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Biochemical and Biophysical Research Communications 340 (2006) 13–22
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Prion protein induced signaling cascades in monocytes

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Received 23 November 2005
Available online 6 December 2005

Abstract

Prion proteins play a central role in transmission and pathogenesis of transmissible spongiform encephalopathies. The cellular prion protein (PrP^C), whose physiological function remains elusive, is anchored to the surface of a variety of cell types including neurons and cells of the lymphoreticular system. In this study, we investigated the response of a mouse monocyte/macrophage cell line to exposure with PrP^C fusion proteins synthesized with a human Fc-tag. PrP^C fusion proteins showed an attachment to the surface of monocyte/macrophages in nanomolar concentrations. This was accompanied by an increase of cellular tyrosine phosphorylation as a result of activated signaling pathways. Detailed investigations exhibited activation of downstream pathways through a stimulation with PrP fusion proteins, which include phosphorylation of $ERK_{1,2}$ and Akt kinase. Macrophages opsonize and present antigenic structures, contact lymphocytes, and deliver cytokines. The findings reported here may become the basis of understanding the molecular function of PrP^C in monocytes and macrophages.

Keywords: Prion protein; Prion signaling; Prion function; Monocytes; Syk; Pyk2; Cbl; Fyn; MAPK; Akt

Prion diseases are transmissible fatal neurodegenerative disorders that include Creutzfeldt–Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle, and scrapie in sheep. The infectious agent, the prion, is considered to consist largely or exclusively of the conformationally altered scrapie isoform (PrP^{Sc}) of the host cellular prion protein (PrP^C). The PrP^{Sc} isoform is self-propagating in an autocatalytic manner [1]. Prion diseases can be acquired by ingestion of contaminated food, followed by a primary prion replication and accumulation in lymphoreticular tissues that may be of importance for peripheral pathogenesis [2–9]. In the later stages of the disease, PrP^{Sc} deposits and neurodegeneration are observed in the brain [10–13].

Despite decades of intensive research, the physiological function of PrP^Cremains enigmatic. Prion proteins are highly conserved throughout the evolution of mammals and therefore are thought to play a significant role. The cellular prion protein can be variably glycosylated at two N-glycosylation sites and is C-terminally attached to the cell surface by a glycosyl phosphatidylinositol (GPI) anchor [12,14–16]. GPI-anchored proteins are found in lipid rafts, highly cholesterol- and glycolipid-enriched membrane domains associated with a large number of signaling molecules such as G-protein-coupled receptors and Src-family kinases [17–20]. Several studies have dealt with PrP^C-specific activation [21,22] and the identification of potential interaction partners, e.g., the neuronal cell adhesion molecule (N-CAM) [23] or the laminin receptor and laminin receptor precursor protein [24–26]. PrP has been implicated in the physiology of neurons, affecting neurite outgrowth, neuronal survival, and synaptic function [27–29]. It has been also reported to be involved in the acti-

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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.11.158

vation of lymphocytes, which present a remarkable expression level of PrP^C that is elevated by activation [30,31]. The most relevant expression of PrP^C outside the CNS is found in lymphoid tissues, where it is detectable on lymphocytes, macrophages, and follicular dendritic cells (FDCs) within the germinal centers [6,32–34]. Interestingly, it has recently been reported that PrP^C modulates phagocytosis in macrophages of wild-type and Prnp knockout mice [35]. However, the mechanisms of PrP interaction with putative ligands are poorly understood and it is unclear whether there is a paracrine or autocrine function of PrP.

In order to study a possible binding of extracellular PrP to ligands on the cell surface of macrophages, we established a cell culture model in which murine monocyte/macrophages (P388D-1) were exposed to different prion fusion proteins. The synthesized prion fusion proteins were (I) specifically detectable by their tag and therefore immunologically distinguishable from host PrP^C; (II) produced in vivo (in mammalian cells) and therefore very closely related to naturally occurring PrP^C and fully post-translationally modified, to prevent any modifications that might influence binding activities. For this approach, we used the Fc-portion of human IgG_1 as a tag, thus allowing the isolation of post-translationally modified fusion prion proteins from the supernatant of mammalian cell lines.

In this study, we show the attachment of certain PrP fusion proteins to the surface of P388D-1 monocytes in nanomolar concentrations. We were able to demonstrate Src-dependent activation of P388D-1 monocytes shortly after incubation with PrP fusion proteins. Consecutive activation of multiple downstream pathways, including MEK/ERK and PI3K/Akt, suggests that PrP^C may have a role in transducing signals involved in macrophage function, such as phagocytosis, cell migration, and cytokine production.

