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Activation of phosphatidylinositol 3-kinase by cellular prion protein and its role in cell survival

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Abstract

The cellular prion protein (PrP^C) is thought to be involved in protection against cell death, however the exact cellular mechanisms involved are still controversial. Herein we present data that strongly indicate a functional link between PrP^C expression and phosphatidylinositol 3-kinase (PI 3-kinase) activation, a protein kinase that plays a pivotal role in cell survival. Both mouse neuroblastoma N2a cells and immortalized murine hippocampal neuronal cell lines expressing wild-type PrP^C had significantly higher PI 3-kinase activity levels than their respective controls. Moreover, PI 3-kinase activity was found to be elevated in brain lysates from wild-type mice, as compared to prion protein-knockout mice. Recruitment of PI 3-kinase by PrP^C was shown to contribute to cellular survival toward oxidative stress by using 3-morpholinosydnonimine (SIN-1) and serum deprivation. Moreover, both PI 3-kinase activation and cytoprotection by PrP^C appeared to rely on copper binding to the N-terminal octapeptide of PrP^C. Thus, we propose a model in which the interaction of copper(II) with the N-terminal domain of PrP^C enables transduction of a signal to PI 3-kinase; the latter, in turn, mediates downstream regulation of cell survival.

Keywords: Prion protein; Phosphatidylinositol 3-kinase; Copper; Cellular survival; Oxidative stress

The cellular prion protein (PrP^C) is a glycosylphosphatidylinositol (GPI)-anchored membrane protein that is highly conserved in mammalian species [1]. The molecule is expressed most abundantly in the brain, but has also been detected in other non-neuronal tissues as diverse as lymphoid cells, lung, heart, kidney, gastrointestinal tract, muscle, and mammary glands [2,3]. Although PrP^C is likely to have a basic biological function in cells, its precise physiological role is still unknown. Over the past years, evidence has been accumulating consistent with an involvement of PrP^C in signal transduction pathways [4]. This is not surprising as PrP^C is targeted to cholesterol-rich microdomains of the plasma membrane, regions that are abundant in receptors and signaling molecules [5] Another well-studied aspect of PrP^C is its ability to selectively bind copper(II) ions via an octarepeat domain in the N-terminal region [6,7]. Several studies have indicated that deletion of this domain (consisting of four sequential copies of the sequence PHGGGWGQ) interferes with PrP^C function [8–10]. In addition, the capacity of PrP^C to interact with superoxide ions and hydrogen peroxide has been found to be copper-dependent [11]. Hence, copper(II) binding also confers redox properties to the

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protein. Finally, there is clearly an emerging role for prion protein in cellular survival, particularly as regards protection against copper [12], oxidative stress [12,13], serum deprivation [14], and the regulation of intracellular bcl-2/bax levels [15]. Nevertheless, the precise links between the copper binding, signal transduction, and cytoprotective roles of PrP^C have not yet been determined.

One multifunctional protein kinase that plays a pivotal role in neuronal cell survival and apoptosis, and which also associates with lipid microdomains, is phosphatidylinositol 3-kinase (PI 3-kinase) [16]. PI 3-kinase is known to be sensitive to redox signaling by superoxide and hydrogen peroxide [17], and to be activated by copper(II) ions [18]. Upon recruitment to the vicinity of the plasma membrane PI 3-kinase catalyzes the formation of D3-phosphorylated phosphoinositides, notably phosphatidylinositol 3,4,5-triphosphate (PIP_3). PIP_3 has several downstream target molecules, in particular the serine/threonine protein kinase B (PKB or Akt). The PI 3-kinase/Akt pathway suppresses cell death by regulating bcl-2 family member activity and mitochondrial function [16].

Given this background, we decided to examine the relationship between PrP^C and PI 3-kinase, and to specifically investigate the roles of copper and the octarepeat region of PrP^C in signal transduction. In the present manuscript, we demonstrated a clear link between PrP^C expression and induction of PI 3-kinase activity, and have shown that this mechanism is likely to involve the octarepeat region of PrP^C. Moreover, activation of PI 3-kinase by copper-bound PrP^C participates in the cellular response to stress.

Materials and methods

Materials and reagents. Sodium diethyldithiocarbamate trihydrate (DDC), 3-morpholinosydnonimine hydrochloride (SIN-1), and insulin were purchased from Sigma (Schnelldorf, Germany). The PI 3-kinase inhibitors wortmannin and LY294002 were from Calbiochem (Schwalbach, Germany). Hydrogen peroxide (H_2O_2) was freshly diluted from a 30% stock solution.

