NADPH oxidase and extracellular regulated kinases 1/2 are targets of prion protein signaling in neuronal and nonneuronal cells

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Putative functions of the cellular prion protein, PrP^C, include resistance to oxidative stress, copper uptake, cell adhesion, and cell signaling. Here, we report NADPH oxidase-dependent reactive oxygen species (ROS) production and extracellular regulated kinases 1/2 (ERK1/2) phosphorylation on PrP^C stimulation in the 1C11 neuroectodermal precursor, in its neuronal differentiated progenies, and in GT1-7 neurohypothalamic and BW5147 lymphoid cells. In neuroprogenitor, hypothalamic, and lymphoid cells, ERK1/2 activation is fully controlled by the NADPH oxidase-dependent ROS production. In 1C11-derived bioaminergic cells, ROS signaling and ERK1/2 phosphorylation are both controlled by Fyn kinase activation, introducing some specificity in PrP^C transduction associated with this neuronal context. These data argue for an ubiquitous function of PrP^C in cell-redox homeostasis through ROS production.

Although the central role of the cellular prion protein (PrP^C) in transmissible spongiform encephalopathies (TSEs) has been recognized for many years, the identification of PrP^C normal function(s) still constitutes a great challenge in the field of prion biology. PrP^C is a ubiquitous glycoprotein anchored to the cell surface by a glycosyl phosphatidyl inositol (GPI) moiety. It is majorly expressed in neuronal cells where TSE-associated damages occur. Knockout experiments (1) have not unraveled any physiological role of PrP^C. However, *ex vivo* studies using cells from PrP null mice or prion-infected cells revealed alterations in copper metabolism and enhanced susceptibility to oxidative stress (2, 3). Biochemical and structural studies (4) of the copper-binding ability of PrP^C bring support to the view of an involvement of PrP^C in copper homeostasis. Altogether, these data have led to the assumption that PrP^C protects neurons from oxidative damage (5). However, the exact relation between PrP^C and oxidative stress remains elusive.

Another set of investigations focuses on the localization of PrP^C on the cell membrane, consistent with a possible role of this protein in cell adhesion or cell signaling. Indeed, PrP^C binds N-CAM (6), laminin, and the laminin receptor (7–9). Such interactions were assumed to reflect some role of PrP^C in neurite extension and maintenance (10) or even in cell survival. The observation that PrP knock-out mice expressing N-terminally truncated PrP suffer from neurodegeneration soon after birth (11) also fits in with the hypothesis that PrP^C may control neuronal functions.

In a recent report (12), we showed a caveolin-dependent coupling of PrP^C to the tyrosine kinase Fyn. This coupling was evidenced by using the murine 1C11 neuronal cell line. The 1C11 clone behaves as a committed neuroepithelial progenitor that lacks neuron-associated functions (13). On induction, nearly all 1C11 cells develop bipolar extensions, express neuronal markers, such as neurofilaments, N-CAM, $\gamma\gamma$-enolase, or synaptophysin, and convert into either $1C11^{5\text{-HT}}$ serotonergic or $1C11^{NE}$ noradrenergic cells. In the 1C11 progenitor and throughout differentiation, PrP^C, caveolin, and Fyn are endogenously expressed (12, 14). However, the PrP^C-dependent Fyn activation is restricted to $1C11^{5\text{-HT}}$ and $1C11^{NE}$ progenies, which have implemented a complete neuronal phenotype including serotonin or noradrenaline synthesis, storage, and transport (12).

In the present study we searched for intracellular targets recruited by PrP^C stimulation. Because of the potential link between the prion protein and the cellular redox state, we investigated whether PrP^C might be coupled with a reactive-oxygen-species (ROS) production pathway in the 1C11 neuronal differentiation model (13) and in GT1-7 hypothalamic (15) and BW5147 lymphoid (16) cells. Indeed, beyond their role in cell toxicity and oxidative stress, ROS may also behave as chemical mediators in signal transduction processes involved in cell proliferation and/or differentiation (17). We identified NADPH oxidase, a major ROS generator in cells, and extracellular regulated kinases 1/2 (ERK1/2), two mitogen-activated protein kinases (MAPKs), as targets of the PrP^C-mediated signaling. Similar responses were observed in neuronal and nonneuronal cells. Moreover, in 1C11 fully differentiated neuronal cells only, an involvement of the Fyn kinase in the control of the PrP^C-induced transduction cascade was established, in agreement with the idea of some specificity of PrP^C signaling related to the expression of a complete serotonergic or noradrenergic phenotype.

