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Regulation of focal adhesion formation and filopodia extension by the cellular prion protein (PrPC)

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While the prion protein (PrP) is clearly involved in neuropathology, its physiological roles remain elusive. Here, we demonstrate PrP functions in cell–substrate interaction in *Drosophila* S2, N2a and HeLa cells. PrP promotes cell spreading and/or filopodia formation when overexpressed, and lamellipodia when downregulated. Moreover, PrP normally accumulates in focal adhesions (FAs), and its downregulation leads to reduced FA numbers, increased FA length, along with Src and focal adhesion kinase (FAK) activation. Furthermore, its overexpression elicits the formation of novel FA-like structures, which require intact reggie/flotillin microdomains. Altogether, PrP modulates process formation and FA dynamics, possibly via signal transduction involving FAK and Src.

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Filopodia/lamellipodia
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1. Introduction

The physiological role of the cellular prion protein (PrPC, henceforth called "PrP") remains poorly understood. However, it is thought to play roles in neuroprotection, cell adhesion and signal transduction [1,2]. Our previous results using T cells have revealed that PrP can induce signal transduction in association with reggie/flotillin microdomains [3], which are thought to serve as platforms for the assembly of multiprotein signalling complexes [4]. For instance, reggies are known to interact with Src tyrosine kinases [5], the adaptor proteins CAP and vinexin [6], and actin [7]. In addition, we recently showed that reggies are required for the recruitment of CAP to focal adhesions (FAs) [8] and the regulation of cell morphology [8,9].

Given the close association of PrP and reggies, we here investigated possible functions of PrP in cell–substrate interaction, FA and process formation. To this aim, *Drosophila* S2, mouse N2a and human HeLa cells were used to study the effect of PrP overexpression and downregulation on cell–substrate interaction. Our results uncover new roles of PrP in cell spreading and process extension. Remarkably, PrP modulates FA dynamics and the formation of FA-like structures, which seem to depend on reggie.

2. Materials and methods

2.1. Reagents and antibodies

Cell culture reagents were purchased from Gibco BRL (Germany). Antibody information is provided under Supplementary material.

2.2. Plasmids

The cloning procedures for the PrP, EGFP-PrP, EGFP-GPI, DsRed-PrP, and ECFP-R1EA constructs are described in Supplementary material. The rat reggie-1-HA construct has been described previously [10]. The GFP-paxillin and β3-integrin-GFP vectors were kindly provided by Yamada and Imhof, respectively.

2.3. Cell culture, transfection, and siRNA

N2a and HeLa cells were cultured in MEM supplemented with 10% FCS, L-glutamine, pyruvate and penicillin/streptomycin, and transfected using Lipofectamine 2000 (Invitrogen). S2 cells cultured in Schneider's Medium supplemented with 10% FCS,

Abbreviations: PrP, prion protein; FA, focal adhesion; FAK, focal adhesion kinase; TIRFM, total internal reflection fluorescence microscopy; R1EA, reggie-1 EA construct; siRNA, small interfering RNA.

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L-glutamine and penicillin/streptomycin, were transfected with Effectene (Qiagen). Duplexed small interfering RNAs (siRNAs) were transfected at 100 pmol/ml. The target sequences for mouse and human PrPs were: 5-CTGATTGAAGGCAACAGGAAA-3 and 5-CAGCAAATAACCATTGGTTAA-3, respectively (Qiagen). siRNA against firefly luciferase (GL2, Dharmacon) served as control.

2.4. Spreading assay

S2 cells were transfected for 24 h, seeded on coverslips coated with alcian blue, poly-lysine, laminin or fibronectin (Sigma–Aldrich) for 1 h, and stained with phalloidin. Cell area (excluding filopodia) was scored from 20 randomly selected fields (>80 cells).

2.5. Fluorescence, filopodia and FA quantification

N2a cells were transfected for 24–48 h, seeded on poly-lysine coated coverslips for 24 h, and stained with anti-GFP or anti-PrP antibodies (non-permeabilizing conditions), or with phalloidin or anti-paxillin antibody (permeabilizing conditions). The membrane expression levels of our constructs were controlled by measuring anti-GFP indirect fluorescence (50 cells/construct). Filopodial length was scored in two independent experiments (50 single cells). FA number and length were scored in four independent experiments (>200 cells).

