. Together, these findings show that cross-linking of PrP^c and its association with the reggie raft microdomains activates and depends on distinct intracellular signaling cascades.

Our analysis of PrP^c capping and function was performed in Jurkat T cells because they represent a well-studied system demonstrating the induction of signaling cascades in response to lateral clustering of specific GPI-anchored proteins or GM1 by coalescing raft microdomains (25, 39, 40). A critical view on these events shows that the underlying cellular mechanisms are poorly understood and are most frequently discussed in the context of T cell activation (i.e., proliferation, cytokine production, etc.). Clearly, however, several distinct and intermittent steps are required, for instance, to guide proteins from distant positions to a specific region, the cap. According to our present results, one outcome of PrP^c signaling is the spatial concentration of PrP^c itself and of proteins communicating with PrP^c in reggie caps. The lipid raft microdomain proteins reggie-1 and reggie-2 on the cytoplasmic face of the plasma membrane seem to act as scaffolds or as organizing centers (20, 24) for the spatial reorganization of specific GPI-anchored proteins (and other molecules) coupling them to signaling events.

Rafts, PrP^c and the reggies

Mouillet-Richard et al. (2000) reported that PrP^c cross-linking led to fyn activation in a neuroectodermal cell line and this interaction involved caveolin-1. Since T lymphocytes as well as most neurons are devoid of caveolin-1 and caveolae (15, 18), we propose that PrP^c communicates with intracellular effectors of T cell activation across the lymphocyte plasma membrane via reggie microdomains. This, most likely, effects the reorganization of the cytoskeleton.

Consistent with the raft hypothesis, an association between PrP^c, the reggies, lck, and fyn also occurs to some extent without AB-mediated activation of PrP^c—as evidenced by coimmunoprecipitation assays on nonactivated Jurkat T cells. Small aggregates of GPI-linked proteins and Src-type kinases are not resolved at the light microscopic level because they tend to reside in rafts smaller in size (estimated at 50 nm) and comprising fewer molecules than clustered rafts (16, 41). Our EM analysis on nonstimulated cells shows detection of reggie-1 and PrP^c by immunogold in form of distinct small clusters of <0.1 μm . Coclusters of reggie-1 and reggie-2 are recognized more easily as distinct circumscribed microdomains at the EM and even LM level. In neurons, reggie-1 and reggie-2 exhibit a punctate distribution with individual puncta occurring in just the 100 to 200 nm size range before and after activation of GPI-linked proteins, which has led to the view that they preexist as “preformed centers” for the assembly of GPI-anchored proteins before and upon activation (20).

Since cellular membranes possess more than one type of raft, because of regional differences in lipid composition and different raft-dependent functions (42, 43), microscopic analysis is needed to determine the spatial organization of specific raft components before and after capping. It was thereby revealed that PrP^c cross-linking was in fact sufficient for recruiting Thy-1 along with PrP^c to the cap. The GPI-anchored protein CD55, however, did not coalesce with PrP^c after PrP^c cross-linking implying a certain degree of specificity underlying the capping process. Apparently, cells have the ability to sort raft components and to discriminate between stimuli arriving from different cross-linked GPI-anchored proteins.

While blocking the increase in $[\text{Ca}^{2+}]_i$ prevents PrP^c capping, globular PrP^c aggregates nevertheless form in response to AB cross-linking. But instead of being concentrated in the cap, they are distributed around the cell's circumference. It is therefore conceivable that a first signal

emerges from aggregate formation upon AB cross-linking of PrP^c in Jurkat T cells, which is amplified to mediate the condensation of the globular aggregates in the caps depending on the remodeling of the actin cytoskeleton (40, 14). The reggies demarcate the sites where the assembly, signaling, and amplification occur and they seem to communicate with the actin cytoskeleton by direct interaction with the adaptor protein CAP (44, 45). Reggies also seem to function as organizing centers for multiprotein complex formation and communication across the plasma membrane in neurons where they are up-regulated during axon growth and regeneration (18, 19, 46), which requires signaling, dynamic rearrangements of cytoskeletal elements, and membrane transport.

