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Clustering of cellular prion protein induces ERK1/2 and stathmin phosphorylation in GT1-7 neuronal cells

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Abstract The physiological role of the prion protein is largely unknown. Here, clustering of prion at the surface of GT1-7 cells was observed upon anti-prion antibody treatments. This clustering was associated with a rapid and transient phosphorylation of the mitogen activated protein kinases (MAPKs) extracellular receptor kinases 1 and 2 (ERK1/2), and also of the microtubule-destabilizing protein stathmin at serine 16. The specificity of this antibody-mediated activation was ascertained by its inhibition by prion small interfering RNA. The phosphorylation of ERK1/2 but not that of stathmin was abolished by the MAPK/ERK kinase 1 inhibitor U0126, whereas both signaling pathways were blocked by the specific inhibitor of the epidermal growth factor receptor AG1478, suggesting the likely recruitment of this receptor upon prion clustering.

Keywords: EGF receptor; MAP kinase; Clustering; Prion signaling; Stathmin; siRNA

1. Introduction

The causing agent of the transmissible spongiform encephalopathies is thought to be a protein called PrP^{sc} which represents an abnormally conformed 'scrapie' isoform of the cellular prion protein (PrP^{c}) [1]. PrP^{c} is a ubiquitous glycoprotein highly expressed in neurons, mostly anchored at the cell surface via a C-terminal glycosylphosphatidylinositol (GPI) moiety. Mice devoid of PrP^{c} ($PrP^{-/-}$) develop normally and are resistant to scrapie inoculation [2] but, like conditional post-natal knockout mice [3], showed no major phenotype that could unravel a clear physiological function of PrP^{c}. The best described property of PrP^{c} is its high affinity for Cu(II), which suggests that PrP^{c} could participate in copper metabolism and protection against oxidative stress [4]. Indeed, primary cultures of neurons from $PrP^{-/-}$ mice exhibit increased susceptibility to oxidative stress [5]. PrP^{c} has also been proposed to be involved in neurite outgrowth and neuronal survival in cell cultures of primary neurons [6]. Moreover, PrP^{c} localization in 'lipid rafts' is compatible with roles in cell adhesion and/or transmembrane signaling [7]. Consistent with these hypotheses, several cellular partners of PrP^{c} have been proposed, including the extracellular matrix protein laminin, laminin receptors and the neural cell adhesion molecule (NCAM) [8,9], but to date there is no functional ligand of PrP^{c} which has been clearly identified and widely accepted. Therefore, antibody cross-linking has been used to identify PrP^{c} induced signaling pathways in differentiated murine 1C11 neuronal cells, revealing a caveolin-dependent coupling of PrP^{c} to the tyrosine kinase Fyn [10]. Recently, the same group also showed the production of NADPH oxidase-dependent reactive oxygen species (ROS) and the phosphorylation of extracellular receptor kinases 1 and 2 (ERK1/2) in 1C11, in GT1-7 neurohypothalamic and BW5147 lymphoid cells [11]. These results suggest a function of PrP^{c} in cell-redox homeostasis through ROS production. In addition, PrP^{c} antibody-mediated cross-linking in vivo was lately found to trigger rapid and extensive apoptosis in hippocampal and cerebellar neurons, further arguing for PrP^{c} functions in the control of neuronal survival [12]. The physiological relevance of PrP^{c} antibody cross-linking remains to be demonstrated but these studies strongly support a role of PrP^{c} in the transduction of intracellular signaling pathways. As it is a GPI-anchored protein, PrP^{c} very likely associates with a transmembrane protein to transduce signals.

In this study, we searched for intracellular targets activated after PrP^{c} antibody-mediated cross-linking in GT1-7 neurohypothalamic cells. In particular, we studied the phosphorylation of stathmin, a tubulin-binding protein regulated by phosphorylation at four serine residues by several protein kinases [13–15]. Treatment of GT1-7 cells with the anti-prion SAF34 and SAF61 monoclonal antibodies (mAbs) resulted in clustering of PrP^{c} at the cell surface as well as in the induction of at least two independent signaling pathways. Our results suggest that PrP^{c} activation might be coupled with the control of microtubule dynamics, but also that the transmembrane tyrosine kinase EGF receptor could be a functional partner of PrP^{c}.