Construction of recombinant plasmids. The generation of the plasmids pBs-*Prnp* and pCI-*Prnp* has been described previously [19]. The construction of pBs-*Prnp*-H/G was performed in two steps. First, the octapeptide repeat region was removed and replaced by a linker fragment containing the cleavage sites for the restriction endonucleases *Bsu*36I, *Apa*I, and *Nae*I by using overlap-extension-PCR. The outer primers were M13-20 (5′-GTAAAACGACGGCCAGT) and rev (5′-AACAGCTATGACCATG), and the overlapping primers were CB1 (5′-GGGAGTGGGCCCAGTTCAGCCGGCGGGGGTACCCATAATC) and CB2 (5′-TGAACTGGGCCCACTCCCTGAGGGGTAACGGTTGCCTCC). In the second step, the resulting construct was cleaved with *Bsu*36I and *Nae*I, treated with S1-nuclease, and ligated with the hybridized primers CB3 (5′-CCTCAGGGTGGCACCTGGGGGCAGCCCGGGGGTGGTGGCTGGGGACAACCCGGTGGGGGCAGCTGGGGACAACCTGGAGGTGGTAGTTGGGGTCAGCCCGGGGGCGGTGGATGGGGCCAA) and CB4 (5′-TTGGCCCCATCCACCGCCCCCGGGCTGACCCCAACTACCACCTCCAGGTTGTCCCCAGCTGCCCCCACCGGGTTGTCCCCAGCCACCACCCCCGGGCTGCCCCCAGGTGCCACCCTGAGG) coding for a murine octapeptide repeat region with all four histidines replaced by glycines. The *Prnp* coding region of pBs-*Prnp*-H/G was then excised with *Xba*I and *Xho*I, and cloned into the corresponding sites of pCI-neo (Promega) to generate pCI-*Prnp*-H/G. The sequences of the modified *Prnp* genes were verified by DNA sequence analysis.

Cell lines and plasmid transfections. N2a mouse neuroblastoma cells that stably overexpress wild-type murine PrP^C have been described previously [19]. N2a cells that permanently overexpress the H/G mutant prion protein were produced by transfection with pCI-*Prnp*-H/G using the calcium-phosphate method. Following transfection, antibiotic-resistant cells were selected in 400 μg/ml geneticin (G418) and a polyclonal cell line was obtained after 4 weeks. The cells were then maintained at 200 μg/ml geneticin. N2a cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 10% CO_2-enriched atmosphere.

Murine hippocampal neuronal cell lines were generated as described in [20], by stable transfection of a PrP-deficient cell line (HpL3-4) with empty vector (HpL3-4-EM), wild-type PrP (HpL3-4-PrP), and N-terminally truncated PrP lacking the octarepeat region (Δ53–94) (HpL3-4-Δ#1). HpL3-4 cells and derivatives were maintained in DMEM containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% CO_2-enriched atmosphere.

PI 3-kinase assay. Cell cultures were first washed three times with ice-cold buffer A, containing 137 mM NaCl, 20 mM Tris–Cl (pH 7.4), 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.1 mM sodium orthovanadate. Following cell lysis in buffer A containing 1% Nonidet P-40 and 1 mM PMSF, extracts were cleared by centrifugation at 12,000*g* for 10 min at 4 °C. The supernatants were removed and assayed for protein content using the Bio-Rad protein assay. For immunoprecipitation, 150 μg of protein from each lysate was mixed with 3 μl anti-PI 3-kinase p85 (Upstate Biotechnology, Lake Placid, NY) and rotated for 5 h at 4 °C. Protein A–Sepharose beads were added and incubated overnight at 4 °C under slow rotation. Immunocomplexes were collected by washing three times with buffer A containing 1% Nonidet P-40, three times with a buffer containing 100 mM Tris–Cl (pH 7.4), 5 mM LiCl, 0.1 mM sodium orthovanadate, and another two times with a buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 0.1 mM sodium orthovanadate. The assay for PI 3-kinase activity was performed using a commercial ELISA kit (Echelon, Salt Lake City, UT), according to the procedure provided by the manufacturer. Briefly, PI 3-kinase assays were carried out for 2 h at room temperature in a reaction buffer containing 50 mM Hepes (pH 7), 250 μM ATP, and 25 mM $MgCl_2$, and phosphatidylinositol 4,5-biphosphate (PIP_2) substrate. The reaction was stopped using 3 μl of 100 mM EDTA. Thereafter, the reaction products were mixed and incubated with 50 μl PIP_3 detector protein, and then added to the PIP_3-coated microplate for competitive binding. After washing, 100 μl of peroxidase-linked secondary detection reagent was used to detect PIP_3 detector protein binding to the plate. The latter reaction was stopped by the addition of 100 μl of 0.5 M H_2SO_4 to each sample. The PI 3-kinase activity in the samples was determined by reading the ELISA plate at 450 nm, after calibrating with a standard curve. The final colorimetric signal is inversely proportional to the amount of PIP_3 produced by PI 3-kinase activity. Absorbance data are expressed as pmol PIP_3/mg protein.