Signaling by PrP^c

It is known that GPI-anchored proteins play an important role in T cell activation. Following the treatment with phosphatidylinositol-specific phospholipase C, the activation of T cells by ConA is impaired but direct activation via the TCR is unaffected (47, 48). Several reports suggest a role of PrP^c in T cell activation: PrP^c expression is up-regulated during T cell activation, and mitogen-induced proliferation was inhibited by ABs against PrP^c (49, 50). More importantly, the ConA-induced proliferation of lymphocytes from PrP^c knockout mice was significantly reduced (51). PrP^c was shown to interact with Grb2, an important regulator of the Ras signaling pathway (52). PrP^c cross-linking on neuronal cell lines induced activation of the Src-kinase fyn (10) and MAPK-activation (53). Moreover, a recent report shows that PrP^c cross-linking induced neuronal apoptosis in vivo (9). Our results show that PrP^c cross-linking leads to the redistribution of PrP^c itself and, more importantly, recruits fyn, lck, CD3, and LAT, molecules important in T cell activation, to the cap region defined by the reggies. PrP^c cross-linking also induces phosphorylation of ERK 1/2. Capping is dependent on dynamic changes of the actin cytoskeleton ((40) and A. Reuter and C. A. O. Stuermer, unpublished data). Our results suggest that these cytoskeletal changes are regulated by a transient intracellular Ca²⁺ signal that is triggered by PrP^c cross-linking, as capping can be blocked by a membrane-permeable, Ca²⁺ chelating compound. The activation of several signaling pathways by PrP^c cross-linking and, more importantly, the concentration of important signaling molecules in the cap might influence the signaling efficiency from the TCR complex thereby modulating T cell responsiveness.

Internalization of PrP^c and the reggies

It has long been appreciated that endocytosis regulates the activity of signaling receptors at the cell surface, and signaling is often turned off by receptor internalization and subsequent degradation in lysosomes (down-regulation) (54). New views propose that GPI-anchored proteins, derived from plasma membrane rafts, are delivered to endocytic compartments along unusual routes, as well as via the clathrin-mediated pathway (55, 56, 57). This may apply to the presently observed joined internalization of PrP^c and the reggies in (limp-2 positive) endosomes/lysosomes, which occurs most markedly after these GPI proteins have coclustered with the lipid raft proteins reggie-1 and reggie-2 at the plasma membrane. The reggies may be indirectly or directly involved in this internalization, especially since reggie-2 (alias flotillin-1) has been implied in intracellular trafficking (44, 58). PrP^c has been shown to be rapidly recycled, and this may involve the reggies and occur via the recently reported lipid raft-based endocytotic pathway (55, 56, 59) or by its transfer from rafts to clathrin-coated pits (57). We can exclude, however, that in T cells PrP^c is internalized by caveolae as suggested by Peters et al. (60).

CONCLUSION AND PERSPECTIVE

Altogether, we have uncovered a novel functional aspect of PrP^c by showing that clustering of PrP^c by AB-mediated cross-linking in Jurkat T lymphocytes, as well as in primary lymphocytes, leads to its redistribution to the cap defined by reggie rafts and to the recruitment of various signaling molecules to the cap. Capping is dependent on a Ca²⁺ signal elicited by PrP^c-clustering, which acts most probably on the actin cytoskeleton. Furthermore, PrP^c cross-linking leads to an activation of the MAP kinases ERK 1/2, and finally to stimulated endocytosis of PrP^c. Taken together all these events may modulate T cell activation.

This could imply that during cell contact formation, PrP^{sc} on the membrane of “infected” cells might act as receptor or ligand for PrP^c on the uninfected cell (61, 62), or even that shedded microparticles exert such functions (63), so that cell activation through PrP^c clustering in reggie rafts and subsequent reggie-associated internalization might contribute to PrP^{sc} propagation.