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Abbreviations: EGFR, epidermal growth factor receptor; ERK, extracellular receptor kinase; GPI, glycosylphosphatidylinositol; mAb, monoclonal antibody; MAPK, mitogen activated protein kinase; MKK, MAPK/ERK kinase; PBS, phosphate-buffered saline; PrP^{c}, cellular prion protein; PrP^{sc}, scrapie prion protein; ROS, reactive oxygen species; siRNA, small interfering RNA

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2. Materials and methods

2.1. Cell culture and small interfering RNA transfection

Mouse GT1-7 cells [16] were grown at 37 °C, 5.5% CO_2, in Dulbecco's modified Eagle's medium with 4.5 g/l glucose (DMEM, Life Technologies, Cergy-Pontoise, France) supplemented with 10% v/v fetal bovine serum (FBS, Life Technologies) and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin, Life Technologies). For small interfering RNA (siRNA) transfection, cells were plated at 6×10^4 cells/cm^2 and transfected 24 h after plating with the Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen, Cergy-Pontoise, France). Briefly, 2 μl Lipofectamine 2000 was mixed with siRNAs in 100 μl of Opti-MEM medium and, after 30 min at room temperature, was added to the cells without antibiotics. Antibody treatments were performed 48 h after transfection on serum-deprived cells. PrP^c siRNA used for prion silencing and the scramble dsRNA sequence used as a control were as in [17] and were synthesized by Proligo (Paris, France).

2.2. Antibody treatments and cell lysis

Cells were grown in 12-well plates and, at 60–70% confluence, were serum starved for 18 h before treatment with purified mouse anti-PrP^c mAbs SAF34, SAF61 [18] or anti-HA-Tag mAb 12CA5 [19] at 10 μg/ml in the presence of 0.1% bovine serum albumin (BSA cell culture tested, Sigma, St. Quentin, France) for 10 min at 37 °C. Control cells were treated with BSA alone. When indicated, cells were incubated with inhibitors (U0126, 10 μM, Cell signaling technology, or AG1478, 100 nM, Calbiochem) for 20 min at 37 °C prior to antibody treatment. After treatment, the culture medium was removed and the cells were washed twice with ice-cold phosphate-buffered saline (PBS) before being lysed in 100 μl loading buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, bromophenol blue).

2.3. Electrophoresis and immunoblot analysis

Samples diluted in loading buffer were heated for 5 min at 95 °C and loaded onto a 13% SDS–PAGE gel. Electrotransfer and immunoblot analysis were performed as described previously [20]. The primary antibodies used were the following: mouse monoclonal anti-P-ERK1/2 (clone mitogen activated protein kinase (MAPK)-YT, Sigma), rabbit anti-ERK1/2 (Upstate), mouse monoclonal anti-PrP^c SAF83 [20] and rabbit anti-phosphostathmin at serine 16 [14]. Immunoblots were developed using the ECL kit (Amersham) and quantification was performed with the SCION software (Scion Corporation). All experiments were repeated three times independently and statistical analyses were performed using Student's t tests: * corresponds to $P < 0.05$.

2.4. Immunofluorescence

GT1-7 cells were grown on glass coverslips coated with poly-D,L-ornithin (0.75 μg/cm^2, Sigma, St. Louis, MO). After antibody treatments for 10 min, the cells were washed with cold PBS and fixed at 4 °C for 30 min with 2% formaldehyde/30 mM sucrose and for 20 min in methanol. Cells were washed in PBS and blocked for 1 h with 3% BSA (fraction V, PAA, Linz, Austria). Control cells were incubated after fixation with purified anti-PrP^c mAb SAF34 or SAF61 (10 μg/ml) for 1 h at room temperature and washed with PBS. PrP^c immunolocalization was revealed with alexafluor 546-conjugated goat anti-mouse immunoglobulins (Molecular Probes Europe, Leiden, Holland) incubated for 1 h at room temperature. After extensive washes, the slides were mounted in mowiol 4-88 (Calbiochem, La Jolla, CA) and observed under a confocal microscope (TCS, Leica) set on sequential mode with 200 nm optical sections. Images were analyzed with the MetaMorph software (Universal Imaging).