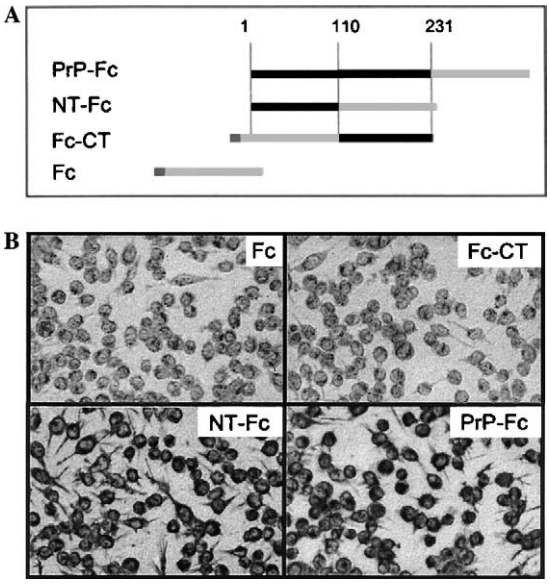

Fig. 1. Fusion proteins of PrP and the IgG1 CH-CH3 -domain (Fc). (A) The design of the different fusion proteins is shown schematically. PrP-derived amino acids are indicated in black, the Fc domain in light gray, and the signal peptide in dark gray. Amino acid positions of the GPI anchorage site (231) and the physiological cleavage site (110) of PrP are marked [42]. (B) Staining of P388D-1 monocytes for PrP-fusion proteins. P388D-1 monocytes were incubated (90 min) with 20 nM Fc as control, 10 nM Fc-CT, 10 nM NT-Fc or 20 nM PrP-Fc in normal growth media containing 5% serum. After fixation of cells and heat inactivation of endogenous phosphatases, binding of Fc or Fc fusion proteins was detected using an alkaline phosphatase-conjugated antibody directed against human Fc.

Materials and methods

Cell lines and cell culture. N2a neuroblastoma cells [36] and HEK293 cells [37] were maintained in DMEM, supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 200 IE/ml penicillin, and 20 μg/ml streptomycin. P388D-1 monocytes [38] were cultivated in RPMI medium containing 4.5 g/L glucose, 20% heat-inactivated horse serum, 2 mM glutamine, 200 IE/ml penicillin, and 20 μg/ml streptomycin.

Cloning procedures and production of fusion proteins. The coding sequences of murine PrP and the CH_2CH_3 part of human IgG_1 (Fc) were cloned in-frame into the pRK5 expression vector [39]. For the production of PrP-Fc protein, aa 1–231 of PrP were fused at the C-terminus to IgG_1. The denoted NT-Fc contains aa 1–110 of PrP only. Fc-CT includes aa 111–231 of PrP. The Fc protein and EphrinA5-Fc protein used as controls have been described previously[40]. To allow secretion of N-terminal fusion proteins, the signal peptide (aa 1–31) of EphA7 was aminoterminally included in the Fc-fragment. A schematic overview is given in Fig. 1A.

Transfection of HEK293 cells was performed as described [41]. N2a cells were transfected using Effectene or SuperFect (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. To generate stably transfected cells, the cDNAs were cotransfected with an expression vector for neomycin transferase in a ratio of 19:1. Transfected cells were selected by the addition of G418 (400 μg/ml) to the media.

Confluent N2a and HEK293 cells secreting Fc fusion proteins were transferred to media containing 0.5% FCS. After 5–6 days, the conditioned media were collected and precleared by a short centrifugation at 4000*g* to remove contaminating cells. Then 20 mM Hepes, pH 7.5, was added to the media and incubated with an appropriate amount of protein A–agarose beads (10 μl/ml, Roche Diagnostics GmbH, Mannheim, Germany) at 4 °C for 16 h. The suspension was then transferred into a column and washed several times. Bound Fc fusion proteins were released with elution buffer (150 mM NaCI, 100 mM glycine, adjusted to pH 2.5 with HCl). Protein concentrations were determined using the Bradford assay (Bio-Rad, Munich, Germany) and Western blot analysis was routinely performed to check the quality of purified proteins using PrP and Fc specific primary antibodies.

Staining of cells for PrP binding activity. Subconfluent P388D-1 monocytes (80%) grown on coverslips were incubated for 1.5 h at room temperature with different PrP fusion proteins added to the respective growth media (10 nM Fc-CT, 10 nM NT-Fc, 20 nM PrP-Fc, and 20 nM Fc as control). Varying amounts of fusion proteins were applied in consideration of the physiological cleavage of PrP [42,43]. After extensive washing, the cells were fixed in 4% formaldehyde and 60% acetone for 90 s at room temperature and proteases were heat-inactivated. Afterwards, unspecific protein binding sites were blocked by incubating the cells with HBSS containing 10% goat serum. Binding of Fc-proteins was detected using secondary goat-anti-human-Fc antibody conjugated to alkaline phosphatase. The staining followed conventional NBT/BCIP staining protocols.

Cell lysis and immunoprecipitation. After incubation with different fusion proteins, the P388D-1 monocytes were lysed in ice-cold lysis buffer (150 mM NaCI, 50 mM Hepes, pH 7.5, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride). Lysates were precleared and the protein concentrations were determined.

Immunoprecipitation of precleared lysates was performed with the appropriate antibody (1 μl/ml) and protein A–agarose (10 μl/ml). After incubation (at least 4 h at 4 °C), the immunoprecipitates were washed extensively with washing buffer (150 mM NaCI, 20 mM Hepes, pH 7.5, 0.1% Triton X-100, 10% glycerol, 10 mM NaF, and 1 mM sodium orthovanadate) and subjected to Western blotting.