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REFERENCES

1. Weissmann, C. (1999) Molecular genetics of transmissible spongiform encephalopathies. *J. Biol. Chem.* **274**, 3–6
2. Madore, N., Smith, K. L., Graham, C. H., Jen, A., Brady, K., Hall, S., et al. (1999) Functionally different GPI proteins are organized in different domains on the neuronal surface. *EMBO J.* **18**, 6917–6926
3. Durig, J., Giese, A., Schulz-Schaeffer, W., Rosenthal, C., Schmucker, U., Bieschke, J., et al. (2000) Differential constitutive and activation-dependent expression of prion protein in human peripheral blood leucocytes. *Br. J. Haematol.* **108**, 488–495
4. Aguzzi, A., Montrasio, F., and Kaeser, P. S. (2001) Prions: Health scare and biological challenge. *Mol. Cell. Biol.* **2**, 118–126
5. Prusiner, S. B. (1995) Prions. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 13,363–13,383
6. Brown, D. R., Qin, K., Herms, J. W., Madlung, A., Manson, J., Strome, R., et al. (1997) The cellular prion protein binds copper in vivo. *Nature* **390**, 684–685
7. Brown, D. R. (2001) Prion and prejudice: normal protein and the synapse. *Trends Neurosci.* **24**, 85–90

8. Chiarini, L. B., Freitas, A. R. O., Zanata, S. M., Brentani, R. R., Martins, V. R., and Linden, R. (2002) Cellular prion protein transduces neuroprotective signals. *EMBO J.* **21**, 3317–3326
9. Solfrosi, L., Criado J.R., McGavern D.B., Wirz S., Sanchez-Alavez M., Sugama S. et al. (2004) Cross-linking cellular prion protein triggers neuronal apoptosis in vivo. *Science* 10.1126/science.1094273
10. Mouillet-Richard, S., Ermonval, M., Chebassier, C., Laplanche, J. L., Lehmann, S., Launay, J. M., et al. (2000) Signal transduction through prion protein. *Science* **289**, 1925–1928
11. Vey, M., Pilkuhn, S., Wille, H., Nixon, R., DeArmond, S. J., Smart, E. J., et al. (1996) Subcellular colocalization of the cellular and scrapie prion proteins in caveolae-like membranous domains. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 14,945–14,949
12. Naslavsky, N., Stein, R., Yanai, A., Friedlander, G., and Taraboulos, A. (1997) Characterization of detergent-insoluble complexes containing the cellular prion protein and its scrapie isoform. *J. Biol. Chem.* **272**, 6324–6331
13. Friedrichson, T., and Kurzchalia, V. (1998) Microdomains of GPI-anchored proteins in living cells revealed by cross-linking. *Nature* **394**, 802–805
14. Simons, K., and Toomre, D. (2000) Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* **1**, 31–39
15. Simons, K., and Ikonen, E. (1997) Functional rafts in cell membranes. *Nature* **387**, 569–572
16. Simons, K., and Ehehalt, R. (2002) Cholesterol, lipid rafts, and disease. *J. Clin. Invest.* **110**, 597–603
17. Fra, A. M., Williamson, E., Simons, K., and Parton, R. G. (1994) Detergent-insoluble glycolipid microdomains in lymphocytes in the absence of caveolae. *J. Biol. Chem.* **269**, 30,745–30,748
18. Lang, D. M., Lommel, S., Jung, M., Ankerhold, R., Petrausch, B., Laessing, U., et al. (1998) Identification of reggie-1 and reggie-2 as plasma membrane-associated proteins which cocluster with activated GPI-anchored cell adhesion molecules in non-caveolar micropatches in neurons. *J. Neurobiol.* **37**, 502–523
19. Schulte, T., Paschke, K. A., Laessing, U., Lottspeich, F., and Stuermer, C. A. O. (1997) Reggie-1 and reggie-2, two cell surface proteins expressed by retinal ganglion cells during axon regeneration. *Development* **124**, 577–587
20. Stuermer, C. A. O., Lang, D. M., Kirsch, F., Wiechers, M. F., Deininger, S., and Plattner, H. (2001) Glycosylphosphatidyl inositol-anchored proteins and fyn kinase assemble in noncaveolar plasma membrane microdomains defined by reggie-1 and -2. *Mol. Biol. Cell* **12**, 3031–3045