2.6. Microscopy

N2a, HeLa and S2 cells were immunostained as previously described [7], and visualized using a Plan-Apochromat 63×/1.4 objective in a confocal microscope (LSM510 META) and/or Axioplan2 equipped with an AxioCam HRm (widefield images). For total internal reflection fluorescence microscopy (TIRFM), the TIRF slider system and a α-Plan Fluar 100×/1.45 objective were used with an Axiovert 200M (all Zeiss). Images were processed using the Axiovision 4.6 or LSM510 softwares (Zeiss).

2.7. Immunoblotting

N2a cells were lysed using ice-cold lysis buffer (20 mM Tris–HCl pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA, 1% Triton X-100, 10% Glycerin) supplemented with protease and phosphatase inhibitor cocktails (Calbiochem). Hundred microgram proteins were loaded per lane and immunoblotted as previously reported ($n = 4$) [8].

3. Results and discussion

3.1. PrP induces cell spreading and filopodia formation

To assess if PrP affects cell–substrate interaction, we used *Drosophila* S2 cells, a PrP-negative non-adhesive cell line routinely used to characterize adhesion molecules. Upon transfection, PrP-expressing cells showed remarkable spreading on alcian blue, and produced abundant filopodia (Fig. 1A). These effects were also observed upon expression of an EGFP-PrP fusion protein but not of the EGFP-GPI control construct (Fig. S1). Since PrP interacts with laminin but not fibronectin [11], we performed spreading assays on these substrates. For all substrates tested, the area covered by cells expressing non-tagged PrP or EGFP-PrP was 4-fold larger than that of control cells (transfected with EGFP-GPI or non-transfected; Fig. 1B). Thus, heterologous PrP expression in S2 cells triggers cell spreading and filopodia extension in a substrate-independent manner.

3.2. PrP levels regulate process formation

The results obtained in S2 cells prompted us to analyze if PrP would also affect the morphology of mammalian cells. For this, we used mouse neuroblastoma N2a cells, a cell line frequently employed for the characterization of PrP cellular properties. Upon expression of EGFP-PrP, the spontaneous levels of spreading in N2a cells were not altered (data not shown). However, we observed a significant increase in the number of filopodia >10 μm length (11.2 ± 3.3 filopodia/cell), as well as in the length of the longest filopodium (18.4 ± 2.8 μm), compared to EGFP-GPI transfected cells (2.0 ± 1.3 filopodia/cell and 11.4 ± 2.7 μm, respectively) (Fig. 2A–D). Interestingly, PrP often accumulated in filopodial tips (Fig. 2A). Corresponding controls showed that both EGFP-fusion proteins were efficiently expressed (Fig. S2) and equally presented on the plasma membrane (Fig. S3A–E), and also that the endoge-

