# Role of glycosylphosphatidylinositols in the activation of phospholipase $A_2$ and the neurotoxicity of prions

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Prion-induced neuronal injury *in vivo* is associated with prostaglandin $E_2$ production, a process that can be reproduced in tissue-culture models of prion disease. In the present study, neuronal phospholipase $A_2$ was activated by glycosylphosphatidylinositols (GPIs) isolated from the cellular prion protein ($PrP^c$) or from disease-associated isoforms ($PrP^{Sc}$), resulting in prostaglandin $E_2$ production, but not by GPIs isolated from Thy-1. The ability of GPIs to activate neuronal phospholipase $A_2$ was lost following the removal of acyl chains or cleavage of the phosphatidylinositol–glycan linkage, and was inhibited by a mAb that recognized phosphatidylinositol. In competition assays, pretreatment of neurons with partial GPIs, inositol monophosphate or sialic acid reduced the production of prostaglandin $E_2$ in response to a synthetic miniprion (sPrP106), a synthetic correlate of a $PrP^{Sc}$ species found in Gerstmann–Sträussler–Scheinker disease (HuPrP82–146), prion preparations or high concentrations of PrP-GPIs. In addition, neurons treated with inositol monophosphate or sialic acid were resistant to the otherwise toxic effects of sPrP106, HuPrP82–146 or prion preparations. This protective effect was selective, as inositol monophosphate- or sialic acid-treated neurons remained susceptible to the toxicity of arachidonic acid or platelet-activating factor. Addition of PrP-GPIs to cortical neuronal cultures increased caspase-3 activity, a marker of apoptosis that is elevated in prion diseases. In contrast, treatment of such cultures with inositol monophosphate or sialic acid greatly reduced sPrP106-induced caspase-3 activity and, in co-cultures, reduced the killing of sPrP106-treated neurons by microglia. These results implicate phospholipase $A_2$ activation by PrP-GPIs as an early event in prion-induced neurodegeneration.

## INTRODUCTION

The transmissible spongiform encephalopathies (TSEs) or prion diseases are fatal, neurodegenerative disorders that include Creutzfeldt–Jakob disease (CJD) in man, bovine spongiform encephalopathy in cattle and scrapie in sheep and goats. They are thought to develop following the conversion of a normal host protein, designated $PrP^c$, into disease-associated isoforms, $PrP^{Sc}$ (Prusiner, 1998). This process results in a profound change in the biochemical properties of the PrP protein, such that the $\alpha$-helical structure of $PrP^c$ is converted to the $\beta$-sheet-rich forms of $PrP^{Sc}$ (Pan *et al.*, 1993). This conformational change also confers partial resistance to digestion with proteinase K, a property that defines disease-associated $PrP^{Sc}$ from the normal cellular $PrP^c$ isoform (Prusiner, 1982). The subsequent accumulation of insoluble, fibrillar aggregates of $PrP^{Sc}$ is thought to lead to neuronal dysfunction, degeneration (Williams *et al.*, 1997a; Jeffrey *et al.*, 2000) and death via caspase-3-associated apoptosis (Giese *et al.*, 1995; Jamieson *et al.*, 2001).

The cellular mechanisms leading to neuronal death can be studied in tissue-culture systems to determine the functional significance of *in vivo* observations. Thus, neuronal culture systems developed to investigate interactions between prions and neurons have demonstrated that synthetic peptides derived from the prion protein are neurotoxic (Forloni *et al.*, 1993; Salmona *et al.*, 2003), provided that the peptides contain substantial $\beta$-sheet content (Hope *et al.*, 1996). One of the important events leading to the degeneration of cultured neurons following the addition of toxic PrP peptides is the activation of phospholipase $A_2$ ($PLA_2$) and the subsequent metabolism of arachidonic acid to prostaglandins (PGs) by the cyclo-oxygenases (COXs) (Bate *et al.*, 2004b). This observation is consistent with observations that levels of $PGE_2$ are raised in brain areas showing neuronal death in murine scrapie (Williams *et al.*, 1994, 1997b) and that raised levels of $PGE_2$ are detected in the cerebrospinal fluid of patients with CJD (Minghetti *et al.*, 2000, 2002). However, little is known about the processes by which prions or neurotoxic PrP peptides activate $PLA_2$.

Cellular $PrP^c$ is required for the process by which PrP peptides induce apoptosis (Brown *et al.*, 1994), suggesting that there are specific interactions between PrP peptides, $PrP^c$, $PLA_2$ and apoptotic pathways. Most $PrP^c$ molecules are linked to membranes via a glycosylphosphatidylinositol (GPI) anchor (Stahl *et al.*, 1992); the presence of the GPI anchor affects the properties of $PrP^c$ (Taraboulos *et al.*, 1995). GPI anchors contain a conserved core that consists of ethanolamine phosphate in an amide linkage to the carboxyl terminus of the protein, three mannose residues, glucosamine and phosphatidylinositol (Mayor & Riezman, 2004). However, many variations on this core structure are possible and the GPIs isolated from $PrP^c$ in hamster brains contain high amounts of galactose, mannose and sialic acid (Stahl *et al.*, 1992). We therefore investigated the ability of GPIs extracted from both $PrP^c$ and $PrP^{Sc}$, obtained from uninfected and infected cells of a murine neuroblastoma cell line, and GPIs extracted from Thy-1 to activate $PLA_2$, as measured by the induction of $PGE_2$. We also examined the ability of GPIs from these sources to induce caspase-3 activity in primary cultures of cortical neurons, as this enzyme is known to be involved in apoptotic cell death. To determine which moiety of the GPI molecules was responsible for the effects observed in each case, the inhibitory effect of some GPI-related molecules on the induction of $PGE_2$ and on the neuronal toxicity of PrP peptides was investigated.