3. Results

3.1. Anti-prion antibody treatment induces clustering of PrP^c at the surface of GT1-7 cells

We first analyzed by immunofluorescence the effect of PrP^c antibody-mediated cross-linking on its distribution at the cell surface of GT1-7 cells which, as we showed previously, express high levels of endogenous PrP^c [20]. We used two mouse monoclonal anti-prion antibodies, SAF34, directed against the flexible octarepeat region of PrP^c (amino acids 59–89), and SAF61, recognizing PrP^c residues 144–152. Serum-deprived GT1-7 cells were treated in culture with the relevant mAb (at 10 μg/ml for 10 min), then fixed without permeabilization, and PrP^c localization was revealed with fluorescent secondary antibodies. The immunostaining specificity of the anti-PrP^c antibodies was verified on primary cortical neuron cultures from PrP$^{-/-}$ mice, where they revealed no staining (not shown). Immunofluorescence after anti-PrP^c antibody treatments of GT1-7 cells revealed the formation of small clusters of PrP^c at the surface of the cells, which were not detected on non-treated control cells immunostained with the same mAbs after fixation (Fig. 1). These results show that the SAF34 and SAF61 mAbs induce a clustering of PrP^c at the surface of GT1-7 cells. We thus further used these mAbs and, in particular, SAF34, which had not been used for such studies before, to characterize the effect of anti-PrP^c treatment on intracellular signaling in GT1-7 cells.

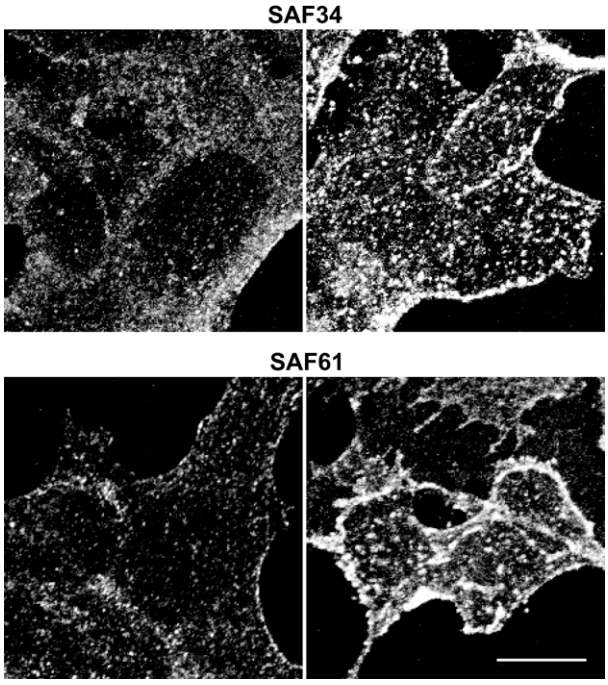

Fig. 1. PrP^c immunofluorescence staining on control GT1-7 cells (left), or after treatment in culture with the SAF34 or SAF61 mAbs (10 μg/ml) for 10 min (right) (see Section 2). PrP^c immunolocalization was revealed with Alexa 546-conjugated goat anti-mouse immunoglobulins. Images were acquired and processed using the same protocol with a confocal microscope and represent a projection of stacks. Bar: 10 μm.

3.2. PrP^c siRNA knockdown inhibits anti-PrP^c antibody induced activation of ERK1/2

We assessed the specificity of the responses of GT1-7 cells to anti-PrP^c antibody treatments by inhibiting the expression of the PrP^c protein with PrP^c specific siRNA transfection (Fig. 2). The expression of PrP^c was followed by Western blot analysis revealing the typical, highly glycosylated isoforms pattern described in GT1-7 cells [20]. We used previously described siRNA duplexes specific for mouse PrP^c (PrP^c siRNA), as well as scrambled double stranded RNA (dsRNA) unrelated