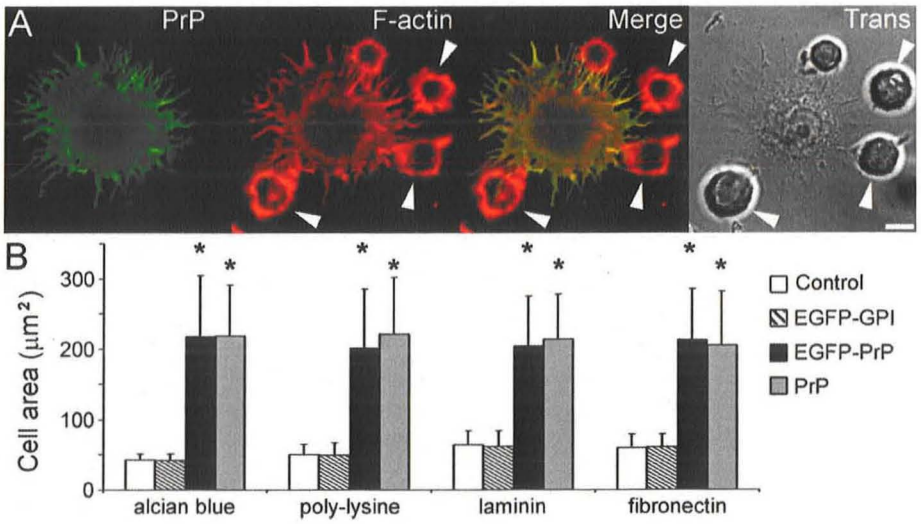

Fig. 1. PrP induces spreading and filopodia formation in S2 cells. (A) Transfected S2 cells were transferred to alcian blue coated coverslips, and stained with anti-PrP antibody (green) and phalloidin to visualize F-actin (red). Confocal images of a cell expressing non-tagged PrP show increased cell area and formation of filopodia compared to non-transfected cells (arrowheads). Scale bar: 5 μm. (B) Quantification of the area shows that S2 cells expressing non-tagged PrP or EGFP-PrP spread equally well on alcian blue, poly-lysine, laminin or fibronectin coated coverslips. Non-transfected (Control) and transfected cells expressing EGFP-GPI do not spread under these conditions. Values shown are the mean ± S.D. $^{*}P < 0.05$, One-Way ANOVA.

nous PrP expression was not affected by the EGFP-GPI construct (Fig. S2F and G). Thus, PrP overexpression positively influences filopodia formation and extension. To strengthen this observation, we used siRNAs to knockdown PrP in N2a cells (>95% efficiency; Fig. S4). Indeed, siRNA-treated cells showed significantly fewer filopodia but extensive lamellipodial veils (Fig. 2E) compared to control, wild type or PrP-overexpressing cells (Fig. 2A, B and E). Rescue experiments performed by co-transfecting siRNA and EGFP-PrP (lacking the siRNA binding site) considerably reverted this abnormal cell morphology (Fig. 2F). Altogether, these results indicate that the surface levels of PrP expression regulate process formation: PrP absence elicits lamellipodia formation and increased PrP levels induce filopodia formation and extension.

3.3. PrP is a component of FAs

The PrP morphological phenotypes observed in S2 and N2a cells strongly suggested an involvement of PrP in cell–substrate interaction. Since FAs play a pivotal role in cell–cell matrix interactions, we took advantage of TIRFM to analyze the localization of PrP at the cell–substrate interface using FA markers such as paxillin and vinculin. Non-transfected N2a cells grown on poly-lysine exhibited few paxillin/PrP-positive FAs (data not shown). Notably, overexpression of EGFP-PrP, but not EGFP-GPI, induced strong accumulation of EGFP-PrP at discrete dotted and streak-shaped structures (Fig. 3A and B, and Fig. S5A). While some of these structures co-localized with paxillin, some others did not (Fig. 3A), and were therefore termed FA-like structures. To further analyze the presence of PrP in FAs, we immunostained human epithelial HeLa cells, which form quite prominent FAs. In non-transfected cells, we observed a distinct accumulation of endogenous PrP at vinculin-positive FAs on poly-lysine (Fig. 3C), laminin and fibronectin (data not shown). Particularly, PrP-positive FAs were localized at the end of stress fibers (Fig. 3D, and Fig. S6G) and exhibited additional FA markers, such as β3-integrin, pFAK, pSrc, and reggie-1 (Fig. S6A–F). PrP accumulation in FAs was not observed in HeLa cells treated with siRNA, confirming the specificity of the immunostaining (Fig. S7). Like in N2a cells, overexpression of EGFP-PrP, but not of EGFP-GPI, resulted in the appearance of FA-like structures in HeLa cells (Fig. 3E and F, and Fig. S5B), some of which partially co-localized with paxillin (Fig. 3E). Moreover, in both cell types PrP often resided at the distal end of FAs, much like pERK in fibroblasts [12], suggesting a signalling role of PrP in these structures. Altogether, these data indicate that PrP is a component of FA and that novel FA-like structures are induced upon PrP overexpression.

The PrP/paxillin-positive FAs observed here appear to represent "classical" FAs. On the other hand, the paxillin-negative FA-like structures might reflect different maturational states of FAs. Alternatively, they may constitute functionally distinct FAs, as it is known that FAs can differ in size and protein composition [13]. Hence, PrP may be present in "exploratory" and early FA-complexes with high turnover rates, and may thus play a role in the exploratory activity of filopodial tips.