## METHODS

**Cell lines.** Murine neuroblastoma NB4-1A3 cells were grown in neurobasal medium supplemented with 2 mM glutamine, 100 U penicillin $ml^{-1}$, 100 μg streptomycin $ml^{-1}$ and 1 % B27 components (Invitrogen). Uninfected N2a neuroblastoma cells and prion-infected ScN2a cells were grown in Hams F12 medium containing 2 mM glutamine, 200 nM retinoic acid, 5 % fetal calf serum (FCS), 100 U penicillin $ml^{-1}$ and 100 μg streptomycin $ml^{-1}$. NB4-1A3 cells were plated into 96-well plates at $5 \times 10^4$ cells per well and allowed to adhere overnight. The following day, cells were pretreated with test compounds for 3 h before addition of PrP peptides or prion preparations for a further 24 h. Cell survival was determined by treating cultures with WST-1 (Roche Diagnostics); percentage cell survival was calculated by reference to untreated cells incubated with WST-1 (100 %). $PLA_2$ activity was determined by measuring $PGE_2$ in NB4-1A3 cells plated in 24-well plates at $5 \times 10^5$ cells per well that had adhered overnight. Cells were then treated with test compounds for 3 h before the addition of PrP peptides or prion preparations. After a further 24 h, total $PGE_2$ levels were determined by using a competitive enzyme immunoassay kit (Amersham Biosciences) according to the manufacturer's instructions. All experiments were performed in triplicate with each of three separate batches of GPI preparations.

**Primary neuronal cultures.** Primary cortical neurons were prepared from the brains of mouse embryos as described previously (Bate *et al.*, 2002) and plated in 48-well plates at $2 \times 10^6$ cells per well. Cultures were pretreated with test compounds for 3 h before the addition of PrP peptides. Caspase-3 activity was measured 24 h later by using a fluorometric immunosorbent enzyme assay kit according to the manufacturer's instructions (Roche Diagnostics). For cell-survival assays, microglia [prepared by dissociating the cerebral cortices of newborn mice, as described previously (Bate *et al.*, 2002)] were added to peptide-treated neurons in the ratio of 1 microglial cell : 10 neurons. After 4 days, microglia were removed by shaking (260 r.p.m for 30 min) and survival of neurons was determined by treating cultures with WST-1.

**Prion peptides.** The toxic peptide HuPrP82–146, containing aa 82–146 of the human prion protein found in Gerstmann–Sträussler–Scheinker disease (Salmona *et al.*, 2003), and the synthetic miniprion sPrP106 derived from the murine PrP sequence were synthesized by solid-phase chemistry and purified by reverse-phase HPLC (Bonetto *et al.*, 2002).

**Prion preparations.** PrP molecules resistant to limited protease digestion (10 μg proteinase K $ml^{-1}$ for 1 h at 37 °C) were partially purified by reverse-phase chromatography on a C18 Sep-Pak column (Waters) and quantified by a PrP-specific ELISA, as described previously (Bate *et al.*, 2004a).

**Reagents.** Phosphatidylinositol, inositol monophosphate, inositol, inositol-1,4-bisphosphate, inositol-1,4,5-triphosphate, arachidonic acid, platelet-activating factor (PAF), hydrogen peroxide, mannose, glucosamine, galactose, sialic acid (*N*-acetylneuraminic acid) and staurosporine were obtained from Sigma.

**Isolation of GPI anchors.** GPIs were isolated from uninfected N2a cells that were lysed in water, passed through a 26-gauge needle to solubilize cellular debris and centrifuged (10 min at 14 000 ***g***). Insoluble material was suspended in a buffer containing 10 mM Tris/HCl, 100 mM NaCl, 10 mM EDTA, 0·5 % Nonidet P-40, 0·5 % sodium deoxycholate and 2 mM PMSF, pH 7·2. $PrP^c$ was immunoprecipitated following the addition of mAb SAF53 (a gift from Professor J. Grassi, CEA, Saclay, France) and protein G–agarose (Sigma). A sample of immunoprecipitated $PrP^c$ was retained for analysis by Western blot. The depleted lysate was subsequently incubated with an anti-Thy-1 mAb (Serotech) and protein G–agarose and immunoprecipitated. Precipitates were washed five times with PBS containing 0·02 % Tween 20, suspended in PBS containing 100 μg proteinase K $ml^{-1}$ and digested at 37 °C for 24 h to release GPIs. Insoluble material was collected by centrifugation at 14 000 ***g*** and the pellet was washed five times with water. The released GPIs were extracted with water-saturated butan-1-ol, washed with water, split into two and lyophilized. One sample was dissolved in ethanol at 2 μg $ml^{-1}$ and applied to silica gel 60 high-performance TLC (HPTLC) plates (Whatman) for analysis; the other sample was dissolved in tissue-culture medium for bioassays. Controls were prepared by incubating mAb SAF53 in buffer (in the absence of cellular lysates) and protein G–agarose. Control preparations were treated as above.

**Isolation of $PrP^{Sc}$-GPI.** GPIs were also isolated from prion-infected ScN2a cells that were lysed in water and treated as described above before solubilization in extraction buffer that did not contain PMSF. As ScN2a cells contain both $PrP^c$ and $PrP^{Sc}$, cell lysates were predigested with 10 μg proteinase K $ml^{-1}$ for 1 h at 37 °C to remove $PrP^c$. Digestion was stopped with 5 mM PMSF and protease-resistant $PrP^{Sc}$ was immunoprecipitated with mAb SAF53 and protein G–agarose. After extensive washing, immunoprecipitated $PrP^{Sc}$ was split into two samples. One sample was retained for Western blot analysis, whilst the other was further digested with 100 μg proteinase K $ml^{-1}$ at 37 °C for 24 h to release the GPIs. GPIs were subsequently extracted with water-saturated butan-1-ol, as described above.

**Western blotting.** Samples were dissolved in 50 μl Laemmli buffer (Bio-Rad), boiled and subjected to electrophoresis on a 15 % polyacrylamide gel. Proteins were transferred onto a Hybond-P PVDF membrane (Amersham Biosciences) by semi-dry blotting. Membranes were blocked by using 10 % milk powder in Tris-buffered saline, pH 7·2, containing 0·2 % Tween 20. PrP was detected by incubation with mAb SAF53, followed by a secondary anti-mouse IgG conjugated to peroxidase. Bound antibody was visualized by using an enhanced chemiluminescence kit (Amersham Biosciences).