21. Neumann-Giesen, C. B., Falkenbach, C. S., Beicht, P., Claasen, S., Lüers, G., Stuermer, C. A. O., et al. (2004) Membrane and raft association of reggie-1/flotillin2: role of myristoylation, palmitoylation and oligomerization. Induction of filopodia by overexpression. *Biochem. J.* **378**, 509–518
22. Bickel, P. E., Scherer, P. E., Schnitzer, J. E., Oh, P., Lisanti, M. P., and Lodish, H. F. (1997) Flotillin and epidermal surface antigen define a new family of caveolae-associated integral membrane proteins. *J. Biol. Chem.* **272**, 19,793–19,802
23. Montixi, C., Langlet, C., Bernard, A. M., Thimonier, J., Dubois, C., Wurbel, M. A., et al. (1998) Engagement of T cell receptor triggers its recruitment to low-density detergent-insoluble membrane domains. *EMBO J.* **17**, 5334
24. Rajendran, L., Masilamani, M., Solomon, S., Tikkanen, R., Stuermer, C. A. O., Plattner, H., et al. (2003) Asymmetric localization of flotillins/reggies in preassembled platforms confers inherent polarity to hematopoietic cells. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 8241–8246
25. Harder, T., Scheiffele, P., Verkade, P., and Simons, K. (1998) Lipid domain structure of the plasma membrane revealed by patching of membrane components. *J. Cell Biol.* **141**, 929–942
26. Hooper, N. M. (1999) Detergent-insoluble glycosphingolipid/cholesterol-rich membrane domains, lipid rafts and caveolae. *Mol. Membr. Biol.* **16**, 145–156
27. Janes, P. W., Ley, S. C., and Magee, A. I. (1999) Aggregation of lipid rafts accompanies signaling via the T cell antigen receptor. *J. Cell Biol.* **147**, 447–461
28. Lewis, R. S. (2001) Calcium signaling mechanisms in T lymphocytes. *Annu. Rev. Immunol.* **19**, 497–521
29. Nel, A. E. (2002) T-cell activation through the antigen receptor. Part 1: Signaling components, signaling pathways, and signal integration at the T-cell antigen receptor synapse. *J. Allergy Clin. Immunol.* **109**, 758–770
30. Shyng, S.-L., Heuser, J. E., and Harris, D. A. (1994) A glycolipid-anchored prion protein is endocytosed via clathrin-coated pits. *J. Cell Biol.* **125**, 1239–1250
31. Kaneko, K., Vey, M., Scott, C., Pilkuhn, S., Cohen, F. E., and Prusiner, S. B. (1997) COOH-terminal sequence of the cellular prion protein directs subcellular trafficking and controls conversion into the scrapie isoform. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 2333–2338
32. Schroeder, W., Stewart-Galetka, S., Perry, D. A., Goldsmith, D., and Duvic, M. (1994) Cloning and characterization of a novel epidermal cell surface antigen (ESA). *J. Biol. Chem.* **269**, 19,983–19,991
33. Vega, M. A., Segui-Real, B., Garcia, J. A., Cales, C., Rodrigues, F., Vanderkerckhove, J., et al. (1991) Cloning, sequencing, and expression of a cDNA encoding rat LIMP II, a novel 74