3.4. PrP regulates FA dynamics

To determine if PrP regulates characteristic FA properties, we quantitatively analyzed paxillin-positive FAs in N2a cells. Interestingly, PrP downregulation caused a significant reduction in FA number (~30%) along with an increase in the size of the longest FA per cell (Table 1). Moreover, while most of the siRNA-treated N2a cells showed an increase in the number of large FAs (>1 μm), the majority of PrP-overexpressing cells exhibited higher numbers of small FAs (<1 μm; Table 1). These data indicate that PrP can affect FA number and size. Because the size of FAs reflects differences in their turnover rates [14], we speculated that PrP might also regulate FA turnover. Accordingly, N2a cells overexpressing PrP showed higher turnover rates of GFP-paxillin FAs compared to PrP downregulated cells (Videos S1A and B).

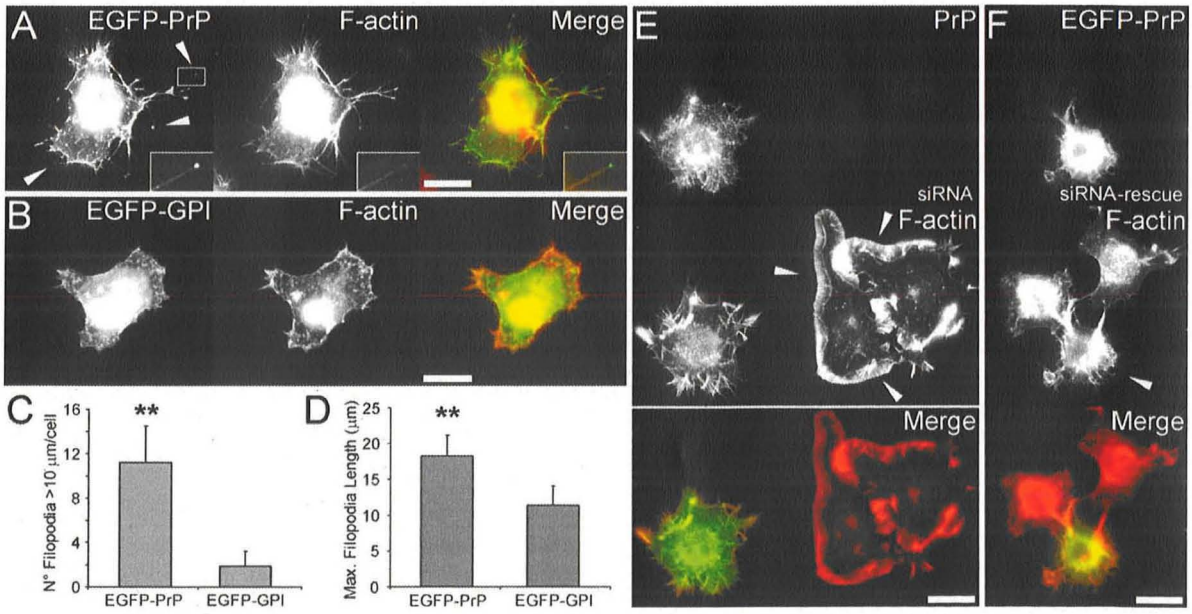

Fig. 2. PrP-dependent filopodia extension in N2a cells. (A and B) N2a cells were transfected for 24 h, transferred to poly-lysine coated coverslips for 24 h, and stained with phalloidin (red). Widefield images of cells expressing EGFP-PrP (A), but not EGFP-GPI (B), show long filopodia with accumulation of PrP at filopodia tips (arrowheads, magnification fields). (C and D) N2a cells, prepared as above, were used to quantify the effects of PrP overexpression on filopodia number (C) and length (D). Values shown are the mean ± S.E.M. $^{**}P < 0.001$, Mann–Whitney Rank Sum Test. (E and F) N2a cells were transfected with siRNA against PrP (E) and co-transfected with EGFP-PrP (F) for 48 h, transferred to poly-lysine coated coverslips for 24 h, and stained with anti-PrP (green in E) and phalloidin (red). Widefield images show a strong reduction of filopodia number and length, and lamellipodia formation upon PrP downregulation (arrowheads in E). siRNA-mediated effects on filopodia and lamellipodia formation are reverted by EGFP-PrP co-transfection (arrowhead in F). Scale bars: 10 μm.