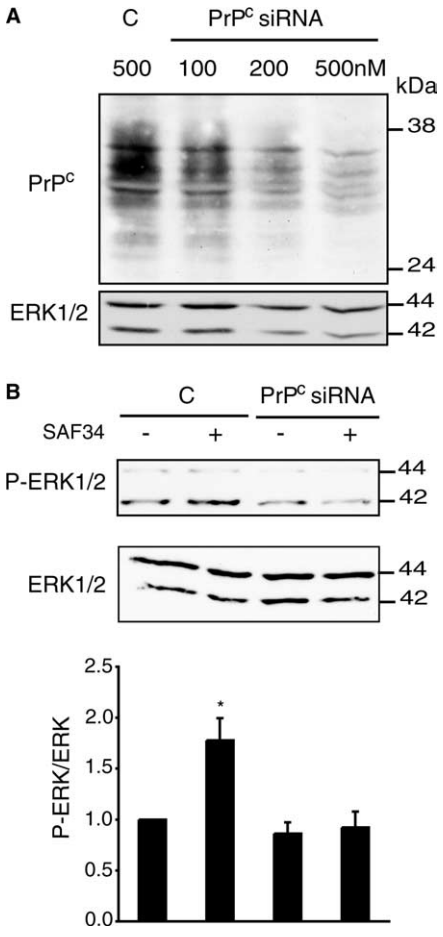

Fig. 2. (A) Dose-dependent siRNA silencing of PrP^c: anti-PrP^c immunoblot with the SAF83 mAb, 48 h after transfection of GT1-7 cells with various concentrations of PrP^c siRNA, compared to control dsRNA (C). (B) Cells transfected with either control dsRNA or PrP^c siRNA duplexes were treated for 10 min without mAb (−) or with (+) the SAF34 mAb (10 μg/ml). ERK1/2 phosphorylation was revealed with anti-P-ERK1/2 antibodies and total ERK was used as a loading control of the gel. Quantification shows an increase of P-ERK1/2 in cells transfected with the control dsRNA and treated with the SAF34 mAb and no activation when the cells are transfected with PrP^c siRNA. Quantification and statistical analyses were performed on three independent experiments (*$P < 0.05$).

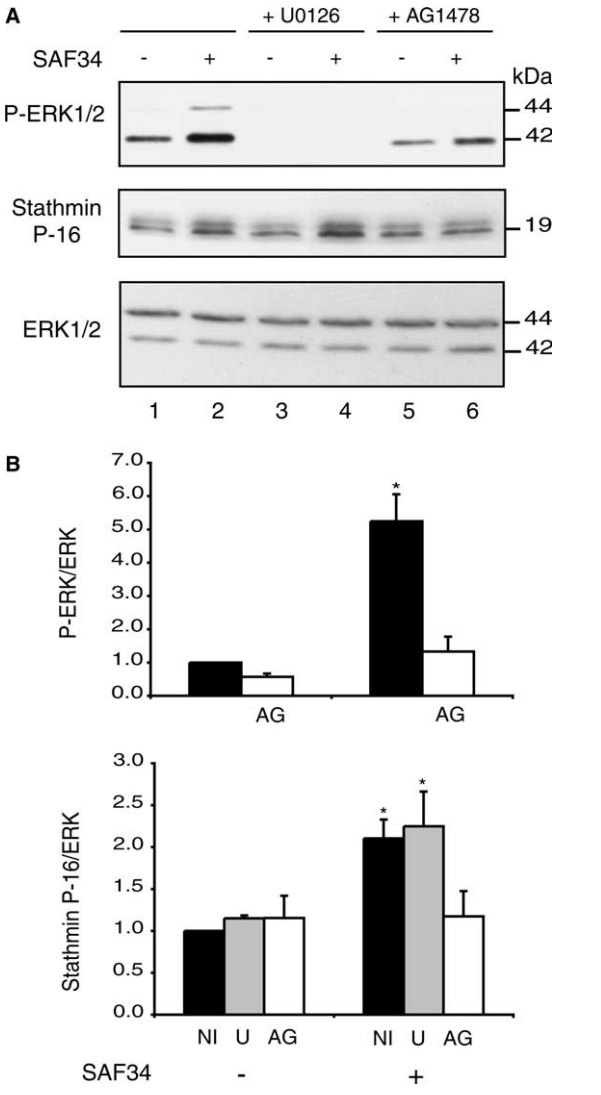

Fig. 3. Serum deprived GT1-7 cells were treated for 10 min without mAb (−) or with (+) the SAF34 mAb (10 μg/ml). When indicated, the MKK1 inhibitor U0126 (10 μM) or the EGFR inhibitor AG1478 (100 nM) was added to the cells 20 min prior to antibody treatment. (A) Immunoblot analyses of phosphorylation of ERK1/2 and stathmin at serine 16. (B) Quantification of the immunoblots for P-ERK1/2 and stathmin P-16. Total ERK1/2 was used as a loading control of the gel. ■ No inhibitor (NI), ▒ U0126 (U, 10 μM) and □ AG1478 (AG, 100 nM). Quantification and statistical analyses were performed on three independent experiments (*$P < 0.05$).