Western blot. Proteins were separated on SDS–polyacrylamide gels (9–12.5%), transferred to PVDF membranes, and probed with the respective primary antibodies (see below). Bound antibodies were visualized using secondary antibodies coupled to horseradish peroxidase or alkaline phosphatase and chemoluminescence (ECL system (Amersham, NY, USA) or CDP-Star (Applied Biosystems, CA, USA)).

The antibody 3B5 has been described before [44]. Monoclonal antibody 6H4 was purchased from Prionics (Zurich, Switzerland), the 4G10 anti-phosphotyrosine and the monoclonal Syk antibodies from Upstate (Chicago, USA), and the polyclonal Syk, Pyk2, fyn, and cbl antibodies from Santa Cruz (Santa Cruz, USA). MAPK-, phospho-MAPK- (pT-Ep-Y motif), and phospho-Akt- (pSer 473) antibodies were purchased from New England Biolabs (Beverly, USA).

Evaluation of phosphorylation levels. Calculation of phosphorylation levels was performed by evaluating the band intensities from five independent experiments using TotalLab v 2.01 software (Nonlinear dynamics, Newcastle upon Tyne, UK). The medians were normalized to the activity of monocytes stimulated with Fc as control.

Results

Generation of Fc-fusion proteins

The prion protein fusion proteins consisted of the Fc portion of human IgG_1 linked either N- or C-terminally to different portions of mouse PrP (Fig. 1A). Due to the physiological cleavage site of PrP [42], these include NT-Fc (aa 1–110 of PrP), Fc-CT (aa 111–231 of PrP), PrP-Fc, Ephrin-A5-Fc, and Fc only. The fusion proteins were expressed in HEK293 (Fc-CT and NT-Fc) and N2a (PrP-Fc, Ephrin-A5-Fc, and Fc) cells, captured from conditioned culture media, and purified by immunoprecipitation.

Binding of fusion proteins to the cell surface of P388D-1 monocytes

In order to investigate cellular binding of different prion fusion proteins, P388D-1 monocytes were incubated with nanomolar concentrations of purified Fc, Fc-CT, NT-Fc, or PrP-Fc. N- and C-terminal parts of the PrP were found as physiological cleavage products next to full-length PrP and therefore Fc-CT and NT-Fc were applied here in a lower concentration than Fc and PrP-Fc [42,43]. Immunohistochemical staining was performed using an antibody against human IgG. As shown in Fig. 1B, the Fc-CT protein shows barely any binding to the cell surface, similar to the control with Fc protein. In contrast, P388D-1 monocytes showed equally strong staining after either incubation with PrP-Fc or the N-terminus of PrP as Fc fusion protein (NT-Fc) already in a concentration of 10 and 20 nM, respectively. The staining of PrP-Fc and NT-Fc was located at cytoplasm processes and the outer cytosol.

Stimulation of monocytes with PrP-Fc induces tyrosine phosphorylation

With regard to the binding of PrP as fusion protein to P388D-1 monocytes, we examined induction of signaling cascades. Serum starved P388D-1 monocytes were treated over 15 min with Fc, PrP-Fc or for positive control with the lectin concanavalin A (Con A), a well-known stimulant of macrophages. Since the activation of signaling cascades is usually accompanied by changes in the phosphorylation state, we analyzed the phosphorylation of tyrosine residues in whole cell lysates [45]. The incubation with 50 nM Fc-protein caused no significant change in comparison to the untreated sample (Fig. 2A). However, P388D-1 monocytes showed enhanced tyrosine phosphorylation of various proteins after stimulation with 50 nM PrP-Fc. The incubation with the lectin concanavalin A (60 pg/ml) used as positive control resulted in a strong tyrosine phosphorylation as well. In addition, a potential induction of tyrosine phosphorylation by stimulation with other fusion proteins was studied. To this purpose, we stimulated P388D-1 monocytes with 50 nM each of Fc, NT-Fc, Fc-CT, and PrP-Fc over 15 min. While PrP-Fc clearly induced tyrosine phosphorylation in these cells, the truncated proteins caused only negligible or no activation (data not shown). As further control, the P388D-1 monocytes were stimulated with