**TLC immunoblotting.** Extracted GPIs were examined by HPTLC on silica gel 60 HPTLC plates by using a mixture of choloroform/methanol/water (10 : 10 : 2·5 by volume). Plates were soaked in 0·1 % poly(isobutylmethacrylate) in hexane, dried and blocked with PBS containing 5 % milk powder. They were probed with 1 μg mAb 5AB3-11 $ml^{-1}$ [which binds to phosphatidylinositol (Bate & Kwiatkowski, 1994)], washed with PBS/Tween and incubated with goat anti-mouse IgG conjugated to peroxidase (Sigma) for 1 h. Bound antibody was washed and visualized by using an enhanced chemiluminescence kit (Amersham Biosciences).

**Chemical manipulation of GPIs.** To remove acyl chains from GPIs (deacylation), extracted GPIs (2 μg $ml^{-1}$) were treated with 0·05 M NaOH at 60 °C for 2 h and the reaction mixture was then neutralized. Extracted GPIs (2 μg $ml^{-1}$) were also deaminated by treatment with a mixture containing 0·1 M sodium acetate, pH 3·8, and 0·5 M sodium nitrite ($NaNO_2$) at room temperature for 24 h, after which time the reaction mixture was neutralized.

**Statistical analysis.** Results were compared by using one- and two-way analysis of variance techniques as appropriate. Statistical significance was set at the 1 % level.

## RESULTS

### Induction of $PGE_2$ by GPIs associated with $PrP^c$ and $PrP^{Sc}$

Neuronal death induced by prions or PrP peptides is accompanied by increased $PGE_2$ production, a marker of the activity of the $PLA_2$/COX pathway (Bate *et al.*, 2004b). Similarly, overnight incubation of NB4-1A3 neuroblastoma cells with GPIs extracted from N2a cells ($PrP^c$-GPI) or from ScN2a cells containing the $PrP^{Sc}$ isoform ($PrP^{Sc}$-GPI) caused a significant increase in the production of $PGE_2$, compared with untreated cells or cells incubated with GPIs isolated from Thy-1 (Table 1). No significant differences were observed in the amounts of $PGE_2$ produced in response to $PrP^c$-GPI from uninfected N2a cells or to $PrP^{Sc}$-GPI from ScN2a cells (both at 20 ng $ml^{-1}$); in each case, the amounts were comparable to those induced by 10 μM arachidonic acid. HPTLC analysis was unable to detect any significant differences between $PrP^c$-GPI and $PrP^{Sc}$-GPI (Fig. 1). Pretreatment with 1 μg mAb 5AB3-11 $ml^{-1}$ (which recognizes phosphatidylinositol; Bate & Kwiatkowski 1994) blocked induction of $PGE_2$ by both $PrP^c$-GPI and $PrP^{Sc}$-GPI preparations, but had no effect on the production of $PGE_2$ in cells incubated with arachidonic acid, indicating that the phosphatidylinositol part of the GPIs was involved specifically in the stimulation of the $PLA_2$ pathway by GPIs.

### GPI structures required to activate neuronal $PGE_2$ production

To determine the structural characteristics of GPIs that are required to activate neuronal $PGE_2$ production, NB4-1A3 cells were incubated with 20 ng PrP-GPI, deaminated PrP-GPI or deacylated PrP-GPI $ml^{-1}$. Treatment with nitrous acid (deamination) releases the phosphatidylinositol component of the GPI from the glycan core by breaking the linkage between phosphatidylinositol and glucosamine; HPTLC analysis showed that the resultant product migrated with similar properties to a control phosphatidylinositol (Fig. 1). In biological assays, $PGE_2$ levels in cells incubated with 20 ng deaminated PrP-GPI $ml^{-1}$ were significantly lower than in cells incubated with 20 ng untreated PrP-GPI $ml^{-1}$ (Fig. 2). Treatment with mild NaOH (deacylation)

**Table 1.** Production of $PGE_2$ by NB4-1A3 cells incubated with GPIs extracted from normal ($PrP^c$-GPI) or prion-infected ($PrP^{Sc}$-GPI) neuroblastoma cells

Cell cultures were incubated overnight with the preparations shown in the presence or absence of 1 μg mAb 5AB3-11 $ml^{-1}$ (which recognizes phosphatidylinositol). Mean ± SD $PGE_2$ production (pg $ml^{-1}$) from triplicate experiments repeated three times (nine observations) is shown.

| Treatment | $PGE_2$ (pg $ml^{-1}$) |
|---|---|
| $PrP^c$-GPI (20 ng $ml^{-1}$) | 178 ± 64* |
| $PrP^c$-GPI (20 ng $ml^{-1}$) + mAb 5AB3-11 | < 20 |
| $PrP^{Sc}$-GPI (20 ng $ml^{-1}$) | 162 ± 51* |
| $PrP^{Sc}$GPI (20 ng $ml^{-1}$) + mAb 5AB3-11 | < 20 |
| Arachidonic acid (10 μM) | 205 ± 20* |
| Arachidonic acid (10 μM) + mAb 5AB3-11 | 212 ± 44* |
| Thy-1-GPI (20 ng $ml^{-1}$) | < 20 |
| None | < 20 |

*$P < 0·01$ compared with untreated cells.

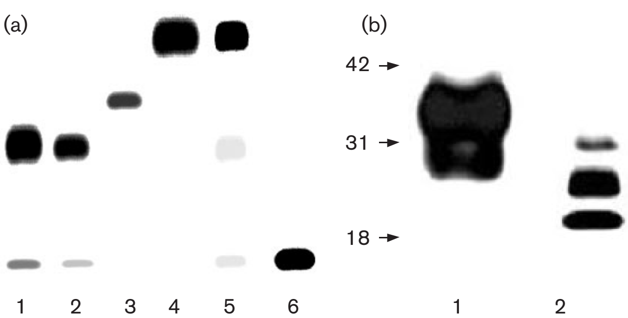


**Fig. 1.** (a) Immunoblot of HPTLC plate containing GPIs from $PrP^c$, $PrP^{Sc}$ or Thy-1. Analysis of GPIs isolated from $PrP^c$, $PrP^{Sc}$ or Thy-1 was determined by using silica gel HPTLC plates and immunoblotting with 1 μg mAb 5AB3-11 $ml^{-1}$. Lane 1, $PrP^c$-GPI; lane 2, $PrP^{Sc}$-GPI; lane 3, Thy-1-GPI; lane 4, phosphatidylinositol; lane 5, $PrP^c$-GPI after deamination by treatment with nitrous acid; lane 6, $PrP^c$-GPI after deacylation by treatment with NaOH. (b) Western blot showing immunoprecipitated $PrP^c$ and $PrP^{Sc}$. Lane 1, $PrP^c$; lane 2: protease-resistant PrP ($PrP^{Sc}$). PrP was demonstrated by immunoblot with mAb SAF53.