- kDa lysosomal membrane protein related to the surface adhesion protein CD36. *J. Biol. Chem.* **266**, 16,818–16,824
34. Guse, A. H., Roth, E., and Emmrich, F. (1993) Intracellular Ca^{2+} pools in Jurkat T lymphocytes. *Biochem. J.* **291**, 447–451
 35. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**, 3440–3450
 36. Legler, D. F., Micheau, O., Doucey, M. A., Tschopp, J., and Bron, C. (2003) Recruitment of TNF receptor 1 to lipid rafts is essential for TNF alpha-mediated NF-kappaB activation. *Immunity* **18**, 655–664
 37. Harder, T., and Simons, K. (1999) Clusters of glycolipid and glycosylphosphatidylinositol-anchored proteins in lymphoid cells: accumulation of actin regulated by local tyrosine phosphorylation. *Eur. J. Immunol.* **29**, 556–562
 38. Föger, N., Marhaba, R., and Zöller, M. (2001) Involvement of CD44 in cytoskeleton rearrangement and raft reorganization in T cells. *J. Cell Sci.* **114**, 1169–1178
 39. Brown, D. A., and London, E. (1998) Functions of lipid rafts in biological membranes. *Annu. Rev. Cell Dev. Biol.* **14**, 111–136
 40. Dykstra, M., Cherukuri, A., Sohn, H. W., Tzeng, S.-J., and Pierce, S. K. (2003) Location is everything: Lipid rafts and immune cell signaling. *Annu. Rev. Immunol.* **21**, 457–481
 41. Dietrich, C., Volovyk, Z. N., Levi, M., Thompson, N. L., and Jacobson, K. (2001) Partitioning of Thy-1, GM1, and cross-linked phospholipid analogs into lipid rafts reconstituted in supported model membrane monolayers. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 10,642–10,647
 42. Gómez-Moutón, C., Abad, J. L., Mira, E., Lacalle, R. A., Gallardo, E., Jiménez-Baranda, S., et al. (2001) Segregation of leading-edge and uropod components into specific lipid rafts during T cell polarization. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 9642–9647
 43. Schuck, S., Honsho, M., Ekroos, K., Shevchenko, A., and Simons, K. (2003) Resistance of cell membranes to different detergents. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 5795–5800
 44. Baumann, C. A., Ribon, V., Kanzaki, M., Thurmond, D. C., Mora, S., Shigematsu, S., et al. (2000) CAP defines a second signaling pathway required for insulin-stimulated glucose transport. *Nature* **407**, 202–207
 45. Kioka, N., Ueda, K., and Amachi, T. (2002) Vinexin, CAP/ponsin, ArgBP2: A novel adaptor protein family regulating cytoskeletal organization and signal transduction. *Cell Struct. Funct.* **27**, 1–7

46. Deininger, S.-O., Rajendran, L., Lottspeich, F., Przybylski, M., Illges, H., Stuermer, C. A. O., et al. (2003) Identification of teleost Thy-1 and association with the microdomain/lipid raft reggie proteins in regenerating CNS axons. *Mol. Cell. Neurosci.* **22**, 544–545
47. Presky, D. H., Low, M. G., and Shevach, E. M. (1990) Role of phosphatidylinositol-anchored proteins in T cell activation. *J. Immunol.* **144**, 860–868
48. Thomas, L. J., DeGasper, R., Sugiyama, E., Chang, H.-M., Beck, P. J., Orlean, P., et al. (1991) Functional analysis of T-cell mutants defective in the biosynthesis of glycosylphosphatidyl anchor. *J. Biol. Chem.* **266**, 23,175–23,184
49. Cashman, N. R., Loertscher, R., Nalbantoglu, J., Shaw, I., Kascak, R., Bolton, D. C., et al. (1990) Cellular isoform of the scrapie agent protein participates in lymphocyte activation. *Cell* **61**, 185–192
50. Li, R., Liu D., Zanusso G., Liu T., Fayen J. D., Huang V., et al. (2001) The expression and potential function of cellular prion protein in human lymphocytes. *Cell. Immunol.* **207**, 49–58
51. Mabbott, N. A., Brown, K. L., Manson, J., and Bruce, M. E. (1997) T-lymphocyte activation and the cellular form of the prion protein. *Immunology* **92**, 161–165
52. Spielhaupter, C., and Schaetzel, H. M. (2001) PrP^C directly interacts with proteins involved in signaling pathways. *J. Biol. Chem.* **276**, 44,604–44,612
53. Schneider, B., Mutel, V., Pietri, M., Ermonval, M., Mouillet-Richard, S., and Kellermann, O. (2003) NADPH oxidase and extracellular regulated kinases 1/2 are targets of prion protein signaling in neuronal and nonneuronal cells. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 13,326–13,331
54. Felberbaum-Corti, M., Gisou, M., Van Der Groot, F. G., and Gruenberg, J. (2003) Sliding doors: clathrin-coated pits or caveolae? *Nat. Cell Biol.* **5**, 382–384
55. Chatterjee, S., Smith, E. R., Hanada, K., Stevens, V. L., and Mayor, S. (2001) GPI anchoring leads to sphingolipid-dependent retention of endocytosed proteins in the recycling endosomal compartment. *EMBO J.* **20**, 1583–1592
56. Fivaz, M., Vilbois, F., Thurnheer, S., Pasquali, C., Abrami, L., Bickel, P. E., et al. (2002) Differential sorting and fate of endocytosed GPI-anchored proteins. *EMBO J.* **21**, 3989–4000
57. Sunyach, C., Jen, A., Deng, J., Fitzgerald, K. T., Frobert, Y., Grassi, J., et al. (2003) The mechanism of internalization of GPI-anchored prion protein. *EMBO J.* **22**, 3591–3601
58. Morrow, I. C., Rea, S., Martin, S., Prior, I. A., Prohaska, R., Hancock, J. F., et al. (2002) Flotillin-1/Reggie-2 traffics to surface raft domains via a novel Golgi-independent pathway; identification of a novel membrane targeting domain and a role for palmitoylation. *J. Biol. Chem.* **277**, 48,834–48,841