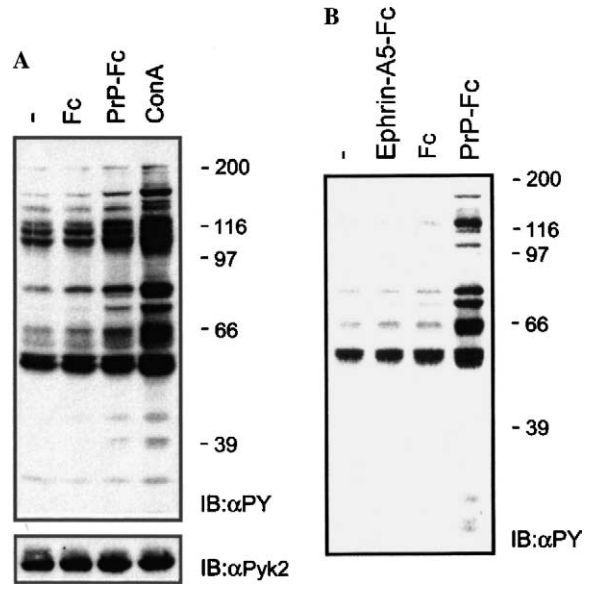

Fig. 2. Induction of tyrosine phosphorylation by PrP-Fc stimulation in the mouse monocytic cell line P388D-1. (A) Serum starved P388D-1 monocytes were stimulated for 15 min with either 60 pg/ml concanavalin A (ConA) or 50 nM Fc or PrP-Fc and subjected to Western blotting. Tyrosine phosphorylation was detected with anti-phosphotyrosine antibody 4G10 (top). Equal protein loading was verified by anti-Pyk2 antibody (bottom). Approximated molecular weights are indicated in kilodalton. (B) Induction of tyrosine phosphorylation by stimulation with different fusion proteins. Serum starved P388D-1 monocytes were left untreated or stimulated for 15 min with 50 nM each of Fc, Ephrin-A5-Fc, and PrP-Fc. While PrP-Fc clearly induced tyrosine phosphorylation in these cells, the Fc and Ephrin-A5-Fc did not affect tyrosine phosphorylation.

the fusion protein Ephrin-A5-Fc and compared to unstimulated cells and cells stimulated with Fc and PrP-Fc. After incubation with 50 nM each over 15 min, an enhanced tyrosine phosphorylation was found in the PrP-Fc stimulated cells only (Fig. 2B). The tyrosine phosphorylation level of the Ephrin-A5-Fc and Fc stimulated cells was as low as in the untreated cells; consequently, controls used did not affect tyrosine phosphorylation.

The dose and time dependence of the stimulation is demonstrated in Fig. 3. Increased concentrations of PrP-Fc (10-200 nM) in the media correlated with enhanced levels of tyrosine phosphorylation, whereas Fc-protein did not have any effect (Fig. 3A). Saturation of stimulation was achieved at PrP-Fc concentrations of about 50 nM, which were therefore used in the following experiments. The kinetics (3–80 min) of the induction of tyrosine phosphorylation after application of 50 nM PrP-Fc, in comparison to Fc, is shown in Fig. 3B. Elevated levels of tyrosine phosphorylation were observed as early as 3 min after stimulation with PrP-Fc and persisted for at least 40 min. A maximum was reached after around 15 min.

Identification of the tyrosine kinases involved in PrP signaling

Tyrosine kinases of the Src-family as well as the kinase Syk represent the principal components mediating the responses of monocytes to extracellular stimuli [46]. To test if Src-like kinases were involved in PrP-Fc signaling, we preincubated P388D-1 monocytes with the specific Src-family kinase inhibitor PP1 (Fig. 4), followed by incubation with either PrP-Fc or a control medium (unconditioned or Fc-conditioned medium). Again, the incubation with 50 nM PrP-Fc over 15 min induced increased tyrosine phosphorylation level (lane 3) in contrast to the controls with untreated or Fc-treated monocytes (lanes 1 and 2). Pretreatment with 2 μM PP1 over 45 min (lanes 4–6) had a strong effect on the overall tyrosine phosphorylation of PrP-Fc as well as Fc stimulated cells. Phosphorylation was practically absent in the controls (lanes 4 and 5). In the PrP-Fc stimulated cells (lanes 3 and 6), PP1 altered not only the tyrosine phosphorylation level but also the tyrosine phosphorylation pattern. In particular, phosphorylation of proteins with molecular weights of around 115, 97, 76/72, and 50 kDa was reduced, whereas phosphorylation in proteins with molecular weights of about 60 and 120 kDa was completely lacking.

Identification of activated kinases

To identify tyrosine kinases that may be phosphorylated upon stimulation with PrP-Fc, lysates of stimulated cells were subjected to immunoprecipitation with specific antibodies against Syk, Pyk2, Cbl, and Fyn.