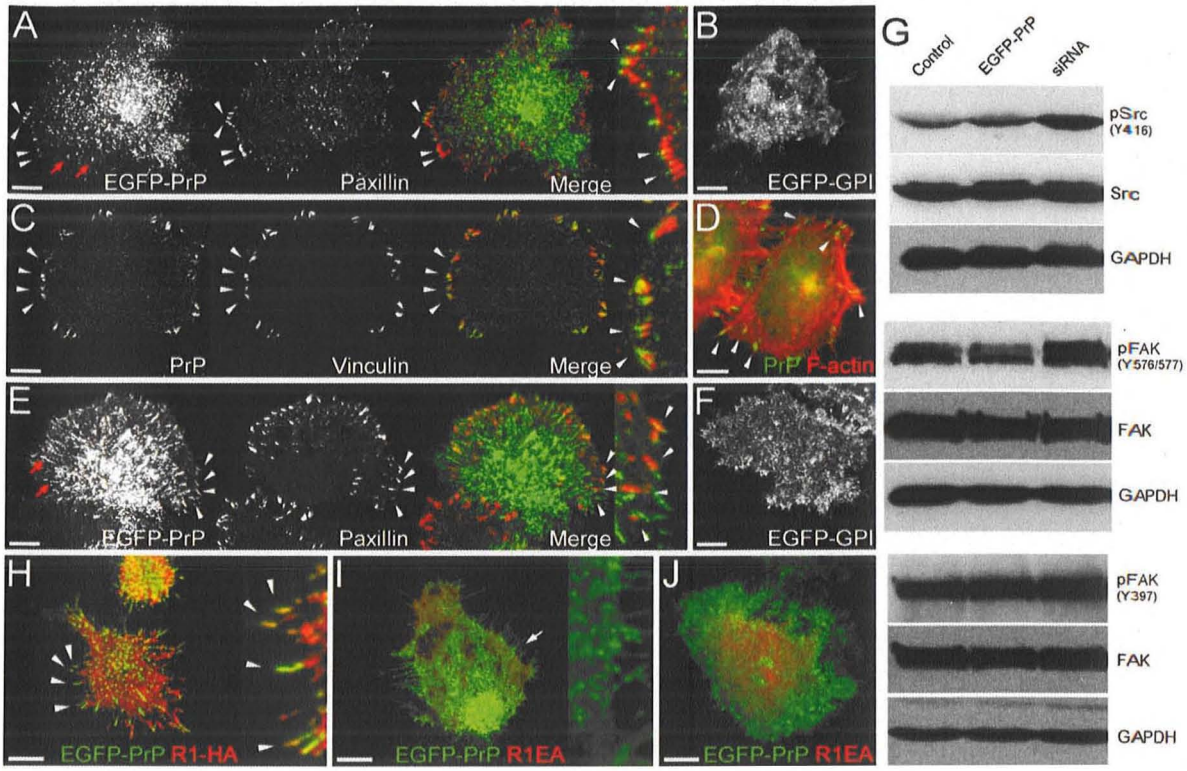

Fig. 3. PrP resides in FAs. (A and B) N2a cells were prepared as described in Fig. 2, stained with anti-paxillin antibody (red) and visualized by TIRFM. Expression of EGFP-PrP (A), but not EGFP-GPI (B), induces the formation of FA-like structures (red arrows). PrP localizes at distal ends of FAs (arrowheads, magnification field in A). (C and D) Non-transfected HeLa cells were grown on poly-lysine coated coverslips, and stained with antibodies against PrP (green) and vinculin (red in C) or phalloidin (red in D). TIRFM images show that endogenous PrP co-localizes with vinculin in FAs (arrowheads in C). A merged widefield image shows the localization of endogenous PrP at the tips of stress fibers (arrowheads in D). (E and F) Transfected HeLa cells, prepared as N2a cells in Fig. 2, were analyzed by TIRFM. Overexpression of EGFP-PrP (E), but not of EGFP-GPI (F), induces formation of FA-like structures (red arrows in E). Note that in HeLa cells endogenous PrP and EGFP-PrP also localize at the distal ends of FAs (arrowheads in magnification fields in C and E). (G) Immunoblots from N2a cells transfected with EGFP empty vector (control), EGFP-PrP or siRNA against PrP (siRNA). The signals of phosphorylated Src (pSrc Y416) and FAK (pFAK Y576/577), but not pFAK Y397, increase upon PrP downregulation. (H) N2a cells were transfected with the EGFP-PrP and reggie-1-HA vectors, cultured as above, stained with anti-HA antibody (red) and recorded by TIRFM. PrP and reggie-1 clearly co-localize in FA-like structures (arrowheads). (I and J) EGFP-PrP FA-like structures observed by TIRFM are lost upon co-expression of the DSRed-R1EA construct in N2a (I) and HeLa (J) cells (region with arrow is enlarged in I). Scale bars: 10 μm.

Since Src kinase activation and the subsequent phosphorylation of focal adhesion kinase (FAK) by Src are involved in the regulation of FA turnover [15], we examined if PrP affects the phosphorylation states of both kinases. In fact, immunoblots using phospho-specific antibodies revealed a significant increase in the levels of pSrc and pFAK Y576/577 upon PrP downregulation (Fig. 3G, and Fig. S8); the levels of FAK autophosphorylation at Y397 were not affected. Taken together, these results indicate that PrP modulates FA dynamics, possibly by regulating Src and FAK phosphorylation.