to any known gene as a control (C) [17]. After 2 days, GT1-7 cells transfected with PrP^c siRNA displayed a dose-dependent inhibition of PrP^c expression, as compared to control dsRNA transfected cells (Fig. 2A). The most effective concentration of siRNA tested (500 nM) yielded a 70–80% PrP^c depletion and was used to examine the effects of anti-PrP^c treatments on such PrP^c depleted GT1-7 cells. On cells transfected with control dsRNA, immunoblots revealed that treatment with the SAF34 mAb (at 10 μg/ml for 10 min) induced, as expected [11], the phosphorylation of ERK1/2 (Fig. 2B). A 2-fold increase in ERK1/2 phosphorylation was observed in these transfected cells (Fig. 2B), whereas a nearly 5-fold activation was observed in non-transfected cells (see below and Fig. 3), probably reflecting a side effect of the membrane-destabilizing transfection protocol. This activation of ERK1/2 was totally abolished in cells treated with PrP^c siRNA (Fig. 2B), thus demonstrating the PrP^c specificity of the signaling pathway induced by SAF34. Similar results were observed also with the SAF61 anti-PrP^c mAb (not shown).

3.3. Anti-PrP^c antibody treatment induces rapid and transient phosphorylation of ERK1/2 and stathmin

The increase of phospho-ERK1/2 (P-ERK1/2) detected by immunoblot in response to treatment of GT1-7 cells with the SAF34 mAb was rapid, with a peak at 10 min (Fig. 3), and transient, as no residual activation was detected after 45 min (not shown). As a further control, we used an isotypic unrelated IgG2a mAb (clone 12CA5) which did not induce activation of ERK1/2 (not shown).

In addition to its 5-fold effect on ERK1/2 phosphorylation, the SAF34 mAb also induced the phosphorylation of stathmin

at serine 16 as shown by immunoblot with a phosphospecific rabbit polyclonal antibody (Fig. 3A). The phosphorylation of stathmin followed the same time course as P-ERK1/2 but its induction was relatively less pronounced, slightly higher than 2-fold (Fig. 3B). Stathmin can be phosphorylated at four residues but serine 16 was the only residue where we detected an increased phosphorylation after anti-PrP^c antibody treatment. This phosphorylation is known to inhibit the microtubule destabilizing activity of stathmin [21,22].

Treatment of the cells with the compound U0126 (10 μM), a specific inhibitor of MAPK kinase 1 (MKK1), prior to activation with the SAF34 mAb, completely blocked the phosphorylation of ERK1/2 (Fig. 3A). The basal level of ERK1/2 was strongly inhibited by the U0126 compound, but no activation was detected even after longer exposure of the immunoblot. This result confirms previous observations with PD98059 [11], another specific inhibitor of MKK1/2, showing that MKK1/2 mediates the activation of ERK1/2 upon anti-PrP^c treatment. In contrast, the phosphorylation of stathmin is completely independent of the MKK/ERK pathway as the inhibitor U0126 had no effect on its induction by SAF34 (Fig. 3A and B).

Because the phosphorylation of stathmin serine 16 is known to be induced by epidermal growth factor (EGF) [21], we used tyrphostin AG1478 (100 nM), a specific inhibitor of the EGF receptor (EGFR), to examine its potential involvement in response to anti-PrP^c treatments. In basal conditions, AG1478 slightly decreased the level of P-ERK1/2 and increased the phosphorylation of stathmin at serine 16 (Fig. 3A, lane 5 compared to lane 1). On the other hand, AG1478 completely blocked the phosphorylation of stathmin at serine 16 induced by the SAF34 mAb and also strongly inhibited the activation of ERK1/2 (Fig. 3A, lane 6 and 3B). The inhibition of P-ERK1/2 was not complete, leaving about 15% of residual phosphorylation, which indicates that there could be a partly independent pathway involved in the activation of ERK. These results suggest that the EGFR plays an essential role in the signaling induced by anti-PrP^c antibodies in GT1-7 cells. We observed similar results, albeit with less pronounced effects, with the SAF61 mAb in all experiments performed, indicating that both antibodies have similar effects.