59. Nichols, B. J. (2002) A distinct class of endosome mediates clathrin-independent endocytosis to the Golgi complex. *Nat. Cell Biol.* **4**, 374–378
60. Peters, P. J., Mironov, A., Peretz, D., van Donselaar, E., Leclerc, E., Erpel, S., et al. (2003) Trafficking of prion proteins through a caveolae-mediated endosomal pathway. *Proc. Natl. Acad. Sci. U.S.A.* **162**, 703–717
61. Chesebro, B. (1999) Prion protein and the transmissible spongiform encephalopathy diseases. *Neuron* **24**, 503–506
62. Chesebro, B. (1998) BSE and prions: uncertainties about the agent. *Science* **279**, 42–43
63. Baron, G. S., Wehrly, K., Dorward, D. W., Chesebro, B., and Caughey, B. (2002) Conversion of raft associated prion protein to the protease-resistant state requires insertion of PrP-res (PrP^{Sc}) into contiguous membranes. *EMBO J.* **21**, 1031–1040

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Fig. 1

Figure 1. Lipid raft association of PrP^c, reggie-1, reggie-2, fyn, and lck. Unstimulated Jurkat T cells were lysed in 1% Triton-X 100 and subjected to sucrose density centrifugation to isolate lipid rafts. Fractions were analyzed by Western blotting. **A, B)** Reggie-1 and reggie-2 are detected in the lipid raft fractions 3–5 as well as in the soluble fractions (6 to 11). **C)** PrP^c is found selectively in the lipid raft fractions (3 and 4). **D, E)** Fyn and lck are localized to the lipid raft fractions and in the detergent soluble fractions. Molecular weight markers appear to the right.

Fig. 2

Figure 2. Labeling of cell surface PrP^c, Thy-1, intracellular reggie-1, reggie-2, F-actin, lck, fyn, CD3, LAT, and phosphotyrosine in unstimulated Jurkat T cells. *A–C*) Exposure of live cells to anti-PrP^c (*A*, green) and anti-Thy-1 (*B*, red) ABs results in punctate and patchy cell surface labeling, with regions of substantial double labeling (*C*, yellow) but no caplike condensations were apparent without AB-mediated cross-linking (compare **Figure 3**). *D – I*) F-actin (*A*c), detected by phalloidin (*D*), lck (*E*), fyn (*F*), CD3 (*G*), phosphotyrosine (*H*) and LAT (*I*) are distributed along the cells circumference (but compare **Figure 3**). *J–L*) Immunostainings with anti-reggie-1 (*J*, green) and anti-reggie-2 (*K*, red) reflect the plasma membrane-associated punctate and in many cells (caplike) concentrated accumulation of the reggie proteins and show a substantial degree of colocalization (m, yellow). Intracellular dispersed vesicle-like staining and clusters of endosomal structures (arrowheads; see text) in which reggie-1 and reggie-2 are partially colocalized exist in addition to the plasma membrane-associated distribution. *M–O*) A proportion of the Jurkat T cells exhibit per se a caplike condensation of both reggie-1 (*N*, red) and reggie-2 (*N*, green) at the plasma membrane where both are colocalized (*O*, yellow). Intracellular endosome-like clusters (see text) are partially double labeled by reggie-1 and -2 (arrowhead).