Preparation of whole mouse brain tissue lysate. Lysates were derived from prion protein-knockout ($Prnp^{-/-}$) mice and their wild-type control littermates. The wild-type mice are descendants of an F1 generation mouse produced by interbreeding C57BL/6J × 129sv mice. C57BL/6J and 129sv mice are the same two strains initially used to generate the $Prnp^{-/-}$ mice [21]. Mice were sacrificed and the brains were removed, following which lysates were prepared by homogenization in freshly prepared buffer consisting of 50 mM Tris–

HCl (pH 7.4), 125 mM NaCl, 1% Nonidet P-40, 0.25% DOC, 1 mM EDTA, 125 mM NaF, 12.5 mM sodium pyrophosphate, 1.25 mM sodium orthovanadate, and a protease inhibitor cocktail (Roche Molecular Biochemicals). Tissue and cell debris were removed by centrifugation. The protein concentration of the cleared lysate was determined with Bio-Rad protein assay. Freshly prepared brain lysate was then used to determine PI 3-kinase activity, as described above.

Western blot analysis. Protein extract was prepared from confluent cells as described under 'PI 3-kinase assay.' Following determination of protein concentration, equal amounts (30 μg) of total protein were separated on 12% SDS–polyacrylamide minigels (Novex NuPage, Invitrogen Life Technologies, Basel, Switzerland), transferred to polyvinylidene difluoride membranes (Millipore), and probed with anti-PrP monoclonal antibody 6H4 (Prionics, Zurich, Switzerland). Visualization of bound antibodies was performed using the Amersham enhanced chemiluminescence (ECL) system.

Measurement of cell viability. Cell viability was determined by measuring the redox potential of the cells using a commercially available MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)-based assay (Cell Proliferation Kit I—Roche Molecular Biochemicals). Briefly, 3×10^5 cells per well were plated in a 24-well plate and grown in 1 ml of complete medium. The MTT assay was performed according to the manufacturer's instructions. Absorbance at 570 nm (A_{570}) was quantified using a plate reader. The A_{570} in control (untreated) cells was taken as 100% of viability. At least three independent samples were assayed for each experiment.

Results

The involvement of PI 3-kinase signaling in cell death regulation by PrP^C was first investigated using mouse N2a neuroblastoma cells. We established N2a cells stably expressing wild-type murine PrP^C (N2a-PrP), and another N2a cell line stably transfected with a construct in which all four existing histidines in the octapeptide repeat region of the molecule had been replaced with glycines (N2a-H/G). The N2a-PrP and N2a-H/G cells overexpressed their respective prion protein to similar levels, as demonstrated by Western blot in Fig. 1A.

We compared baseline PI 3-kinase activities in this panel of N2a cell lines; in view of the fact that serum-derived signals are known to stimulate PI 3-kinase activity [22], cells were harvested after culturing for 16 h in serum-free medium. As shown in Fig. 2A, the baseline PI 3-kinase activity in N2a-PrP cells was more than two-fold (2.39×) higher than that found in non-transfected control N2a cells ($p < 0.004$). N2a-H/G cells had baseline PI 3-kinase levels that did not significantly differ from controls (Fig. 2A). The latter observation implied that the function of octarepeat histidine residues was directly related to stimulation of PI 3-kinase activity by PrP^C. Since it has already been shown that the octarepeat histidines function primarily in the binding of copper(II) ions to PrP^C [6,23], we wanted to determine the effect of copper removal from the medium on PI 3-kinase activity. PI 3-kinase levels were therefore measured following addition of the copper(II) chelator DDC [24] to the culture medium for 16 h. This lowered the PI 3-kinase activation of N2a-PrP cells to a level not statisti-