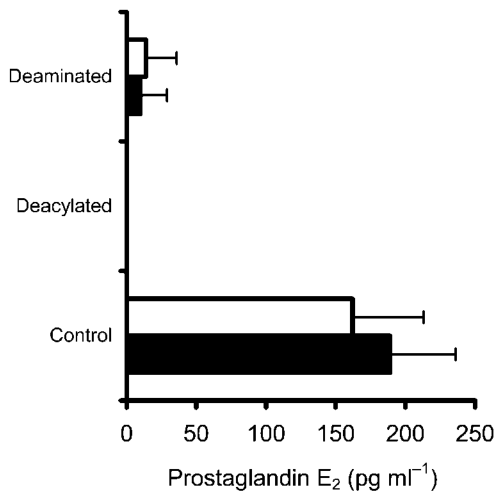


**Fig. 2.** $PGE_2$ production in response to GPIs. Neuroblastoma cells were treated with 20 ng $PrP^c$-GPI $ml^{-1}$ (filled bars) or 20 ng $PrP^{Sc}$-GPI $ml^{-1}$ (empty bars). Control, untreated PrP-GPI; deacylated, PrP-GPI treated with NaOH; deaminated, PrP-GPI treated with $NaNO_2$. Each value is the mean ± SD level of $PGE_2$ (pg $ml^{-1}$) from triplicate experiments repeated three times (nine observations).

removes ester-linked acyl chains, resulting in a water-soluble product. The production of $PGE_2$ by cells incubated with 20 ng deacylated PrP-GPI $ml^{-1}$ was significantly lower than that of cells incubated with 20 ng untreated PrP-GPI $ml^{-1}$ (Fig. 2).

## Partial GPIs inhibit $PGE_2$ production induced by PrP peptides

Competition studies were used to further identify components of GPIs that are required for activation of the $PLA_2$/PG pathways. Levels of $PGE_2$ in cells incubated with 100 μM GPI-related molecules (partial GPIs), such as inositol monophosphate, glucosamine, mannose or sialic acid, were not raised above those of untreated cells. However, production of $PGE_2$ by NB4-1A3 cells incubated with 10 μM HuPrP82–146, 10 μM sPrP106, 10 ng prion preparation $ml^{-1}$ or 20 ng PrP-GPI $ml^{-1}$ was reduced significantly in cells that had been pretreated with inositol monophosphate or sialic acid, but not in cells pretreated with glucosamine, inositol or mannose (Table 2). As the production of $PGE_2$ is a two-stage process that requires the release of arachidonic acid by $PLA_2$ and the conversion of arachidonic acid to PGs by the COX enzymes, we examined whether inositol monophosphate or sialic acid had an effect on $PGE_2$ production in cells exposed to 10 μM arachidonic acid. Pretreatment with inositol monophosphate or sialic acid did not reduce $PGE_2$ production in response to arachidonic acid, showing that they had no effect on the COX enzymes (Table 2). Treatment with these partial GPIs did not compromise cell survival and neurons could be grown in 100 μM inositol monophosphate or 100 μM sialic acid for weeks without any adverse effects on cell survival or growth rates (unpublished results).

## Inhibition of the toxic effect of PrP peptides on NB4-1A3 cells by partial GPIs

To determine the effect of partial GPIs on the toxicity of PrP peptides, NB4-1A3 cells were pretreated with selected partial GPIs at 100 μM before the addition of 10 μM HuPrP82–146, 10 μM sPrP106 or 10 ng prion preparation $ml^{-1}$. Incubation of NB4-1A3 neuroblastoma cells overnight with HuPrP82–146, sPrP106 or prion preparation reduced cell survival by approximately 50 % (Table 3). Pretreatment of the cultures with inositol monophosphate or sialic acid protected cells in each case, whereas pretreatment with galactose, glucosamine or mannose had no effect. The efficacy of these compounds was examined by pretreating cells with different concentrations of the partial

**Table 2.** Inositol monophosphate and sialic acid reduce $PGE_2$ production

Levels of $PGE_2$ (pg $ml^{-1}$) produced by NB4-1A3 cells pretreated with 100 μM partial GPIs as shown and incubated thereafter with 10 ng prion preparation $ml^{-1}$, 10 μM HuPrP82–146, 10 μM sPrP106, 20 ng PrP-GPI $ml^{-1}$ or 10 μM arachidonic acid. Each value is the mean ± SD level of $PGE_2$ produced from triplicate experiments repeated three times (nine observations).

| Pretreatment | $PGE_2$ produced (pg $ml^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | None | Prion | HuPrP82–146 | sPrP106 | PrP-GPI | Arachidonic acid |
| None | <20 | 376 ± 48 | 338 ± 50 | 409 ± 36 | 192 ± 39 | 226 ± 27 |
| Galactose | <20 | 354 ± 42 | 302 ± 44 | 385 ± 58 | 178 ± 33 | 188 ± 45 |
| Glucosamine | <20 | 328 ± 76 | 336 ± 48 | 394 ± 35 | 169 ± 38 | 213 ± 34 |
| Inositol monophosphate | <20 | 188 ± 62* | 139 ± 53* | 145 ± 50* | <20* | 215 ± 38 |
| Inositol | <20 | 391 ± 52 | 313 ± 43 | 403 ± 43 | 202 ± 29 | 233 ± 54 |
| Mannose | <20 | 380 ± 44 | 319 ± 44 | 376 ± 62 | 180 ± 25 | 199 ± 39 |
| Sialic acid | <20 | 216 ± 45* | 188 ± 54* | 259 ± 58* | 37 ± 15* | 205 ± 41 |

*$P < 0·01$ compared to cells incubated with peptides or PrP-GPI alone.