Fig. 3

Fig. 3 (cont)

Figure 3. Jurkat T cell capping induced by PrP^c cross-linking and colocalization with reggie-1 and -2, Thy-1, CD3, LAT, fyn, lck, F-actin, and phosphotyrosine residues of proteins. The middle vertical row of images shows PrP^c after cross-linking by specific primary and multivalent secondary ABs (PrP^cX) in living cells (green); arrowheads mark labeled and double-labeled sites in the cap. **Aa–Af)** AB-mediated cross-linking of PrP^c leads to the condensation of PrP^c in globular aggregates in caps (**b, e**) which are also labeled by reggie-1 (**a**, red) and reggie-2 (**d**, red), so that PrP^c and reggie-1 (**c**) and PrP^c and reggie-2 (**f**) are colocalized to a significant extent. **g–i)** After AB cross-linking of PrP^c and PrP^c accumulation in the cap (**h**), Thy-1 is detected in the same restricted cap region (**g**, red) and co-localized with PrP^c to a substantial extent (**i**). **j–l)** PrP^c condensed into globular aggregates after cross-linking (**l**, green) exhibits a significant degree of colocalization with CD3 (**j**, red), which shows a similar contraction into globular aggregates as PrP^c, and so both are colocalized to a significant degree (**l**). **m–o)** Anti-lck staining (**m**) coclusters with PrP^c (**n**) in the cap upon PrP^c cross-linking. (**n, o**). **p, q, r)** F-actin (Ac) detected by phalloidin (**p**), increases in the cap after PrP^c cross-linking (**q, r**). **Ba–c)** Anti-fyn staining is increased in the region of the cap (**a**, red) that is occupied by PrP^c (**b**) and colocalization is evident (**c**). **d–f)** Anti-phosphotyrosine (PTY) immunolabeling (**d**, red) is partially colocalized in the cap region occupied by PrP^c after cross-linking (**e, f**). **g–i)** Anti-LAT staining (**g**) is clustered in the cap of cross-linked PrP^c (**h, i**). **j–l)** CD55 (**j**) is not contracted to the cap upon PrP^c cross-linking (**k, l**).

Fig. 4

Figure 4. PrP^c capping and cocapping with reggie and lck in peripheral blood lymphocytes. (A–I) mAB-induced PrP^c cross-linking results in capping of PrP^c (B, E, H), and partial colocalization with reggie-1 (A–C), reggie-2 (D–F) and lck (G–I).

Fig. 5

Figure 5. EM colocalization of PrP^c, reggie, and lck. *A–C*) Cryosections in orientations from perpendicular (*A*) to almost parallel (*C*) to the cell surface. Scale bars, 0.1 μ m. *A*) Au₆ gold (black arrowheads), detecting PrP^c in unstimulated Jurkat T cells occurs in small distinct clusters, <0.1 μ m, along the plasma membrane. *B*) Au₁₀ gold (black arrowhead), detecting PrP^c after cross-linking, and Au₆ gold labels (white arrowheads) detecting reggie-1, occur as small (<0.1 μ m) or larger (>0.1 μ m) coclusters (encircled) along the plasma membrane. *C*) Au₆ gold (black arrowheads), detecting PrP^c after cross-linking and Au₁₀ (white arrowheads) detecting reggie-1, are seen as coclusters (roughly 0.1 μ m across), here in a grazing section along the cell surface. *D–F*) Detection of coclusters of PrP^c and reggie-1 (*D*), reggie-2 (*E*), and lck (*F*) in plastic sections using Au₁₀ gold (black arrowheads) for the detection of PrP^c after cross-linking and Au₅ gold (white arrowheads) for reggie-1, reggie-2, and lck, respectively. Scale bars, 0.1 μ m.