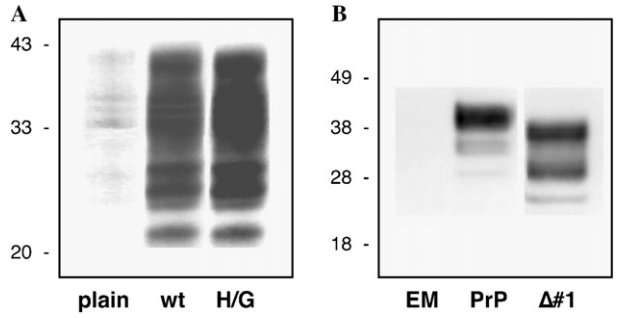

Fig. 1. PrP^C expression in N2a and HpL3-4 cell lines. Western blot analysis was carried out with the monoclonal PrP^C antibody 6H4 of 30 μg total protein extract (A) from non-transfected N2a cells (plain), N2a cells stably expressing wild-type PrP^C (wt), and N2a cells stably expressing mutant PrP^C devoid of any histidine in the octapeptide repeat region (H/G); (B) from HpL3-4 cells transfected with empty vector (EM: HpL3-4-EM), wild-type PrP^C (PrP: HpL3-4-PrP), and N-terminal truncated PrP^C (Δ#1: HpL3-4-Δ#1).

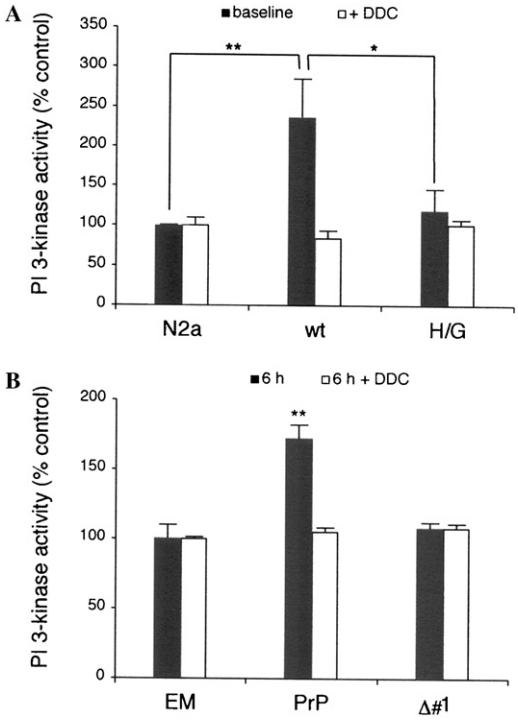

Fig. 2. Increased PI 3-kinase activity correlates with wild-type PrP^C expression and is dependent on copper bound to the N-terminal octapeptide repeats. (A) N2a cell lines were grown to ~80% confluence and cultured in serum-free medium for 16 h. PI 3-kinase activity was then measured in control N2a (N2a), N2a-PrP (wt), and N2a-H/G (H/G) cells, at basal levels and after addition of the copper(II) chelator DDC. (B) HpL3-4 cell lines were grown to ~80% confluence and cultured in serum-free medium for 6 h. PI 3-kinase activity was then measured in HpL3-4-EM (EM), HpL3-4-PrP (PrP), and HpL3-4-Δ#1(Δ#1) cells, at basal levels and after addition of DDC. Preparation of cell lysates and PI 3-kinase assay procedures are described under Materials and methods. PI 3-kinase data are expressed as a percentage of control N2a or HpL3-4 cells. Data are means ± SD (bars) of three independent experiments. Statistical significance by Student's t test: $*p < 0.05$; $**p < 0.004$.

cally different from those of control N2a and N2a-H/G cells (Fig. 2A). It should be noted that treatment of the three N2a cell lines with 40 μM DDC alone was not cytotoxic to the cells (results not shown). In order to further substantiate the link between PrP^C and PI 3-kinase, kinase activity levels were assessed in murine hippocampal neuronal cells lacking PrP^C (HpL3-4-EM), expressing wild-type PrP^C (HpL3-4-PrP), and transfected with a plasmid expressing an N-terminally truncated PrP^C (Δ53–94; HpL3-4-Δ#1) (Fig. 1B). Importantly, these neuronal cell lines have been shown to express PrP^C at levels equivalent to those from wild-type mouse brain [20]. Following culture in serum-free medium for 6 h, HpL3-4-PrP cells had a 72% higher PI 3-kinase activity than HpL3-4-EM cells; the cells expressing PrP^C lacking the octarepeat region (HpL3-4-Δ#1) had levels similar to the cells transfected with vector only (Fig. 2B). Akin to the N2a cells, addition of DDC lowered the PI 3-kinase activity of HpL3-4-PrP to that of empty vector transfected cells (Fig. 2B).