Materials and Methods

Antibodies. Monoclonal PrP-targeted antibodies, SAF61, SAF32, and Bar221, were kindly provided by the Service de Pharmacologie et d'Immunologie (Commissariat à l'Energie Atomique, Saclay, France). The polyclonal 1A8 antibody was from C. Farquhar. SAF61, Bar221, and 1A8 target C-ter epitopes, and SAF32 encompasses the 79–92 PrP-epitope. C-20, a monoclonal antibody against the $p47^{PHOX}$ NADPH oxidase subunit, was purchased from Santa Cruz Biotechnology. A polyclonal antibody recognizing the dually phosphorylated region of the active form of ERK1/2 [pTpY185/187] was from BioSource International (Camarillo, CA).

Cell Culture and Enzyme Inhibition. 1C11 cells were cultured and differentiated along the serotonergic ($1C11^{5\text{-HT}}$) or the catecholaminergic ($1C11^{NE}$) pathway as described (13). GT1-7 cells (15) were grown in DMEM/10% FCS. T lymphocytes, BW5147, and the BW5147-derived $Thy1^{-e}$ mutant deficient for GPI synthesis (16), were grown in RPMI/7% FCS.

Abbreviations: ROS, reactive oxygen species; ERK1/2, extracellular regulated kinases 1/2; PrP^C, cellular prion protein; DPI, diphenyleneiodonium chloride; PMA, phorbol 12-myristate 13-acetate; $1C11^{5\text{-HT}}$, 1C11 serotonergic cells; $1C11^{NE}$, 1C11 noradrenergic cells; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase.

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Specific enzyme inhibition was performed by pretreating cells at 37°C for 45 min in their culture medium with the inhibitor. The NADPH oxidase activity was inhibited with diphenyleneiodonium (DPI, Sigma). PP2 and PD98059 (Calbiochem) were used to inhibit the Fyn kinase and the MAPK kinase (MEK), respectively.

Reactive Oxygen Species (ROS) Detection by Fluorescence. Extracellular release of ROS from 1C11 precursor cells, their neuronal progenies ($1C11^{5\text{-HT}}$ and $1C11^{NE}$), GT1-7 cells, or T lymphocytes was followed by using the fluorogenic reagent OxyBurst Green H_2HFF BSA (Molecular Probes). In a typical assay, cells were washed twice with PBS, supplemented with 1 mM Ca^{2+} and Mg^{2+}, and preincubated for 2 min at 37°C in the presence of the fluorogenic reagent (10 $\mu g \cdot ml^{-1}$). Along cell stimulation with PrP antibodies, the fluorescence was continuously recorded at $\lambda = 528$ nm (slit width = 10 nm) with excitation at $\lambda = 488$ nm (slit width = 10 nm) by using a Cary Eclipse fluorometer (Varian). Fluorescence measurements with 1C11 or GT1-7 adherent cells and with lymphoid cells were performed in a 96-well microculture plate and in a 1-ml, stirred, thermostated four-optical fluorescence cuvette, respectively.

Preparation of Cytosolic Extracts. 1C11 precursor, $1C11^{5\text{-HT}}$, $1C11^{NE}$, GT1-7, and lymphoid cells were washed in PBS with 1 mM Ca^{2+} and Mg^{2+} and incubated for 30 min at 4°C in NET lysis buffer [50 mM Tris·HCl (pH 7.4)/150 mM NaCl/5 mM EDTA/1% Triton X-100/1 mM Na_3VO_4 and a mixture of protease inhibitors; Roche]. Extracts were centrifuged at 14,000 × *g* for 15min. Protein concentrations in the supernatant were measured by using the bicinchoninic acid method (Pierce). Cytosolic extracts were stored at −80°C before analysis.

Assessment of ERK1/2 Phosphorylation by Western Blotting. Twenty micrograms of cytosolic proteins were resolved by SDS/10% PAGE and transferred to Immobilon membranes (Millipore). Membranes were blocked in 5% nonfat dried milk and Tris-buffered saline with 0.1% Tween 20 for 1 h at room temperature and then incubated overnight at 4°C with 0.5 $\mu g \cdot ml^{-1}$ ERK1/2 [pTpY185/187] primary antibody. Bound antibody was revealed by enhanced chemiluminescence detection (Amersham Pharmacia).