**Table 3.** Partial GPIs protect neurons against PrP peptides/prion preparations

Survival of NB4-1A3 cells pretreated with partial GPIs (100 μM) and subsequently incubated with 10 μM HuPrP82–146, 10 μM sPrP106 or 10 ng prion preparation $ml^{-1}$. Each value is the mean ± SD percentage cell survival from triplicate experiments repeated four times (12 observations).

| Pretreatment | Cell survival compared to control (%) | | |
|---|---|---|---|
| | HuPrP82–146 | sPrP106 | Prion preparation |
| Control medium | 64 ± 4 | 52 ± 5 | 44 ± 6 |
| Galactose | 65 ± 4 | 50 ± 5 | 42 ± 4 |
| Glucosamine | 63 ± 5 | 53 ± 7 | 41 ± 5 |
| Inositol monophosphate | 99 ± 4* | 101 ± 5* | 87 ± 7* |
| Mannose | 68 ± 4 | 54 ± 12 | 46 ± 9 |
| Sialic acid | 94 ± 5* | 95 ± 5* | 82 ± 7* |

*Cell survival is significantly greater ($P < 0{\cdot}01$) than in cells incubated with peptides/prion preparation alone.

GPIs before the addition of 10 μM sPrP106. Pretreatment with phosphatidylinositol, inositol monophosphate or sialic acid resulted in a dose-dependent increase in cell survival (Fig. 3); however, pretreatment with inositol did not inhibit the toxicity of sPrP106, indicating that the presence of a phosphate group on the inositol ring was necessary for the protective effect. In addition, the survival of cells incubated with 10 μM sPrP106 (52 ± 5 % cell survival; $n = 9$) was not affected by pretreatment with 10 μM inositol-1,4-bisphosphate (50 ± 7 %) or 10 μM inositol-1,4,5-triphosphate (54 ± 4 %).

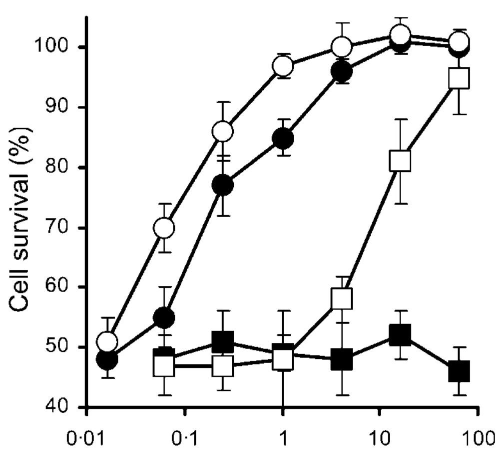


**Fig. 3.** Protection of NB4-1A3 cells against the toxic effect of sPrP106. Survival of NB4-1A3 neuroblastoma cells pretreated with different concentrations of phosphatidylinositol (●), inositol monophosphate (○), inositol (■) or sialic acid (□) before incubation with 10 μM sPrP106. Each value represents the mean ± SD cell survival (%) from triplicate experiments repeated four times (12 observations).

We have shown previously that the toxicity of PrP peptides requires activation of $PLA_2$ and the subsequent release of neurotoxins, such as arachidonic acid and PAF (Bate *et al.*, 2004b). To determine whether the partial GPIs inhibited the activity of such neurotoxins, NB4-1A3 cells were treated with 10 μM arachidonic acid or 10 μM PAF in the presence or absence of partial GPI anchors. The survival of cells incubated with arachidonic acid alone (54 ± 6 %; $n = 9$) was not significantly different from that of cells incubated with arachidonic acid and 10 μM inositol monophosphate (53 ± 6 %) or arachidonic acid and 50 μM sialic acid (55 ± 5 %). Similarly, the survival of cells incubated with PAF (60 ± 6 %) was not significantly different from that of cells incubated with PAF and 10 μM inositol monophosphate (62 ± 5 %) or PAF and 50 μM sialic acid (59 ± 5 %). Partial GPIs had no effect on the survival of NB4-1A3 cells incubated with 20 ng staurosporine $ml^{-1}$ (40 ± 3 %) or 5 μM hydrogen peroxide (46 ± 5 %). Such observations suggest that inositol monophosphate and sialic acid inhibit PrP-induced activation of $PLA_2$ specifically, rather than by blocking neurotoxins directly or by altering apoptotic pathways in general.

## Inhibition of caspase-3 production in neurons

Caspase-3 activity was measured in primary cortical neurons as an indication of apoptosis. Caspase-3 activity was increased significantly above that of untreated cells following 24 h incubation with 10 μM sPrP106, 10 μM HuPrP82–146 or 20 ng PrP-GPI $ml^{-1}$, but not in cells incubated with 20 ng Thy-1-GPI $ml^{-1}$ or 100 μM partial GPIs. Pretreating $PrP^{c}$-GPI with 1 μg mAb 5AB3-11 $ml^{-1}$ reduced the caspase-3 activity response to GPIs, but caspase-3 activity in neurons pretreated with sPrP106 or HuPrP82–146 was not affected (Table 4). Competition experiments showed that caspase-3 activity in response to sPrP106, HuPrP82–146 or PrP-GPI was reduced significantly in neurons that had been pretreated with inositol monophosphate or sialic acid, but was not affected by pretreatment with galactose, glucosamine, inositol or mannose.

## Killing of sPrP106-damaged neurons by microglia is blocked by partial GPIs

Previous studies showed that microglia kill neurons that have been affected sublethally by PrP peptides (Brown *et al.*, 1996). In the present study, primary cortical neurons were pretreated with partial GPIs for 3 h before the addition of 10 μM sPrP106. A further 3 h later, microglia were added and the survival of neurons was measured after 4 days. The survival of neurons in co-cultures containing sPrP106 and 10 μM inositol monophosphate or 100 μM sialic acid was significantly higher than that of untreated neurons or of neurons pretreated with 100 μM glucosamine or 100 μM

**Table 4.** PrP-GPIs induce caspase-3 activity in primary cortical neurons

Caspase-3 activity in primary cortical neurons incubated for 24 h with 10 μM sPrP106, 10 μM HuPrP82–146, 20 ng PrP-GPI $ml^{-1}$ or 20 ng Thy-1-GPI $ml^{-1}$, in the presence or absence of 1 μg mAb 5AB3-11 $ml^{-1}$ or 100 μM partial GPIs, as shown. Each value is the mean ± SD level of caspase-3 activity in cells from triplicate experiments repeated twice (six observations).