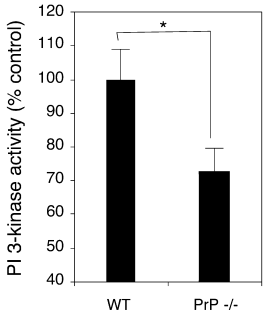

Fig. 3. PI 3-kinase activity in wild-type and $Prnp^{-/-}$ mouse brain lysates. PI 3-kinase activity was determined in whole brain lysates prepared from prion-knockout ($Prnp^{-/-}$) and wild-type (WT) littermate control mice. As with the cell culture model, expression of PrP^C correlated with significantly increased PI 3-kinase activity. Preparation of brain lysates and PI 3-kinase assay procedures are described under Materials and methods. PI 3-kinase data are expressed as a percentage of wild-type. Data are means ± SD (bars) of four independent experiments. Statistical significance by Student's t test: $^*p < 0.002$.

Finally, we turned to comparing kinase activity levels in whole brain lysates from $Prnp^{-/-}$ and wild-type littermate control mice of the same genetic background. In agreement with the results obtained from the cell culture models, PI 3-kinase activity was a significant 30% lower in the brain lysate from transgenic mice lacking PrP^C expression ($p < 0.002$; Fig. 3). In summary, our findings so far gave a strong indication that PrP^C expression correlated with increased PI 3-kinase activity.

Several studies have argued that expression of cellular prion protein promotes cellular survival, in particular towards oxidative stress. We therefore decided to investigate whether the PrP^C/PI 3-kinase cascade could play a role in the protection against free radical toxicity by H_2O_2 and 3-morpholinosydnonimine (SIN-1). SIN-1 is an oxidative stress agent that decomposes and yields superoxide ions, nitric oxide species, and peroxynitrite [26]. MTT assays were performed on N2a, N2a-PrP, and N2a-H/G cells challenged for 24 h with the indicated concentrations of H_2O_2 and SIN-1 (Fig. 4A). Our results showed that PrP^C increased resistance to 3 mM SIN-1 ($p < 0.005$ with respect to N2a-H/G), but increased susceptibility to higher doses of H_2O_2 (300 μM; $p < 0.005$).

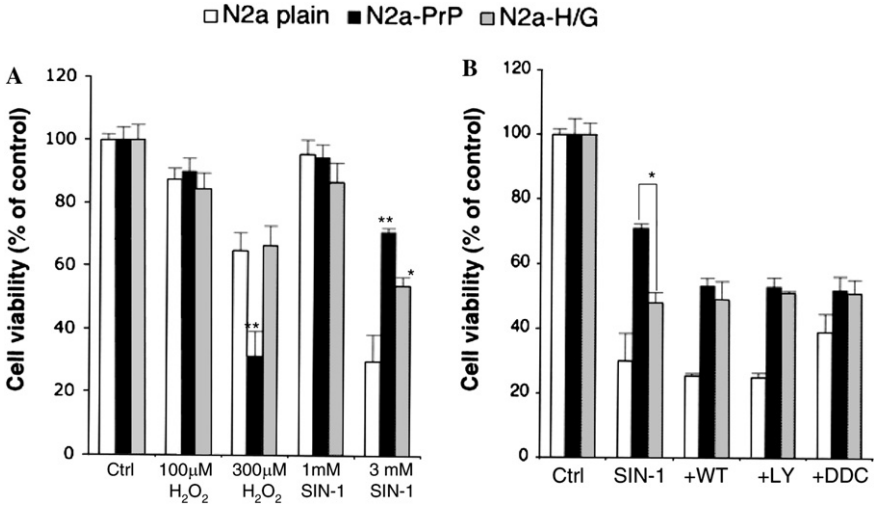

Fig. 4. Resistance of N2a cell line overexpressing PrP^C to SIN-1 is partly dependent on PI 3-kinase activity and copper. Cell lines were grown to 80% confluence, cultured in serum-free medium for 16 h, and subsequently exposed to the indicated H_2O_2, and SIN-1 concentrations for 24 h (A); where indicated in (B), cells were also treated with the PI 3-kinase inhibitors 300 nM wortmannin (WT) or 40 μM LY294002 (LY), or the copper(II) chelator diethyldithiocarbamate (DDC). After this time, cell viability was determined by a modified MTT assay, as described under Materials and methods. Data are presented as percentages of untreated control cultures. Standard deviations are indicated. $^*p < 0.009$; $^{**}p < 0.005$.