Immunoprecipitation of ^{32}P-Labeled $p47^{PHOX}$ NADPH Oxidase Subunit. 1C11 precursor, $1C11^{5\text{-HT}}$, or $1C11^{NE}$ cells were washed in PBS and incubated in phosphate-free DMEM containing 50 μCi of ^{32}P per ml per 10^6 cells for 1 h at 37°C. After cell stimulation with PrP antibodies, ^{32}P-labeled cells were scrapped off into ice-cold NET lysis buffer and centrifuged at 14,000 × *g* for 15 min at 4°C. The $p47^{PHOX}$ NADPH oxidase subunit was immunoprecipitated overnight at 4°C under gentle mixing by using Sepharose G beads (Amersham Pharmacia) coupled to C-20 antibody. Immunocomplexes were resolved by SDS/10% PAGE and transferred to Immobilon membranes. ^{32}P-labeled $p47^{PHOX}$ was detected by using a PhosphorImager (Molecular Dynamics). For each immunoprecipitate, equal amounts of $p47^{PHOX}$ subunit were loaded on the gel, as estimated by Western blotting by using the C-20 antibody.

Results

PrP^C-Dependent NADPH Oxidase Activation Causes ROS Production in Neuronal, Hypothalamic, and Lymphoid Cells. Antibody-mediated ligation was used to mimic an extracellular signal acting on PrP^C. On binding of distinct antibodies against PrP, ROS markedly accumulated in 1C11 precursor cells, as revealed by monitoring the fluorescence of OxyBurst Green H_2HFF BSA (Molecular Probes) (Fig. 1*a*). The PrP^C-mediated ROS release became detectable as soon as 5 min after antibody addition and reached a plateau after 40 min. This response resembles that observed on exposure of 1C11 cells to the phorbol ester PMA, a potent activator of NADPH oxidase through PKCs (ref. 18 and data not shown). ROS production was also observed with $1C11^{5\text{-HT}}$ and $1C11^{NE}$ differentiated cells (Fig. 1*a*). PrP^C ligation also elicited a ROS signal in GT1-7 hypothalamic cells (Fig. 1*a*). The intensity of the ROS response was in the same order of magnitude as that monitored in the 1C11 cell line.

To possibly enlarge this observation to cell systems of non-neuronal origin, experiments were performed with BW5147, a T lymphoid cell line, which expresses PrP^C at its surface (data not shown), and with the $Thy1^{-e}$ cell line, a glycosylation mutant derived from the BW5147 cell line and deficient for GPI synthesis (16). PrP^C activation on BW5147 cells triggered a burst of ROS (Fig. 1*b*). No significant response was obtained with the $Thy1^{-e}$ cell line lacking PrP^C at its cell surface (Fig. 1*b*). The PrP^C-dependent coupling therefore appears not to be restricted to neuronal or neuroendocrine cells but to also participate to the signaling networks of lymphoid cells.

Because NADPH oxidase is known to be a powerful ROS generator, we investigated whether this enzyme was involved in the PrP^C-mediated ROS response. NADPH oxidase is a multicomponent protein, whose recruitment involves phosphorylation steps and participation of members of the Ras superfamily of small G proteins (19). We observed that the binding of specific antibodies to PrP^C on 1C11 cells markedly raised the level of phosphorylation of $p47^{PHOX}$, a NADPH oxidase subunit described as a substrate of PKCs (20). Similar effects were obtained whatever the differentiation state of the 1C11 cells (Fig. 1*c*). Exposure to diphenyleneiodonium (DPI), a selective inhibitor of NADPH oxidase, cancelled the PrP^C-induced ROS production in 1C11 precursor cells (Fig. 1*d*). Comparable results were obtained with 1C11-derived bioaminergic neurons and with GT1-7 and BW5147 cells (data not shown). Altogether, these data identify a NADPH oxidase-dependent ROS production pathway as a feature of PrP^C signaling activity.

PrP^C Stimulation Promotes Phosphorylation of ERK1/2 in Neuronal and Lymphoid Cells. A large number of signaling pathways appears to be regulated by ROS. The broad range of ROS action includes kinase activation, down-regulation of phosphatase activity and direct regulation of transcription factors (17). We focused our search of downstream targets of the PrP^C-NADPH cascade on members of the MAPK family of serine/threonine kinases. Indeed, the redox-mediated activation of MAPK has been established in a number of studies (17). Three distinct groups of kinases belong to the MAPK family: ERKs or MAPK which are involved in cell proliferation and differentiation, c-Jun NH_2-terminal kinase and p38MAPK. The two latter kinases are implicated in inflammatory responses, cell cycle arrest, DNA repair, and cell death.

In 1C11 cells, the antibody-mediated ligation of PrP^C did not elicit any modification in the phosphorylation level of c-Jun NH_2-terminal kinase or of p38MAPK, at least within 30 min (data not shown). By contrast, by 5 min after PrP^C ligation by various antibodies, phosphorylation of both $p44^{ERK1}$ and $p42^{ERK2}$ was promoted (Fig. 2*a*). This ERK stimulation was transient. ERK phosphorylation returned to its basal level by 30 min after antibody ligation (data not shown). Similar effects on phosphorylation of $p44^{ERK1}$ and $p42^{ERK2}$ were observed in $1C11^{5\text{-HT}}$ and $1C11^{NE}$ neuronal cells (Fig. 2*b*). PrP^C-induced ERK activation also occurred in GT1-7 hypothalamic cells and in the BW5147 lymphoid cell line (Fig. 2*b*). As expected, PrP-antibodies had no effect on the $Thy1^{-e}$ cell line (Fig. 2*b*).