| Treatment | Caspase-3 activity (fluorescence units) | | | | |
|---|---|---|---|---|---|
| | None | sPrP106 | HuPrP82–146 | PrP-GPI | Thy-1-GPI |
| None | 1·2±0·5 | 7·3±0·7 | 6·2±0·8 | 4·7±0·7 | 1·1±0·4 |
| +mAb 5AB3-11 | 0·9±0·5 | 6·9±1·1 | 6·6±0·8 | 0·9±0·3 | 0·8±0·7 |
| +Isotype control | 1·0±0·2 | 7·4±0·9 | 6·3±1·1 | 5·0±0·8 | 1·0±0·6 |
| Galactose | 1·1±0·4 | 7·2±1·1 | 5·9±1·2 | 4·4±1·4 | 0·6±0·4 |
| Glucosamine | 0·7±0·5 | 7·7±1·4 | 6·0±0·9 | 4·9±1·0 | 1·2±0·7 |
| Inositol monophosphate | 1·2±0·5 | 2·7±1·4* | 2·2±0·4* | 1·2±0·6* | 1·2±0·5 |
| Inositol | 1·4±0·7 | 7·5±0·8 | 5·6±1·3 | 4·5±1·0 | 1·0±0·5 |
| Mannose | 0·8±0·4 | 7·0±1·6 | 6·5±1·0 | 5·2±1·6 | 0·9±0·3 |
| Sialic acid | 0·9±0·4 | 3·5±2·2* | 3·1±0·7* | 2·0±1·2* | 0·8±0·5 |

*Caspase-3 activity is significantly lower ($P<0{\cdot}01$) than in untreated cells incubated with peptide or PrP-GPI.

mannose before the addition of 10 μM sPrP106 (Fig. 4). Further experiments showed that inositol monophosphate and sialic acid did not affect the production of interleukin 6 by microglia stimulated with lipopolysaccharide, demonstrating that these compounds did not have a direct effect on microglia (unpublished data).

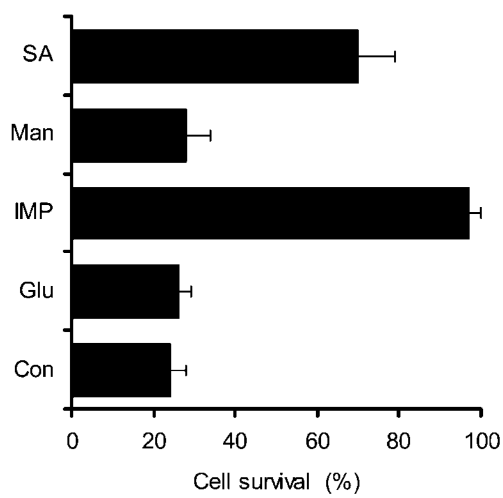


**Fig. 4.** Inositol monophosphate and sialic acid protect sPrP106-treated neurons against microglia. Primary cortical neurons were treated with 10 μM sPrP106 and either a vehicle control (Con), 100 μM glucosamine (Glu), 10 μM inositol monophosphate (IMP), 100 μM mannose (Man) or 100 μM sialic acid (SA). After 3 h, microglia were added in the ratio of 1 microglial cell : 10 neurons; survival of neurons was determined 4 days later. Each value represents the mean ± SD cell survival (%) from triplicate experiments repeated four times (12 observations).

## DISCUSSION

The major neuropathological features of TSEs or prion disease include the accumulation of $PrP^{Sc}$, glial cell activation and the degeneration and apoptotic death of neurons (Giese *et al.*, 1995; Jeffrey *et al.*, 2000; Jamieson *et al.*, 2001). Factors that are increased in affected areas of the brain include $PGE_2$ (Williams *et al.*, 1994, 1997b) and raised levels of $PGE_2$ are detectable in the cerebrospinal fluid of patients with CJD (Minghetti *et al.*, 2000, 2002). The present data show that the GPI anchors attached to $PrP^c$ and $PrP^{Sc}$ stimulate $PGE_2$ production and induce caspase-3 activity in neurons in a similar manner to PrP peptides, the miniprion sPrP106 and prion preparations (Bate *et al.*, 2004b). These results are consistent with reports that concentrated GPI anchors can activate some cell types; for example, GPIs activate lipogenesis in adipocytes (Frick *et al.*, 1998) or induce cytokine production from macrophages (Vijaykumar *et al.*, 2001). The GPIs isolated from $PrP^c$ and $PrP^{Sc}$ migrate with similar $R_f$ values in HPTLC and we were unable to detect any significant differences in their biological activity. These observations suggest that conversion from $PrP^c$ to $PrP^{Sc}$ does not entail significant changes to the GPI anchor, results that are consistent with previous data (Stahl *et al.*, 1992). However, as the exact structure of these GPIs was not determined, it remains possible that subtle differences between GPI-$PrP^c$ and GPI-$PrP^{Sc}$ exist. It is worth noting that the GPI anchor attached to Thy-1 is modified differently from that of $PrP^c$ (Rudd *et al.*, 2001) and that GPI anchors isolated from Thy-1 failed to stimulate $PGE_2$ production or to activate apoptotic pathways. Thus, although it is not known whether $PGE_2$ production and the initiation of apoptotic pathways in neurons is a unique property of GPIs attached to $PrP^c$, this activity is not shared by all GPIs.

The studies reported here provide indications of the structures required of the GPI for biological activity. Thus, the compounds generated following deamination, which releases phosphatidylinositol from the glycan component of GPI, did not stimulate $PGE_2$ production. Deacylated GPIs failed to run on HPTLC, indicating a hydrophilic compound consistent with the removal of acyl chains. Deacylated GPIs also failed to stimulate $PGE_2$ production, demonstrating that the ester-linked acyl chains on the phosphatidylinositol are required for biological activity. In addition, mAb studies demonstrated that a phosphatidylinositol moiety of GPIs is essential for biological activity. The effects of mAb 5AB3-11 were specific in that it did not affect $PGE_2$ produced by cells treated with arachidonic acid, indicating that mAb 5AB3-11 did not affect COX. Such observations suggest that the biological activity of GPI anchors requires both phosphatidylinositol and another, unspecified glycan component.