To test for the role of PI 3-kinase in the acquisition of resistance to SIN-1 toxicity, we employed two selective inhibitors of PI 3-kinase that act via distinct mechanisms, wortmannin (300 nM) and LY294002 (40 μM). We found that simultaneous incubation with either wortmannin or LY294002 decreased the cell viability of N2a-PrP cells exposed to SIN-1 by a significant 20%, and hence lowered MTT viability within the range of N2a-H/G cells (Fig. 4B). Non-transfected control N2a cells and N2a-H/G cells were largely unaffected by the PI 3-kinase inhibitors. Also, non-transfected control N2a cells are more sensitive to SIN-1 than either of the other two transfected cell lines.

Since induction of PI 3-kinase by PrP^C seemed to be copper-dependent (Fig. 2), and the PrP^C/PI 3-kinase signal was protecting from SIN-1-induced stress, we wanted to examine whether chelation of copper(II) from the culture medium would also reduce the protective effect of PrP^C overexpression against SIN-1. Thus, we carried out an experiment in which the three cell lines (N2a, N2a-PrP, and N2a-H/G) were pre-incubated with 40 μM DDC for 16 h, prior to treatment with SIN-1. Remarkably, the effect of copper(II) chelation on N2a-PrP cells mirrored that of the PI 3-kinase inhibitors, i.e., a significant reduction of ~20% in viability (Fig. 4B).

To further support the neuroprotective function of PrP^C-mediated signaling to PI 3-kinase, we turned to a well-documented model of PrP^C-mediated survival, namely that of serum deprivation of immortalized hippocampal neurons. Serum deprivation has been shown to induce apoptotic cell death in PrP-deficient neuronal cells but not in PrP-expressing cells [14,20]. In agreement with a postulated PrP^C/PI 3-kinase cytoprotective signal, the PI 3-kinase inhibitors LY294002 and wortmannin blocked the rescuing effect of PrP^C against serum removal for 24 h (69% with LY294002 and 74% with wortmannin vs. 100% without any treatment; Fig. 5). Treatment with the copper(II) chelator DDC had an inhibitory effect on HpL3-4-PrP survival comparable to the use of the PI 3-kinase inhibitors (64% with DDC vs. 100% without any treatment; Fig. 5), thereby further confirming the relationship between copper and PrP^C/PI 3-kinase signaling. In contrast, DDC resulted in a significant increase in the survival of PrP-deficient (HpL3-4-EM) cells (91% with DDC vs. 70% without any treatment; Fig. 5).

In order to demonstrate that PrP-deficient cells were more susceptible to serum withdrawal because of a failed activation of PI 3-kinase, we incubated serum-deprived HpL3-4-EM neuronal cells with insulin (5 μM) for 24 h. Insulin is a recognized stimulator of the PI 3-kinase pathway [25]. Indeed, insulin completely rescued the PrP-deficient cells from serum withdrawal-induced cell death (100% insulin-treated vs. 71% without treatment; Fig. 6). In addition, both PI 3-kinase inhibitors counteracted and abolished the protective activity of insulin (Fig. 6). Conclusively, these data demonstrate the central role of PI 3-kinase in PrP^C-dependent regulation of survival following the loss of trophic support.

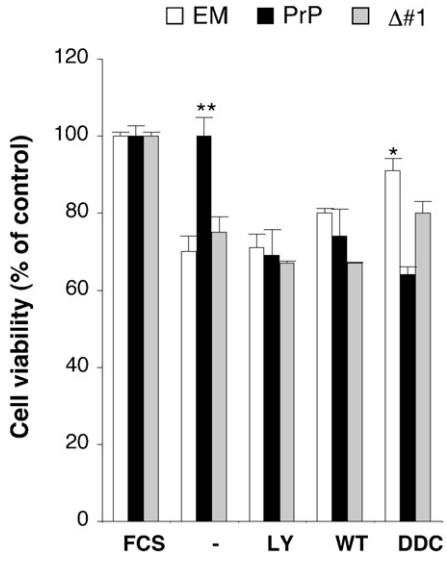

Fig. 5. Inhibitors of PI 3-kinase and the copper chelator DDC impair PrP^C-dependent neuroprotection. HpL3-4 cells were serum deprived for 24 h in the absence or presence of 20 μM LY294002 (LY), 1 μM wortmannin (WT) or 100 μM diethyldithiocarbamate (DDC). Cell survival was measured by the MTT method, as described under Materials and methods. MTT reduction was expressed as a percentage of control cells cultured in FCS-containing medium. Values shown are means ± SD of two independent experiments. Statistical significance by Student's t test: $^*p < 0.008$; $^{**}p < 0.004$.