To examine a possible role of MEKs in the PrP^C-induced ERK1/2 signaling cascade, we used PD98059, a specific inhibitor of MEK1/2. With 1C11 precursor cells and their bioaminergic progenies, as well as with GT1-7 hypothalamic and BW5147

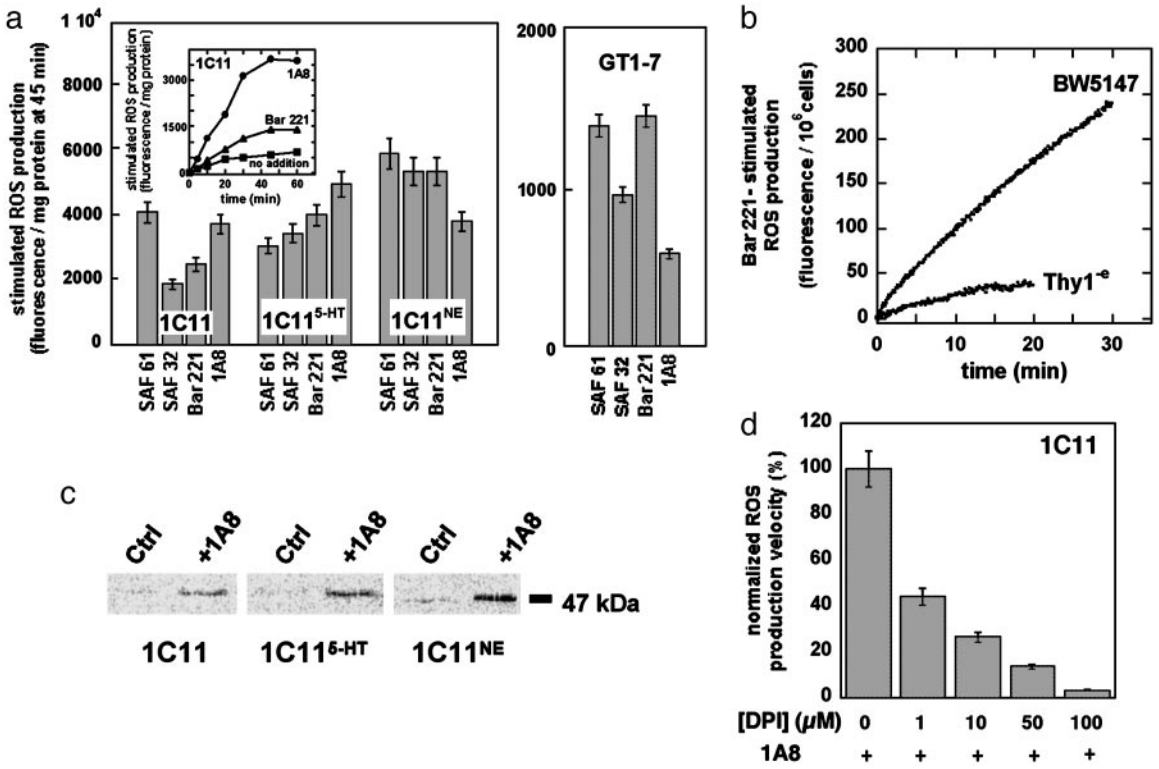

Fig. 1. PrP^C-induced ROS production through NADPH oxidase activity. Extracellular release of ROS was detected by using the fluorogenic reagent OxyBurst Green H_2HFF BSA. (*a Inset*) The PrP^C-mediated ROS production was followed during 60 min with 1C11 precursor cells stimulated by 10 μg·ml^{-1} of PrP-targeted antibodies 1A8 (●) or Bar221 (▲). Background ROS production was also recorded with unstimulated cells (■). (*a*) Antibodies SAF61, Bar221, 1A8, and SAF32 were used at 10 μg·ml^{-1} to elicit ROS production with 1C11 precursor cells, either 1C11$^{5\text{-HT}}$ serotonergic or 1C11^{NE} noradrenergic cells, and with GT1-7 hypothalamic cells. ROS release was completed within 45 min of stimulation. ROS levels at 45 min are expressed in fluorescence intensity per mg of protein. The basal level of ROS production was substracted to the shown signals of PrP^C-induced ROS release. (*b*) ROS release induced by Bar221-mediated PrP^C ligation was also monitored with the BW5147 lymphoid cell line. As a control, ROS production was followed in the BW5147-derived Thy1^{-e} mutant, deficient for GPI synthesis. (*c*) PrP^C stimulation induces NADPH oxidase p47^{PHOX} phosphorylation. After metabolic labeling with [^{32}P]PO_4^{3-}, 1C11 precursor, 1C11$^{5\text{-HT}}$ and 1C11^{NE} cells were stimulated with 1A8 antibody for 1 h. ^{32}P-labeled p47^{PHOX} (47 kDa) was immunoprecipitated from cytosolic extracts and detected as described in *Materials and Methods*. Control experiments (Ctrl) without antibody stimulation are also shown. (*d*) Involvement of NADPH oxidase in the PrP^C-induced ROS production was confirmed by using DPI, a specific inhibitor of NADPH oxidase activity. 1C11 precursor cells were preincubated with DPI concentrations ranging from 1 to 100 μM and exposed to PrP antibodies in the presence of DPI. ROS release was followed over a period of 45 min. Shown are the mean ROS release velocities deduced from these experiments. Values of fluorescence intensity per mg of protein per time unit are normalized with respect to the value in the absence of DPI (100%). Data shown are representative of a set of three independent experiments.

lymphoid cells, the addition of this inhibitor completely cancelled ERK1/2 phosphorylation (Fig. 2*c*). We therefore concluded that MEK1/2 activation mediates the PrP^C signal targeting ERK1/2.