Little is known about the process by which prions or PrP peptides activate neuronal $PLA_2$. One possibility is that high concentrations of GPI anchors bind directly to $PLA_2$. In these assays, high concentrations of GPI anchors were required to activate $PLA_2$, perhaps mimicking the high concentration of GPI anchors that occurs following the aggregation of $PrP^{Sc}$ molecules or the cross-linkage of $PrP^c$ by specific mAbs (Solforosi *et al.*, 2004) or by PrP peptides. The clustering of GPI-anchored proteins is thought to occur in specific membrane microdomains that are known as lipid rafts (Mayor & Riezman, 2004), which are essential for the activation of $PLA_2$ and the toxicity of prions (Bate *et al.*, 2004a). The results presented here are consistent with exogenous GPI anchors inserting into membranes and trafficking to lipid rafts, where high concentrations activate $PLA_2$. It is of interest to note that, although the addition of exogenous GPI anchors activated $PLA_2$ and increased caspase-3 activity in neurons, it did not cause neuronal death. It remains to be seen whether prion-induced neuronal death requires additional, non-GPI signals that are inherent in the prion protein structure or whether the lack of neuronal death in response to GPI anchors is simply a concentration effect.

The neurotoxicity of PrP peptides or prion preparations was reduced by pretreatment with some compounds that are common to all GPI anchors. Initial studies showed that whilst phosphatidylinositol, inositol monophosphate and sialic acid reduced the neurotoxicity of sPrP106 and prion preparations, other components of GPI anchors, namely galactose, glucosamine and mannose, had no effect. The presence of a single phosphate on the inositol ring was essential, as inositol alone and inositols containing more than one phosphate did not affect neurotoxicity. Whilst the protective effects of phosphatidylinositol and inositol monophosphate on sPrP106-induced neurotoxicity were evident at micromolar concentrations, the protective effect of sialic acid was only observed at higher concentrations.

The toxicity of prions or PrP peptides involves the activation of neuronal $PLA_2$ and the production of bioactive second messengers, including arachidonic acid and PAF (Bate *et al.*, 2004b). Treatment of neurons with inositol monophosphate or sialic acid did not affect the toxicity of arachidonic acid or PAF, indicating that these compounds prevent the formation, rather than the action, of such neurotoxins. The production of $PGE_2$ that is associated closely with PrP-induced neurotoxicity is a two-stage process that requires the release of arachidonic acid by $PLA_2$ and the conversion of arachidonic acid to PGs by the COX enzymes. The addition of inositol monophosphate or sialic acid reduced $PGE_2$ production in response to HuPrP82–146, sPrP106 and PrP-GPIs, but did not affect $PGE_2$ production in response to arachidonic acid, showing that these compounds had no direct effect on COX. In regard to prion-induced toxicity, these observations identify $PLA_2$ as the target of inositol monophosphate and sialic acid, a result that is compatible with previous reports that sialic acid inhibits $PLA_2$ (Yang *et al.*, 1994).

The presence of inositol monophosphate or sialic acid greatly reduced microglial killing of neurons damaged by sPrP106. Microglia respond to changes in neurons that are induced by PrP peptides (Bate *et al.*, 2002) and our data are consistent with the concept that the presence of inositol monophosphate or sialic acid prevents the PrP-induced neuronal changes that activate microglia. An alternative explanation, i.e. that inositol monophosphate and sialic acid have a direct effect on microglia, was discounted, as these compounds did not affect the production of interleukin 6 from microglia incubated with lipopolysaccharide (unpublished data).

To our knowledge, this is the first report to demonstrate that high concentrations of PrP-GPIs result in activation of $PLA_2$ and neuronal apoptotic pathways (caspase-3). The activity of GPIs was dependent on a phosphatidylinositol moiety, on ester-linked acyl chains and on an unspecified glycan component. There were no obvious physical differences between GPIs isolated from $PrP^c$ or $PrP^{Sc}$, nor any significant differences in their biological activity. We propose that the high concentrations of GPIs added here mimic the locally high concentrations of GPIs that occur when $PrP^c$ molecules cluster following the addition of PrP peptides, or when the GPI anchors are concentrated following the aggregation of $PrP^{Sc}$ molecules. Pretreatment with some partial GPIs, including inositol monophosphate or sialic acid, protected neurons against the toxicity of PrP peptides, sPrP106 and prion preparations. These partial GPIs prevented the activation of $PLA_2$, rather than inhibiting neurotoxins generated following $PLA_2$ activation. Inositol monophosphate and sialic acid also reduced the HuPrP82–146- or sPrP106-induced activation of apoptotic pathways in cortical neurons and prevented HuPrP82–146- or sPrP106-treated neurons from activating microglia, resulting in increased neuronal survival. The present results are compatible with the hypothesis that inositol monophosphate and sialic acid compete with the complete GPI anchors of $PrP^{c}$ or $PrP^{Sc}$ for cellular receptors and prevent $PLA_2$ activation. Whilst neuronal death in response to prions *in vivo* is undoubtedly a complex process that may include other mechanisms, these observations provide insight into the signalling processes that result in prion-induced neuronal loss.

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## REFERENCES

**Bate, C. A. W. & Kwiatkowski, D. (1994).** A monoclonal antibody that recognizes phosphatidylinositol inhibits induction of tumor necrosis factor alpha by different strains of *Plasmodium falciparum*. *Infect Immun* **62**, 5261–5266.

**Bate, C. A., Boshuizen, R. S., Langeveld, J. P. M. & Williams, A. (2002).** Temporal and spatial relationship between the death of PrP-damaged neurones and microglial activation. *Neuroreport* **13**, 1695–1700.

**Bate, C., Salmona, M., Diomede, L. & Williams, A. (2004a).** Squalestatin cures prion-infected neurons and protects against prion neurotoxicity. *J Biol Chem* **279**, 14983–14990.

**Bate, C. A., Salmona, M. & Williams, A. (2004b).** The role of platelet activating factor in prion and amyloid-$\beta$ neurotoxicity. *Neuroreport* **15**, 509–513.