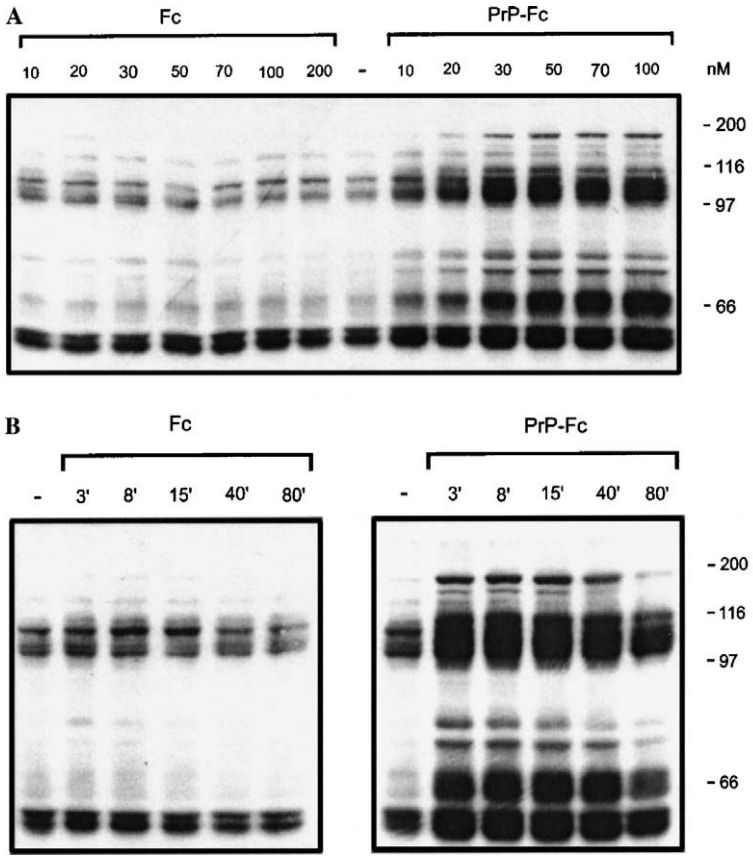

Fig. 3. Kinetics of PrP-Fc induced tyrosine phosphorylation. Serum starved cells were stimulated for 15 min with different concentrations of Fc (10–200 nM) and PrP-Fc (10–100 nM) in (A), or with 50 nM Fc or PrP-Fc for 3–80 min as indicated (B). 4G10 was used as primary antibody.

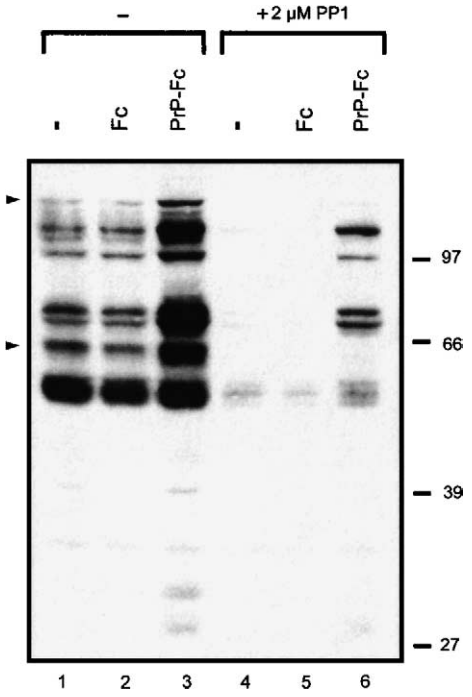

Fig. 4. Effect of src family tyrosine kinase inhibitor PP1 on PrP-Fc induced tyrosine phosphorylation. Serum starved P388D-1 monocytes were preincubated for 45 min with 2 μM PP1 and stimulated with 50 nM Fc or PrP-Fc proteins for 15 min. Tyrosine phosphorylation (activity) was visualized with antibody 4G10. Note the altered tyrosine phosphorylation pattern in lanes 3 and 6 as indicated by arrowheads on the left.

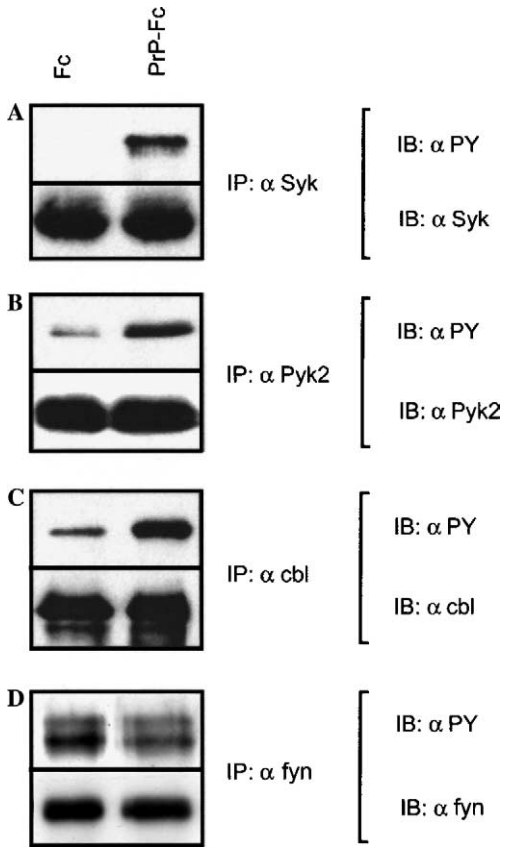

Fig. 5. Identification of tyrosine phosphorylated proteins in monocytes upon PrP-Fc stimulation. Following incubation with 50 nM PrP-Fc or 50 nM Fc proteins as control, P388D-1 monocytes were lysed and equal amounts of proteins were used for immunoprecipitation with antibodies against Syk (A), Pyk2 (B), Cbl (C), and Fyn (D). The tyrosine phosphorylation state (activity) was detected with 4G10 antibody. Equal protein loading was verified by reprobing with Syk, Pyk2, Cbl, and Fyn antibodies, respectively. IP, immunoprecipitation; IB, immunoblot, PY, tyrosine phosphorylation.