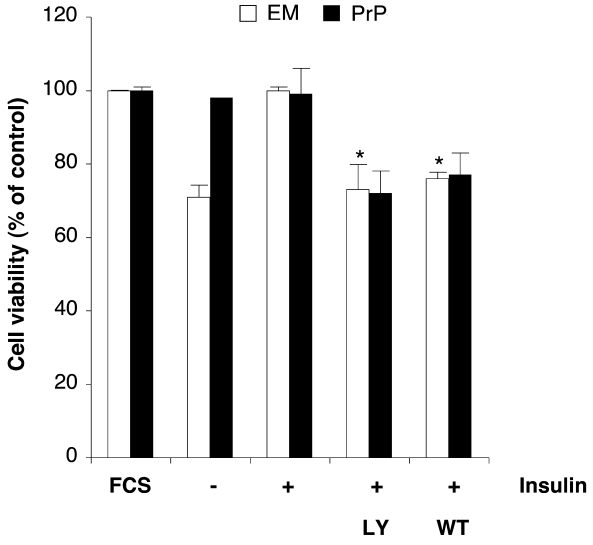

Fig. 6. Protection of PrP^C-deficient cells by insulin is PI 3-kinase-dependent. HpL3-4 cells were serum-starved for 24 h in the presence or absence of 5 μM insulin. In addition, the cells were incubated with the PI 3-kinase inhibitors LY294002 (LY, 20 μM) or wortmannin (WT, 1 μM) as indicated. Cell survival was measured by the MTT method, as described under Materials and methods. MTT reduction was expressed as a percentage of control cells cultured in FCS-containing medium. Values shown are means ± SD of two independent experiments. Statistical significance from insulin-treated cells by Student's t test: $^*p < 0.007$.

Discussion

Our aim in the present work was to gain insight into the physiological function of prion protein, by analyzing the connection between its copper binding, signaling, and neuroprotective roles. We initially used a model based on N2a neuroblastoma cell lines and examined PI 3-kinase, which has a key role in neuronal survival signaling. A link was found between PrP^C overexpression and increased PI 3-kinase activity. This increased PI 3-kinase activity was lost when the N2a-PrP cells were cultured with the copper(II) chelator DDC, or when the N2a cells were expressing a PrP that was unable to bind copper at the octapeptide repeats (N2a-H/G). These findings indicated that PrP^C activation of PI 3-kinase depends on copper binding to the octapeptide repeats. However, since N2a cells have endogenous PrP^C, and overexpression might change the biochemical properties of PrP^C, further studies using PrP-deficient neuronal cells were warranted. Thus, HpL3-4 immortalized mouse hippocampal cells originally derived from Rikn *Prnp*$^{-/-}$ mice [14,20] were used. The advantage of the HpL3-4 model system is that it allowed a direct comparison of PI 3-kinase activity between cells expressing wild-type levels of PrP^C (HpL3-4-PrP) to PI 3-kinase activity in cells that do not express PrP^C at all (HpL3-4-EM). Indeed, PrP-deficient cells had significantly lower PI 3-kinase activity than their PrP-expressing counterparts. Under similar culturing conditions, previous studies observed reduced SOD activity but no caspase-3/9 activation or poly(ADP-ribose) polymerase cleavage in HpL3-4 cells [20]. In addition, our data implied a role for copper-bound PrP^C, since HpL3-4-Δ#1 cells (which have an N-terminal octarepeat deletion mutant of PrP^C) or treatment of HpL3-4-PrP cells with DDC resulted in reduced PI 3-kinase levels. Finally, brain homogenates of *Prnp*$^{-/-}$ mice were also found to have reduced activity of PI 3-kinase compared to littermate controls. In conclusion, three different systems (N2a, HpL3-4, and brain lysates) support the existence of a signaling connection between PrP^C and PI 3-kinase.