3.5. PrP-dependent FA-like structures require reggie microdomains

We have shown that reggie microdomains are involved in PrP clustering and signalling [3], as well as in the activation of Rho-family GTPases and the recruitment of CAP to FAs [8]. Therefore, we examined the contribution of reggie to the formation of PrP-dependent FA-like structures. In N2a cells, EGFP-PrP clearly co-localized with reggie-1 in FA-like structures (Fig. 3H). Notably, in N2a and HeLa cells the formation of PrP FA-like structures was completely abrogated by co-expression of a reggie-1 trans-negative construct (R1EA) (Fig. 3I and J, and Fig. S9), indicating that intact reggie microdomains are necessary for this process.

Altogether, the present data is consistent with our view [4] that PrP requires reggie microdomains for its communication with signal transduction pathways involving Src, FAK, CAP and small GTPases, here regulating filopodia formation and FA dynamics. Interestingly, we have additionally observed that PrP expression induces S2 cell clustering, along with the concomitant accumulation of PrP and reggies at contact sites (G.P. Solis, unpublished), which suggests a role of these molecules in cell contact formation.

Table 1
PrP-mediated regulation of FA number and size.

	EGFP	EGFP-PrP	EGFP-GPI	siRNA PrP	siRNA GL2
FAs/100 μm^2	1.59 ± 0.47	1.46 ± 0.49	1.46 ± 0.44	1.12 ± 0.38*	1.57 ± 0.50
% Of cells with mostly small FAs (<1 μm)	50.66 ± 0.83	67.72 ± 3.00**	48.62 ± 3.46	34.79 ± 1.97**	50.59 ± 1.08
% Of cells with mostly large FAs (>1 μm)	49.34 ± 0.83	32.28 ± 3.00**	51.38 ± 3.46	65.21 ± 1.97**	49.12 ± 1.08
Longest FA (μm)	2.32 ± 0.41	2.32 ± 0.47	2.35 ± 0.41	2.78 ± 0.48*	2.37 ± 0.46

Paxillin-postive FAs were analyzed from N2a cells transfected with EGFP empty vector (EGFP), EGFP-PrP, EGFP-GPI, siRNA against PrP (siRNA PrP) or control siRNA (siRNA GL2). Cells were prepared as described in Fig. 2. Values shown are the mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.001$ (One-Way ANOVA).