The phosphorylation state of precipitated kinases was then detected with a tyrosine phosphorylation-specific antibody (PY), as shown in Fig. 5 (upper panels). Afterwards, the blots were reprobed with respective polyclonal antibodies for Syk, Pyk2, Cbl, and Fyn to ensure equal overall concentrations of kinases (lower panels). We identified a pronounced increase in tyrosine phosphorylation of kinase Syk after stimulation with PrP-Fc (Fig. 5A, upper panel) and a less pronounced increase of kinase Pyk2 (Fig. 5B, upper panel). Adapter protein Cbl revealed a slight increase in tyrosine phosphorylation (Fig. 5C, upper panel), whereas Fyn showed a high basal level of phosphorylation, which was slightly decreased upon stimulation (Fig. 5D, upper panel).

Activation of investigated kinases has been shown to elicit downstream signals involving MEK-$ERK_{1,2}$ and PI3K-Akt pathways [47–51]. We therefore examined the resulting participation of $ERK_{1,2}$ and Akt kinases in these pathways. Equal amounts of proteins of stimulated monocytes were subjected to Western blotting using antibodies directed against activated $ERK_1$and ERK_2 (phosphorylation of threonine and tyrosine residues Thr202/Tyr204 corresponding to human phosphorylated $ERK_{1,2}$) and activated Akt (through phosphorylation at Ser473). Phospho-$ERK_{1,2}$ migrate as double bands at 42 and 44 kDa (Fig. 6A), and Phospho-Akt is detected as a band at about 60 kDa. The stimulation of monocytes with 50 nM PrP-Fc over 15 min resulted in a conspicuous activation of both ERK_1 and ERK_2 in comparison to the control with Fc alone (Fig. 6A, lanes 4 and 8). Akt kinase also showed increased activity after stimulation with PrP-Fc (Fig. 6B, lanes 4 and 8).

Phosphorylation of $ERK_{1,2}$ occurs through upstream activation of MEK and phosphorylation of Akt kinase is due to activation by PI3K [46]. To gain insight into the importance of MEK and PI3K on PrP-Fc-dependent pathways, we investigated the effect of specific inhibitors. Prior to stimulation with PrP-Fc and Fc as described above, the monocytes were preincubated for 45 min by adding inhibitors to the growth media, either inhibitor PP1, specific for Src-like kinase, or Wortmannin specific for PI3K, or U0126 specific for MEK. Corroborating the results shown in Fig. 4, 2 μM PP1 caused a decrease in activation of $ERK_{1,2}$ predominantly in PrP-Fc stimulated cells (Fig. 6A, lanes 3 and 7). This demonstrates that a large portion of $ERK_{1,2}$ is phosphorylated by Src-like-kinases which obviously contribute to the stimulation with PrP-Fc. Moreover, PP1 at the same concentration almost completely inhibited the phosphorylation of Akt in PrP-Fc-stimulated and Fc-stimulated cells (Fig. 6B, lanes 3 and 7). Consequently, phosphorylation of Akt largely