In 1C11 Precursor, GT1-7, and BW5147 Cells, PrP^C-Mediated ERK1/2 Phosphorylation Is Strictly Controlled by NADPH Oxidase Activity. In all cell types examined here, NADPH oxidase and the MEK/ERK1/2 module are downstream intracellular targets of the PrP^C-induced signaling. We wondered whether ROS could take part in ERK activation. The idea of an involvement of ROS in the PrP^C-dependent ERK activation pathway was indeed supported by our observation that PMA increases ERK phosphorylation in the 1C11 and GT1-7 cell lines as well as in T lymphocytes. Moreover, PMA action on ERK phosphorylation was switched off with DPI, an inhibitor of NADPH oxidase (Fig. 3*a*).

In 1C11 precursor, GT1-7 hypothalamic and BW5147 T cells, the PrP^C-dependent ERK phosphorylation was also quenched on addition of DPI (Fig. 3*a*). However, as shown in Fig. 3*a*, in 1C11$^{5\text{-HT}}$ cells, nearly 40% of the PrP^C-induced ERK1/2 phosphorylation persisted despite DPI addition. In 1C11^{NE} cells, >90% of the PrP^C-dependent ERK1/2 phosphorylation escaped the PrP^C-NADPH oxidase cascade. To account for these observations, one must admit that, in 1C11 cells harboring a complete serotonergic or noradrenergic phenotype, alternative signaling pathway(s), independent of NADPH oxidase, also contribute(s) to the PrP^C-induced ERK1/2 phosphorylation.

In 1C11-Derived Serotonergic or Noradrenergic Neurons, the PrP^C-Fyn Coupling Exerts an Upstream Control on ROS Production and ERK1/2 Activation. The identification of a PrP^C-Fyn coupling in the 1C11 cell line prompted us to examine whether Fyn was involved in the PrP^C-ERK signaling pathways. In either 1C11$^{5\text{-HT}}$ or 1C11^{NE} cells, inhibition of the Fyn kinase with PP2 (50 pM) fully cancelled the PrP^C-related ERK signal (Fig. 3*b*). PP2 addition also quenched the PrP^C-mediated ROS production (Fig. 3*c*). In contrast, in 1C11 precursor cells, PP2 treatment (50 pM) neither reduced the amplitude of ERK1/2 phosphorylation (Fig. 3*b*) nor interfered with the PrP^C-dependent NADPH oxidase activation (Fig. 3*c*). These results are in good agreement with our previous observation that the PrP^C-Fyn coupling is restricted to 1C11 cells which have acquired neuronal and neurotransmitter-associated functions (12). In both serotonergic and noradrenergic 1C11-derived cells, recruitment of the tyrosine kinase Fyn hence appears to be a prerequisite to both NADPH oxidase activation and ERK1/2 phosphorylation.