Therefore, we propose that PrP is part of a multiprotein complex centered on reggies and their signalling partners, which initiates the formation of specific membrane domains such as the T cell cap, cell–cell contacts and here cell–substrate contacts.

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References

[1] Aguzzi, A., Sigurdson, C. and Heikenwaelder, M. (2008) Molecular mechanisms of prion pathogenesis. Annu. Rev. Pathol. 3, 11–40.
[2] Linden, R., Martins, V.R., Prado, M.A., Cammarota, M., Izquierdo, I. and Brentani, R.R. (2008) Physiology of the prion protein. Physiol. Rev. 88, 673–728.
[3] Stuermer, C.A.O., Langhorst, M.F., Wiechers, M.F., Legler, D.F., Von Hanwehr, S.H., Guse, A.H. and Plattner, H. (2004) PrPc capping in T cells promotes its association with the lipid raft proteins reggie-1 and reggie-2 and leads to signal transduction. FASEB J. 18, 1731–1733.
[4] Stuermer, C.A.O. and Plattner, H. (2005) The 'lipid raft' microdomain proteins reggie-1 and reggie-2 (flotillins) are scaffolds for protein interaction and signalling. Biochem. Soc. Symp. 72, 109–118.
[5] Liu, J., DeYoung, S.M., Zhang, M., Dold, L.H. and Saltiel, A.R. (2005) The stomatin/prohibitin/flotillin/HflK/C domain of flotillin-1 contains distinct sequences that direct plasma membrane localization and protein interactions in 3T3-L1 adipocytes. J. Biol. Chem. 280, 16125–16134.
[6] 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.
[7] Langhorst, M.F., Solis, G.P., Hannbeck, S., Plattner, H. and Stuermer, C.A.O. (2007) Linking membrane microdomains to the cytoskeleton: regulation of the lateral mobility of reggie-1/flotillin-2 by interaction with actin. FEBS Lett. 581, 4697–4703.
[8] Langhorst, M.F., Jaeger, F.A., Mueller, S., Hartmann, L.S., Luxenhofer, G. and Stuermer, C.A.O. (2008) Reggies/flotillins regulate cytoskeletal remodeling during neuronal differentiation via CAP/ponsin and Rho GTPases. Eur. J. Cell Biol. 87, 921–931.
[9] Langhorst, M.F., Reuter, A., Luxenhofer, G., Boneberg, E.M., Legler, D.F., Plattner, H. and Stuermer, C.A.O. (2006) Preformed reggie/flotillin caps: stable priming platforms for macrodomain assembly in T cells. FASEB J. 20, 711–713.
[10] Solis, G.P., Hoegg, M., Munderloh, C., Schrock, Y., Malaga-Trillo, E., Rivera-Milla, E. and Stuermer, C.A.O. (2007) Reggie/flotillin proteins are organized into stable tetramers in membrane microdomains. Biochem. J. 403, 313–322.
[11] Hajj, G.N., Lopes, M.H., Mercadante, A.F., Veiga, S.S., da Silveira, R.B., Santos, T.G., Ribeiro, K.C., Juliano, M.A., Jacchieri, S.G., Zanata, S.M. and Martins, V.R. (2007) Cellular prion protein interaction with vitronectin supports axonal growth and is compensated by integrins. J. Cell Sci. 120, 1915–1926.
[12] Fincham, V.J., James, M., Frame, M.C. and Winder, S.J. (2000) Active ERK/MAP kinase is targeted to newly forming cell–matrix adhesions by integrin engagement and v-Src. EMBO J. 19, 2911–2923.
[13] Zamir, E. and Geiger, B. (2001) Molecular complexity and dynamics of cell–matrix adhesions. J. Cell Sci. 114, 3583–3590.
[14] Zaidel-Bar, R., Ballestrem, C., Kam, Z. and Geiger, B. (2003) Early molecular events in the assembly of matrix adhesions at the leading edge of migrating cells. J. Cell Sci. 116, 4605–4613.
[15] Webb, D.J., Donais, K., Whitmore, L.A., Thomas, S.M., Turner, C.E., Parsons, J.T. and Horwitz, A.F. (2004) FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. Nat. Cell Biol. 6, 154–161.