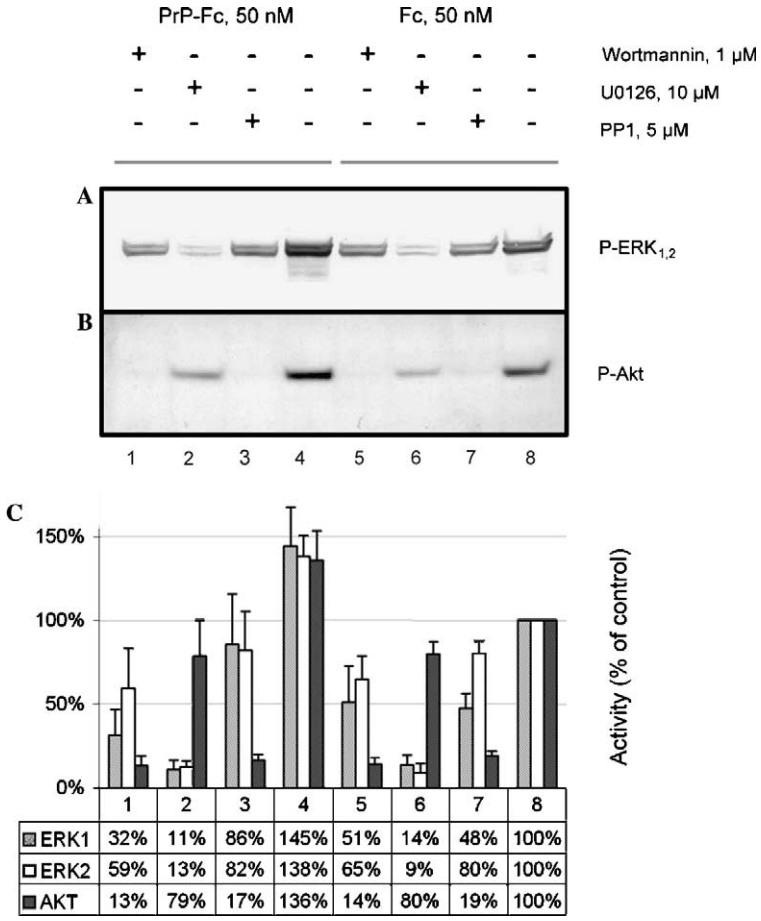

Fig. 6. Activation of $ERK_{1,2}$ and Akt kinase in response to PrP-Fc stimulation and inhibition by PP1, U0126 or Wortmannin. Serum starved P388D-1 monocytes were preincubated for 45 min with 1 μM Wortmannin (lanes 1 and 5), 10 μM U0126 (lanes 2 and 6), 2 μM PP1 (lanes 3 and 7) or left untreated (lanes 4 and 8). Cells were then stimulated with 50 nM PrP-Fc (lanes 1–4) or Fc (lanes 5–8) proteins for 15 min. Activated kinases were detected by using phosphorylation (activity)-specific antibodies against $ERK_{1,2}$ (A) and Akt (B). The medians of the evaluated activities out of three independent experiments are indicated in the graph and table below (C, error bars = SEM).

depends on Src-like kinase, whose inhibition again abolished phosphorylation with PrP-Fc. Furthermore Wortmannin, inhibiting PI3K, strongly decreases the phosphorylation of Akt both in cells stimulated with PrP-Fc and with Fc (Fig. 6B, lanes 1 and 5). The lack of phosphorylation in both cells stimulated with PrP-Fc and cells stimulated with Fc underscores the involvement of PI3K. The use of Wortmannin was accompanied by a slight decrease of $ERK_{1,2}$ phosphorylation (Fig. 6A, lanes 1 and 5), comparable to levels seen with inhibition by PP1. In contrast to the inhibitors Wortmannin and PP1, the MEK-inhibitor U0126 resulted in a clear reduction of $ERK_{1,2}$ phosphorylation (Fig. 6A, lanes 2 and 6), whereas Akt phosphorylation was moderately diminished (Fig. 6B, lanes 2 and 6).

The evaluated phosphorylation levels of $ERK_{1,2}$ and Akt out of five independent experiments are given in the table of Fig. 6C. The values of ERK_1, ERK_2, and Akt phosphorylation of the Fc-control cells without inhibitors were set to 100%. Analysis of variance (ANOVA) revealed a significant ($p < 0.05$) difference between stimulation with PrP-Fc and Fc alone in ERK_1, ERK_2, and Akt phosphorylation. A significant inhibition of both PrP-Fc and Fc-stimulated cells was found by PP1 for ERK_1 and Akt, by Wortmannin for ERK_2 and Akt, and by U0126 for ERK_1 and ERK_2.

In conclusion, upon PrP-dependent stimulation monocytes showed (I) upstream activation of Src-like kinases, as well as activation of Syk and Pyk2; and (II) downstream activation of $ERK_{1,2}$ and, in particular, Akt kinase.

Discussion

The physiological role of PrP^C is still enigmatic. The function of macrophages is thought to be influenced by PrP^C [35,52]. The aim of this study was to gain insight into the physiological function of PrP^C as part of a surface-signaling complex, since several studies suggested that PrP^C binds to the cell surface through a ligand or receptor [21,53,54], although the physiological signals acting on PrP^C have not been identified. In this study, we have investigated the effect of exogenous exposure of PrP fusion proteins on signaling pathways in monocytes/macro-

phages. The monocyte/macrophage cell line P388D-1 was originally established from a mouse with a lymphoid neoplasm and has been reported to be capable of interleukin production and phagocytosis, and therefore seemed to be a suitable model for investigating signaling cascades [38]. Our experiments have shown a strong binding of PrP-Fc and N-terminal PrP fusion proteins (NT-Fc) to the cell surface of monocytes in nanomolar concentrations, which are still about an order of magnitude below the concentrations of PrP in normal mouse brain [55]. In contrast, there was almost no binding of either the C-terminal PrP fusion protein (Fc-CT) or the Fc part originated from human IgG1. Previous studies had described attachment of PrP-Fc fusion proteins to the cerebellar granule layer on mouse brain sections [56] and PrP fusion proteins binding to neurons by showing that cultured cerebellar granule neurons were specifically stained after crosslinking of PrP-Fc with anti-Fc antibodies [27]. Interestingly, histoblots from $Prnp^{0/0}$ mice stained similarly to wild-type mice, which argues for the presence of a potential ligand that is not PrP^C. A number of possible ligands have been described to bind PrP^C, e.g., plasminogen, heparin-like compounds, synapsin Ib, Grb2, Pint 1, laminin receptor, laminin receptor precursor protein, and NCAMs [23–25,57–62]. It is possible that PrP-Fc is bound to a ligand or endogenous PrP^C by forming multimers because (I) PrP-Fc has been shown to stay in a monomer/dimer equilibrium [27] and (II) PrP intrinsically tends to cluster. It has been demonstrated that many other cell surface proteins bind their receptors as a monomer, but induce a signaling cascade only if bound either to membranes or through multimerizing [63]. Further studies will need to clarify the impact of PrP^C aggregation on signaling pathways.