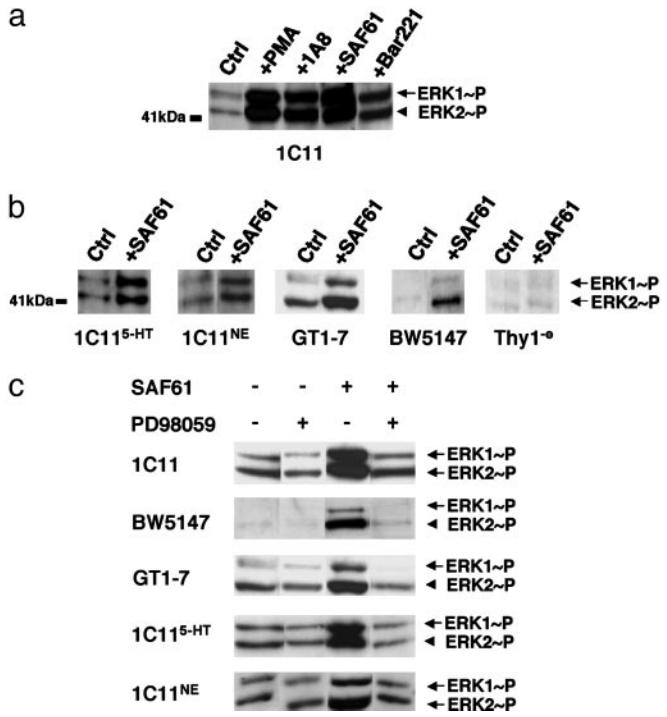

Fig. 2. PrP^C stimulation induces MEK-ERK1/2 phosphorylation. 1C11 precursor, 1C11$^{5\text{-HT}}$ serotonergic and 1C11^{NE} noradrenergic cells, GT1-7 hypothalamic cells, and BW5147 and Thy1^{-e} lymphoid cell lines were stimulated for 5 min by using various PrP-targeted antibodies (10 μg·ml^{-1}) or PMA (1 μM), a potent activator of PKCs. (*a*) Stimulated 1C11 progenitor cells lysates were immunoblotted by using ERK1/2 [pTpY185/187] antibody. Control experiments (Ctrl) with unstimulated cells were also performed. (*b*) ERK1/2 phosphorylation profiles of 1C11$^{5\text{-HT}}$, 1C11^{NE}, and GT1-7 cells and of BW5147 and Thy1^{-e} lymphoid cell lines are compared. All cells were submitted to stimulation by the SAF61 antibody. Also shown are the control experiments without antibody addition. (*c*) Selective inhibition of MEK1/2 abrogates PrP^C-induced ERK activation. Before PrP^C–antibody stimulation, cells were preincubated for 45 min in the presence of PD98059, a selective inhibitor of MEK (5 μM with 1C11 precursor, 1C11$^{5\text{-HT}}$ and 1C11^{NE} cells and with BW5147 T lymphocytes and 20 μM with GT1-7 cells). Cytosolic extracts were immunoblotted by using the ERK1/2 [pTpY185/187] antibody. In all sets of experiments, it was checked that the PD98059 concentration used did not impair cell viability according to trypan blue staining. Data shown are representative of a set of three independent experiments.