4. Discussion

In this study, we show that PrP^c cross-linking with specific antibodies results in clustering of PrP^c at the surface of GT1-7 neurohypothalamic cells. This clustering is associated with a rapid and transient phosphorylation of the MAP kinases ERK1/2 and stathmin at serine 16. This antibody-mediated activation is specific of PrP^c as it is fully inhibited by PrP^c siRNA. The phosphorylation of ERK1/2 but not that of stathmin was abolished by the MKK1 inhibitor U0126, showing that the treatments with the anti-PrP^c SAF34 and SAF61 mAbs induce at least two independent signaling pathways.

Unexpectedly, in the same conditions as for GT1-7 cells, the SAF34 and SAF61 mAbs failed to induce these signaling pathways in primary embryonic neuron cultures (from cortex or hippocampus) or in rat neuroblastoma B104 cells (not shown). We have previously shown that GT1-7 cells highly express hyperglycosylated forms of PrP^c as compared to B104 cells and primary cultures [20]. It is therefore possible that the glycosylation state of PrP^c influences its interaction with functional partners responsible for the coupling of PrP^c with signaling pathways. Comparison of these different cell models should allow to identify the molecular determinants at the basis of the differences observed in terms of signaling pathways induced by anti-PrP^c antibodies.

Interestingly, using immunofluorescence studies, we revealed that antibody treatments in GT1-7 cells induce the formation of PrP^c clusters by an unknown mechanism. Because mAbs have bivalent binding properties, they are able to induce dimerization. We can hypothesize that PrP^c dimerization could activate signaling pathways and result in further clustering of the protein. Clustering could therefore be regarded as a sign of PrP^c activation. In addition, we observed that this antibody-mediated PrP^c clustering induces the activation of ERK1/2, consistent with what was recently described by others [11].

We also identified stathmin as a novel intracellular target phosphorylated in response to this antibody treatment. Stathmin is a cytosolic tubulin-binding protein that can be phosphorylated on up to four serine residues, serine 16, 25, 38 and 63, in response to the activation of various intracellular signaling pathways, in particular when triggered by extracellular signals [15]. Stathmin binds tubulin heterodimers and has a microtubule-destabilizing activity downregulated by phosphorylation [23]. Although serine 25 is mainly activated by ERK1/2 [24], we could not detect significant phosphorylation of this serine despite the strong activation of the ERK1/2 pathway in our experiments. In contrast, we observed phosphorylation of stathmin serine 16 independently of ERK1/2. The phosphorylation at serine 16 is essential for regulating the microtubule-destabilizing activity of stathmin [22]. Our data thus suggest a possible coupling of PrP^c with the dynamics of microtubules via the stathmin protein.

Because the phosphorylation of stathmin on serine 16 is known to be induced upon activation of the EGFR [21,25], it was of interest to test its potential implication. We observed that both signaling pathways were totally or mostly blocked by the specific tyrphostin AG1478 EGFR inhibitor, suggesting a recruitment and transactivation of this receptor upon antibody-mediated PrP^c clustering. Interestingly, it is now well established that the EGFR can be transactivated, by autophosphorylation in the absence of EGF, in response to various stimuli, for instance through the activation of the G protein-coupled receptors (GPCRs), engagement of adhesion molecules or after cholesterol depletion [26–28]. Therefore, it is possible that clustering of PrP^c at the cell surface, as observed by immunofluorescence staining, could locally recruit and activate a fraction of EGFR. The MAPK pathway could be induced following transactivation of the EGFR. Nevertheless, the EGFR inhibitor did not completely abolish the activation of ERK1/2, which could be attributed to the activation of another intracellular signaling pathway independent of the EGFR. Recently, the production of ROS in response to anti-PrP^c antibody has been described in 1C11 and GT1-7 cells [11]. In relation with these findings, it has been reported that the EGFR can induce the generation of ROS, participating in cell survival and proliferation [29]. Altogether, these results argue for a role of PrP^c in normal cell homeostasis, possibly via transactivation of the EGFR.

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