**Bonetto, V., Massignan, T., Chiesa, R. & 8 other authors (2002).** Synthetic miniprion PrP106. *J Biol Chem* **277**, 31327–31334.

**Brown, D. R., Herms, J. & Kretzschmar, H. A. (1994).** Mouse cortical cells lacking cellular PrP survive in culture with a neurotoxic PrP fragment. *Neuroreport* **5**, 2057–2060.

**Brown, D. R., Schmidt, B. & Kretzschmar, H. A. (1996).** Role of microglia and host prion protein in neurotoxicity of a prion protein fragment. *Nature* **380**, 345–347.

**Forloni, G., Angeretti, N., Chiesa, R., Monzani, E., Salmona, M., Bugiani, O. & Tagliavini, F. (1993).** Neurotoxicity of a prion protein fragment. *Nature* **362**, 543–546.

**Frick, W., Bauer, A., Bauer, J., Wied, S. & Müller, G. (1998).** Structure-activity relationship of synthetic phosphoinositolglycans mimicking metabolic insulin action. *Biochemistry* **37**, 13421–13436.

**Giese, A., Groschup, M. H., Hess, B. & Kretzschmar, H. A. (1995).** Neuronal cell death in scrapie-infected mice is due to apoptosis. *Brain Pathol* **5**, 213–221.

**Hope, J., Shearman, M. S., Baxter, H. C., Chong, A., Kelly, S. M. & Price, N. C. (1996).** Cytotoxicity of prion protein peptide ($PrP^{106-126}$) differs in mechanism from the cytotoxic activity of the Alzheimer's disease amyloid peptide, A $\beta$25–35. *Neurodegeneration* **5**, 1–11.

**Jamieson, E. C. A., Jeffrey, M., Ironside, J. W. & Fraser, J. R. (2001).** Activation of Fas and caspase 3 precedes PrP accumulation in 87V scrapie. *Neuroreport* **12**, 3567–3572.

**Jeffrey, M., Halliday, W. G., Bell, J., Johnston, A. R., Macleod, N. K., Ingham, C., Sayers, A. R., Brown, D. A. & Fraser, J. R. (2000).** Synapse loss associated with abnormal PrP precedes neuronal degeneration in the scrapie-infected murine hippocampus. *Neuropathol Appl Neurobiol* **26**, 41–54.

**Mayor, S. & Riezman, H. (2004).** Sorting GPI-anchored proteins. *Nat Rev Mol Cell Biol* **5**, 110–120.

**Minghetti, L., Greco, A., Cardone, F. & 7 other authors (2000).** Increased brain synthesis of prostaglandin E2 and F2-isoprostane in human and experimental transmissible spongiform encephalopathies. *J Neuropathol Exp Neurol* **59**, 866–871.

**Minghetti, L., Cardone, F., Greco, A., Puopolo, M., Levi, G., Green, A. J. E., Knight, R. & Pocchiari, M. (2002).** Increased CSF levels of prostaglandin $E_2$ in variant Creutzfeldt–Jakob disease. *Neurology* **58**, 127–129.

**Pan, K.-M., Baldwin, M., Nguyen, J. & 8 other authors (1993).** Conversion of $\alpha$-helices into $\beta$-sheets features in the formation of the scrapie prion proteins. *Proc Natl Acad Sci U S A* **90**, 10962–10966.

**Prusiner, S. B. (1982).** Novel proteinaceous infectious particles cause scrapie. *Science* **216**, 136–144.

**Prusiner, S. B. (1998).** Prions. *Proc Natl Acad Sci U S A* **95**, 13363–13383.

**Rudd, P. M., Wormald, M. R., Wing, D. R., Prusiner, S. B. & Dwek, R. A. (2001).** Prion glycoprotein: structure, dynamics, and roles for the sugars. *Biochemistry* **40**, 3759–3766.

**Salmona, M., Morbin, M., Massignan, T. & 14 other authors (2003).** Structural properties of Gerstmann-Sträussler-Scheinker disease amyloid protein. *J Biol Chem* **278**, 48146–48153.

**Solforosi, L., Criado, J. R., McGavern, D. B. & 12 other authors (2004).** Cross-linking cellular prion protein triggers neuronal apoptosis in vivo. *Science* **303**, 1514–1516.

**Stahl, N., Baldwin, M. A., Hecker, R., Pan, K.-M., Burlingame, A. L. & Prusiner, S. B. (1992).** Glycosylinositol phospholipid anchors of the scrapie and cellular prion proteins contain sialic acid. *Biochemistry* **31**, 5043–5053.

**Taraboulos, A., Scott, M., Semenov, A., Avraham, D., Laszlo, L. & Prusiner, S. B. (1995).** Cholesterol depletion and modification of COOH-terminal targeting sequence of the prion protein inhibit formation of the scrapie isoform. *J Cell Biol* **129**, 121–132.

**Vijaykumar, M., Naik, R. S. & Gowda, D. C. (2001).** *Plasmodium falciparum* glycosylphosphatidylinositol-induced TNF-$\alpha$ secretion by macrophages is mediated without membrane insertion or endocytosis. *J Biol Chem* **276**, 6909–6912.

**Williams, A. E., van Dam, A.-M., Man-A-Hing, W. K. H., Berkenbosch, F., Eikelenboom, P. & Fraser, H. (1994).** Cytokines, prostaglandins and lipocortin-1 are present in the brains of scrapie-infected mice. *Brain Res* **654**, 200–206.

**Williams, A., Lucassen, P. J., Ritchie, D. & Bruce, M. (1997a).** PrP deposition, microglial activation, and neuronal apoptosis in murine scrapie. *Exp Neurol* **144**, 433–438.

**Williams, A., Van Dam, A.-M., Ritchie, D., Eikelenboom, P. & Fraser, H. (1997b).** Immunocytochemical appearance of cytokines, prostaglandin $E_2$ and lipocortin-1 in the CNS during the incubation period of murine scrapie correlates with progressive PrP accumulations. *Brain Res* **754**, 171–180.

**Yang, H. C., Farooqui, A. A. & Horrocks, L. A. (1994).** Effects of sialic acid and sialoglycoconjugates on cytosolic phospholipases $A_2$ from bovine brain. *Biochem Biophys Res Commun* **199**, 1158–1166.