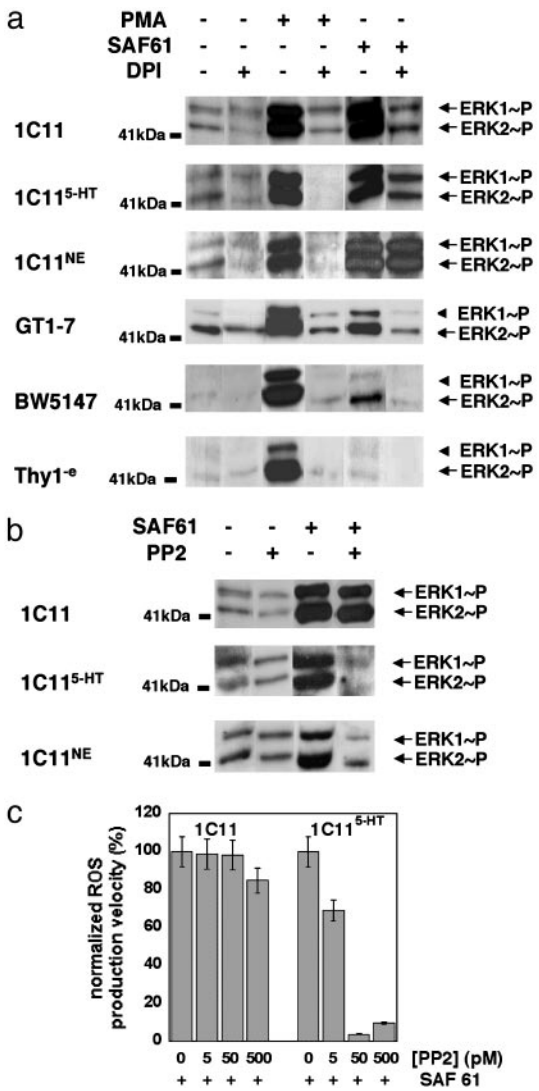

Fig. 3. ROS involvement in the PrP^C-induced ERK1/2 phosphorylation. (*a*) NADPH oxidase implication in the PrP^C-ERK1/2 activation pathway was assessed in 1C11 precursor, 1C11$^{5\text{-HT}}$ and 1C11^{NE} cells, in GT1-7 hypothalamic cells and in BW5147 and Thy1^{-e} lymphoid cell lines. Cells were incubated for 45 min in the presence of DPI (100 μM with 1C11 cells and its differentiated progenies, 25 μM with GT1-7 hypothalamic cells, 5 μM with BW5147, and 1 μM with Thy1^{-e}) and then stimulated for 5 min by addition of SAF61 or PMA in the presence of DPI. Cytosolic extracts were immunoblotted by using the ERK1/2 [pTpY185/187] antibody. (*b*) Fyn tyrosine kinase implication in the PrP^C-ERK1/2 signal transduction pathway was assessed in 1C11 precursor and in either 1C11$^{5\text{-HT}}$ or 1C11^{NE} cells. Cells were preincubated for 45 min with 50 pM PP2 before SAF61-mediated stimulation. ERK1/2 phosphorylation was detected as above. (*c*) Fyn tyrosine kinase involvement in the PrP^C-mediated NADPH oxidase recruitment was assessed in 1C11 precursor and in 1C11$^{5\text{-HT}}$ cells. Before SAF61 stimulation, cells were treated with PP2 concentrations ranging from 5 pM to 0.5 nM. PrP^C-induced ROS production was monitored during 45 min as described in Fig. 1*d*. In all sets of experiments, it was checked that the DPI or PP2 concentrations used did not impair cell viability according to trypan blue staining. Data shown are representative of a set of three independent experiments.

Discussion

The biological function(s) of prion protein is fervently debated and still enigmatic. The prime evidence of a PrP^C-Fyn coupling mediated by caveolin in an *in vitro* inducible neuronal cell line (1C11) lent support to the hypothesis that PrP^C acts as a cell surface signaling molecule. The aim of the present study was to identify ubiquitous intracellular responses mediated by PrP^C stimulation. Because the physiological signals acting on PrP^C remain unidentified, the experimental approach was to mimic an extracellular ligand with PrP-targeted antibodies. We show that antibody-mediated PrP ligation promotes NADPH oxidase activation and ERK1/2 phosphorylation in the 1C11 neuroectodermal progenitor and its neuronal derivatives, in GT1-7 hypothalamic and BW5147 lymphoid cells. These results indicate for the first time that PrP^C signaling activity is not restricted to neuronal cells. Moreover, common intracellular targets are recruited by the PrP^C-mediated signals whatever the cell type studied here. Finally, the nature of the signaling intermediates involved in the PrP^C transduction cascade varies depending on the cell context. In both differentiated 1C11$^{5\text{-HT}}$ and 1C11^{NE} cells, which have acquired the overall neurotransmitter-associated functions of serotonergic or noradrenergic neurons, Fyn activation controls the downstream targets, thereby constituting a level of specificity in PrP^C signaling.

One main observation is the ROS generation through NADPH oxidase activation on PrP^C ligation. ROS act as "second message" signals and control the activation of the

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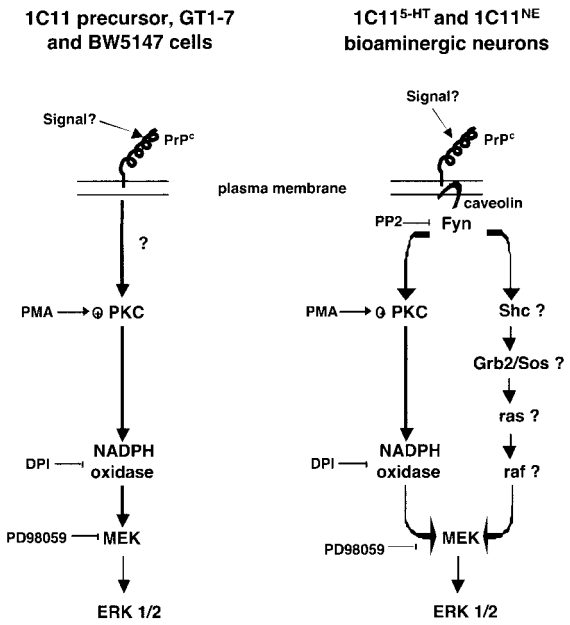

Fig. 4. Model of PrP^C-dependent signaling pathways in 1C11 precursor cells, in 1C11-derived serotonergic ($1C11^{5\text{-HT}}$) or noradrenergic ($1C11^{NE}$) cells, in GT1-7 hypothalamic cells and in BW5147 T lymphocytes. See text for further details.