A functional role for the PrP^C/PI 3-kinase signal with regard to cellular survival was found in both the N2a and HpL3-4 model systems. N2a-PrP cells were dependent on a higher PI 3-kinase activity, and on the presence of physiological copper in the medium, for enhanced survival in response to SIN-1 treatment (which generates various oxyradicals, such as superoxide and peroxynitrite ions). A number of publications have observed that the presence of PrP^C protects cells from oxidative stress induced by H_2O_2 [28]. In contrast, our findings showed that higher expression of PrP^C leads to increased susceptibility at high concentrations of H_2O_2. This is in agreement with the findings of Rachidi et al. [12], and is probably due to generation of excessive Fenton-derived oxyradicals mediated by copper-bound PrP^C. Activation of PI 3-kinase signaling was additionally important for HpL3-4-PrP cells to be rescued from cell death induced by serum deprivation. The beneficial effect of DDC on viability of serum-deprived HpL3-4-EM cells can be explained by considering the high toxicity of extracellular trace copper on PrP-deficient cells [27]. In other words, chelation of copper ions in the culture medium by DDC improved the survival of PrP-deficient cells in view of the fact that these cells are highly susceptible to copper toxicity. Insulin dramatically improved survival of PrP-deficient HpL3-4-EM cells probably by activating the PI 3-kinase pathway. We have therefore shown that in the case of serum withdrawal-induced cell death, HpL3-4-PrP cells are dependent on PI 3-kinase for survival whilst HpL3-4-EM cells are rescued by inducing PI 3-kinase. The connection between PrP^C, PI 3-kinase, and survival is hence clearly upheld.

Bringing our results together, we therefore put forward a model in which PrP^C induces a cellular signal to PI 3-kinase, and this most likely requires the interaction of copper(II) ions with the octapeptide repeat region of PrP^C. PI 3-kinase, in turn, mediates downstream regulation of cell survival. Admittedly, PrP^C might activate additional survival pathways [29], and hence, resistance to SIN-1 was not exclusively dependent on the PrP^C/PI 3-kinase signal. Nevertheless, when addressing the question of cytoprotective signaling through prion protein, it has to be kept in mind that deletion of the N-terminal four octapeptide repeats of PrP^C often eliminates the protective effect [9,10,23]. Such a scenario is in concordance with our model, that is to say, PrP^C lacking the copper-binding domain does not activate the PI 3-kinase signaling pathway. Other signaling cascades reported to be induced by PrP^C are not similarly dependent on its copper-binding region [29] and therefore do not explain why deletion of this region from PrP^C abrogates its neuroprotective function [9,10].

Our hypothesis is further supported by the independent observation that the PI 3-kinase inhibitor wortmannin strongly inhibited survival of cerebellar neurons, induced by application of recombinant PrP^C [15]. Even more recently, it was reported that PrP^C overexpression prevents human breast carcinoma cell line from tumor necrosis factor α-induced cell death, and this may again be linked to the PI 3-kinase/Akt pathway [30]. The model of a PrP^C/PI 3-kinase signal also fits with several other reported findings on prion protein signaling. It lends physiological relevance to the identification of Grb2 as a PrP^C-interacting protein [31], since Grb2 is a known upstream activator of PI 3-kinase [32]. Another emerging component of a PrP^C-dependent signaling complex is the tyrosine kinase Fyn [15,33,35]. PI 3-kinase is known to physically associate with Fyn in transducing protective signals that block β-amyloid neurotoxicity [34]. More downstream, the activation of

Fyn by PrP^C was found to target NADPH oxidase [35], a major site of ROS production in cells. The capacity of PI 3-kinase to stimulate NADPH oxidase as part of signaling cascades triggered at the cell membrane has been extensively documented [36]. Notwithstanding the requirement of copper(II) ions, the exact mechanism by which PrP^C can initiate transmembrane signaling is still unclear. It has been suggested that copper-bound PrP^C acquires redox properties, even weak SOD activity [37]. An intriguing proposition would be that redox signaling mediated by copper-bound PrP^C participates in the activation of PI 3-kinase, which is redox sensitive [17]. Such signaling would therefore take the form of local redox events restricted to the lipid microdomains.

In conclusion, in this manuscript we present the principal idea of signaling of PrP^C through PI 3-kinase, and consequently reinforce the proposition that the physiological function of PrP^C is as an important regulator of neuronal survival. Due to its plasma membrane localization, copper-bound PrP^C can act as a sensor for extracellular stress and transmit signals to the intracellular milieu, the PI 3-kinase cascade having a crucial role in activating neuroprotective mechanisms. Given that adult neurons need to survive for the lifetime of the organism, it might also be interesting to analyze a possible role for PrP^C in other acute and chronic neurological diseases, even aging itself.

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