Fig. 6

Figure 6. Coimmunoprecipitation of PrP^c, reggie-1, lck, and fyn and Western blot analysis of the proteins coimmunoprecipitated by anti-PrP^c ABs from Jurkat cell lysates. The ABs used for detection are indicated above the blots, ABs used for coimmunoprecipitation below each lane. **A)** Anti-reggie-1 mAB detects 47 kD reggie-1 (arrow) after coimmunoprecipitation with PrP^c mAB (and reggie-1 mAB as control). The band above 47 kD reggie-1 is due to the detection of the heavy chain (HC, asterisk) resulting from the use of mABs for precipitation and detection. Anti-reggie-1 mAB also detects reggie-1 in the precipitate after application of PrP^c pAB and cross-linking ABs. Coimmunoprecipitation with mouse IgG₁ and preimmune rabbit antiserum (AS) served as controls and shows, with IgG₁, the HC only. Reggie-1 is also detected after coimmunoprecipitation with lck pAB. **B)** Anti-fyn pAB detects 57 kD fyn in the PrP^c mAB precipitate; **C)** anti-lck pAB detects 55 kD lck. The mouse IgG₁ and rabbit AS controls are blank. **D)** Anti-PrP^c mAB detects the PrP^c specific bands after precipitation with anti-PrP^c mAB. HC, LC, heavy chain and light chain detection (asterisks) due to the use of mABs.

Fig. 7

Fig. 7 (cont)

Figure 7. Ca^{2+} -signaling and activation of the MAP kinases ERK 1/2 by PrP^c cross-linking. A–D) Ligation of the TCR/CD3 complex by OKT3 induces a biphasic Ca^{2+} signal with a transient peak ($\Delta = 450 \text{ nM} \pm 140 \text{ nM}$, $n = 6$) and a long lasting plateau (A). Addition of the PrP^c mAB 6H4 ($2 \mu\text{g/ml}$) alone induces no Ca^{2+} signal, but cross-linking by a secondary goat-anti-mouse antibody induces a transient peak ($\Delta = 143 \text{ nM} \pm 56 \text{ nM}$, $n = 9$) (B). No change in $[\text{Ca}^{2+}]_i$ is observable after the addition of the cross-linking antibody alone (C) (representative tracings of 5–9 independent experiments). A direct comparison of mean $[\text{Ca}^{2+}]_i$ during the peak ($t = 80 \text{ s}$ after stimulation) and at 250 s after stimulation reveals that the Ca^{2+} signal after PrP^c cross-linking was transient, $[\text{Ca}^{2+}]_i$ returned to baseline levels within 250 s (D, data are mean $\pm$ SD). **E)** PrP^c cross-linking induced a slow but sustained phosphorylation of ERK1/2, which was modest in comparison with cross-linking of the TCR/CD3 complex serving as a positive control. The blot was stripped and reprobed with anti-ERK1/2 as loading control. **F)** Inhibition of Ca^{2+} -signaling by preincubation with BAPTA/AM blocked PrP^c redistribution to the cap region after AB-mediated cross-linking in both cells with and without a preformed reggie cap. The percentage of cells showing a preformed reggie cap was unaffected by BAPTA/AM treatment. Likewise Thy-1 failed to concentrate in the cap after BAPTA/AM pretreatment and was widely distributed. PrP^c and Thy-1 rarely formed coclusters under these conditions.

Fig. 8

Figure 8. Internalization of PrP^c and reggie-1 and reggie-2. Arrowheads mark proteins in intracellular structures including endosomes. **A–F)** Cells exposed to PrP^c cross-linking (>10 min) internalize PrP^c into round endosome-like structures (**B**, **E**, green). Similarly reggie-1 (**A**, red) and reggie-2 (**D**, red) are internalized and then no longer detectable at the plasma membrane. Intracellular endosome-like structures are double-labeled by anti-PrP^c and anti-reggie-1 (**C**) and anti-reggie-2 (**F**), respectively. **G–I)** Internalized PrP^c after cross-linking (**H**, green) is partially colocalized (**I**) with anti-limp-2 AB (**G**, red), in late endosomes/lysosomes. **J–O)** Intracellular reggie-1 (**J**, red) and reggie-2 (**M**, red) containing endosome-like structures are partially double-labeled by anti-limp-2 (**K**, green; **N**, green) and thus identified as late endosomes.