MEK-ERK1/2 module (Fig. 4). In 1C11 precursor, GT1-7 and BW5147 cells, the NADPH oxidase-dependent ROS signaling fully governs the PrP^C-induced ERK pathway. By analogy with the mode of action of PMA on ROS production, PKCs are likely to participate to the PrP^C-dependent NADPH oxidase recruitment at the plasma membrane through direct phosphorylation of the NADPH oxidase $p47^{PHOX}$ cytosolic subunit (18). Because PrP^C is anchored to the outer membrane, the PrP^C-dependent signaling mechanisms engaging the linear PKC-NADPH oxidase-MEK-ERK1/2 cascade are likely to also involve yet-to-be identified cellular partners (Fig. 4). In 1C11-derived bioaminergic neurons, we find that Fyn governs all of the PrP^C-induced pathways that converge to the MEK-ERK1/2 module. A first pathway clearly involves the ROS signaling. The second one is NADPH oxidase-independent and possibly involves the Shc-Grb2/SOS-Ras-MEK cascade (21–23) (Fig. 4). In line with this idea, in integrin signaling, a Ras-ERK pathway has been shown to depend on the recruitment of the caveolin-Fyn complex (24). Moreover, Grb2 has been identified as a PrP-interacting protein (25).

In fully differentiated 1C11 progenies, caveolin appears to be one of the protagonists involved in PrP^C-coupling to the tyrosine kinase Fyn (12). Some cell specificity of PrP^C signaling may hence be related to the onset of a PrP^C-caveolin-Fyn complex. Neither GT1-7 hypothalamic (26) nor BW5147 lymphoid cells (27) appear to express caveolin. Therefore, in these cells, the direct control of NADPH oxidase on PrP^C-induced ERK activation, within a single transduction pathway, may be accounted for by the lack of a functional PrP^C-caveolin-Fyn ternary complex. In 1C11 precursor cells, despite the presence of caveolin, the phosphorylation level of Fyn is not sensitive to PrP^C stimulation. Actually, in these cells, PrP^C was shown not to physically and functionally interact with caveolin. In contrast, in fully differentiated serotonergic or noradrenergic progenies, which express the overall neurotransmitter-associated functions of bioaminergic neurons, PrP^C-caveolin interaction and Fyn activation were shown to occur preferentially at neurites (12, 28). Therefore, in these bioaminergic neuronal cells, one can imagine a localization of PrP^C, caveolin and Fyn within specialized microdomains known to concentrate other signaling effectors (29, 30). Potential partners would include the adaptator molecule Shc and the signaling proteins Ras, Raf, MEK and ERK (22, 23). The targeting of PrP^C in such caveolin-rich microdomains would thus expand the repertoire of PrP^C downstream pathways. At this stage, one difficulty is to explain why the PrP^C-dependent signaling is fully governed by Fyn in 1C11-derived neuronal cells. One would expect that the PrP^C molecules on the cell bodies elicit a Fyn–independent response, similar to that observed in the precursor cells. Possibly, in a neuronal fully differentiated context, prion proteins on the cell stroma are switched off from any signaling function or involved in transduction pathway(s) distinct from that described here. Another idea is that, among the PrP^C isoforms, those competent for signaling are selectively targeted to neuritic varicosities known as major sites of signal transduction.

To conclude, by linking ERKs phosphorylation to PrP^C, the present study strengthens the view that PrP^C activation may participate to the survival of neurons and also plays a role in lymphoid cell homeostasis. Indeed, ERKs have emerged as key mediators of cell survival and as proliferation signals (31). Our results also shed some new light on the relationship between PrP^C and ROS. In this work, emphasis has been mainly placed on PrP^C-dependent production of ROS acting as signaling molecules. The demonstration of a link between PrP^C and cellular ROS enables to establish a bridge between PrP and the redox state of the cell. Actually, enhanced susceptibility to oxidants of prion-infected or PrP-deficient cells has been observed (3, 32). Clearly, in such cases, an alteration of PrP^C function is relatable to some loss in the control of the cell redox equilibrium (5, 33). Moreover, induced-oxidative stress has been reported to trigger a sustained activation of ERK, itself promoting neuronal cell death (34, 35). Hence, the occurrence of a PrP^C-mediated NADPH oxidase coupling provides foundation for uncovering whether prion infection contributes to neurodegeneration through modifications of ROS production and ERK stimulation. Hopefully, the 1C11 and the GT1-7 cell systems, which combine PrP^C signaling activity and susceptibility to prions (unpublished data, 36), will help to challenge these ideas.

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