P388D-1 monocytes demonstrate increased tyrosine phosphorylation by incubation with PrP-Fc in comparison with Fc-protein incubation in a dose- and time-dependent manner. The observed fast response argues for an interaction of PrP^C with distinct molecules participating in transmembrane signaling. Interestingly, NT-Fc did not induce a response in this context, although binding to the cell surface as PrP-Fc, which could be interpreted as different spatial binding and activation sequences. To examine the possible PrP^C-induced signaling pathways, we used specific kinase inhibitors. The PrP-Fc-induced tyrosine activation of P388D-1 monocytes was clearly diminished when cells were preincubated with PP1, a potent Src-family kinase inhibitor. This suggests involvement of Src-like kinases in a_PrP^C signaling pathway, in particular since two characteristic bands for Src-like kinases and adapter proteins Cbl were eliminated by the inhibitor PP1. One member of Src-family kinases is Fyn, which has previously been reported as being involved in signal transduction through antibody-mediated crosslinking of host PrP^C [21]. In the differentiated neuronal cells used in that study, the basal activation level of Fyn was low; it was significantly elevated upon stimulation. In our experiments, we used exogenous PrP fusion proteins applied to monocytes, in which a high basal phosphorylation level of fyn kinase was found that was not remarkably changed after stimulation with PrP-Fc.

More notably, we found that PrP-Fc resulted in phosphorylation of Syk and Pyk2. The immunoreceptor-associated Syk tyrosine kinase is part of integrin-mediated signaling, which is essential for cell migration, phagocytosis, and the production of inflammatory mediators [47–49]. Syk tyrosine kinase has been described to be activated by binding of IgG to the Fcγ receptor [64,65]. However, we attribute the activation of the murine P388D-1 monocytes through PrP-Fc to binding of the PrP-portion rather than to an interaction with human Fc protein, since Fc protein alone was used as a control and did not show any binding activity. Pyk2, which is structurally related to focal adhesion kinase (FAK), is known to interact with Src-family kinases and also activates the MAPK pathway [50,51]. Additionally, Pyk2 was found to mediate adhesion and stimulation of cytokines in monocytes [66].

Due to the missing band at 120 kDa after pre-incubation with PP1 and stimulation with PrP-Fc, we investigated Cbl activity. The phosphorylation of Cbl was found to be increased after stimulation with PrP-Fc. Cbl is a multifunctional adapter protein which recruits signaling proteins within Syk and PI3K pathways and could have both positive and negative regulatory effects in downstream signals of tyrosine kinases [67–69]. Therefore, we suggest that phosphorylation of Cbl modulates downstream activity of Syk.

To investigate downstream pathways activated by PrP-Fc, we studied phosphorylation of $ERK_{1,2}$ and Akt kinase. Both revealed an increased phosphorylation after PrP-Fc stimulation in comparison to low basal phosphorylation level of the control (Fc). The Src family kinase inhibitor PP1 reduced $ERK_{1,2}$ phosphorylation to just below basal levels (Fig. 6 A, compare lanes 3 + 4 with 7 + 8), whereas Akt phosphorylation was even more reduced by PP1. This happened regardless of subsequent stimulation with PrP-Fc, arguing for the predominant role of Src-family kinase in the regulation of Akt. Interestingly, Wortmannin, a specific inhibitor of PI3K, not only reduced the activity of Akt kinase, but also that of $ERK_{1,2}$, though to a lesser extent. Recently, a significant influence of Wortmannin and the selective inhibitor of MEK, PD98059, on the PrP-Fc-induced neuronal survival of cerebellar granule neurons has been described [27]. This finding is corroborated by our study, where the alternative MEK-inhibitor U0126 strongly decreased the PrP-Fc-induced phosphorylation of $ERK_{1,2}$. Engagement of PrP^C with antibodies, ligand peptides or its cell surface ligands also leads to an activation of compounds belonging to the ERK signaling pathways [53,59,70,71]. The modulating effect of PrP^C expression on the PI3K-Akt pathway has been shown recently [72]. The use of PI3K and MEK inhibitors thus revealed a cross-talk between MEK-$ERK_{1,2}$ and PI3K-Akt kinase pathways. Despite the different approaches and various stimulation methods of the studies, we suggest that they

have in common a similar PrPC signaling pathway including $ERK_{1,2}$ and Akt kinase.

The activation of $ERK_{1,2}$ and Akt kinase is known to induce diverse biological responses in macrophages such as phagocytosis, migration, and cytokine production. The observed activation of the monocyte/macrophage cell line through stimulation with PrP-Fc could be related to these processes. It has been reported recently that macrophages derived from Prnp wild-type ($Prnp^{+/+}$) and $Prnp^{0/0}$ mice have different capabilities of phagocytosis [35]. Moreover, macrophages contact lymphocytes, deliver cytokines, e.g., TNFα for maturation of follicular dendritic cells [73,74], and present antigen structures. Within this context it was shown that chronic follicular inflammation, induced by a variety of causes, induced a focal high expression of PrP in otherwise prion-free organs [75]. The findings reported here may contribute to the understanding of the molecular function of PrP^C and prove helpful in elucidating the pathological process associated with prion diseases.

Acknowledgments

This work was supported by EU NeuroPrion (FOOD-CT-2004-506579). We wish to thank Gertrud Kwiatkowski for excellent technical assistance and Dorothee Rieger for proofreading the manuscript.

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