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Synthetic Prion Protein

Semisynthesis of a Glycosylphosphatidylinositol-Anchored Prion Protein**

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Proteins are often modified posttranslationally by glycosylation and lipidation.[1] Glycosylphosphatidylinositol (GPI) anchors combine both types of modification and link many proteins to the cell surface.[2] Advances in solid-phase peptide synthesis (SPPS) and recombinant protein engineering, in combination with the development of native chemical ligation (NCL) and expressed protein ligation (EPL), have resulted in numerous total syntheses and semisyntheses of proteins.[3] These approaches facilitate access to homogeneous glyco- and lipoproteins, which serve as defined molecular probes to elucidate the effects of glycosylation and lipidation on the biophysical properties of proteins.[4] Synthetic GPI glycans and lipidated GPI anchors[5] have emerged as valuable tools that allow for the precise dissection of their biological relevance in infectious and metabolic diseases.[6] Efforts towards the assembly of chemically defined GPI-anchored proteins have focused on model studies; no synthetic GPI-anchored protein has been reported to date.[7,8]

A prominent example of a GPI-anchored protein is the prion protein (PrP).[9] Numerous studies have indicated the strong influence of membrane association through the GPI anchor on the conversion of cellular PrP (PrP^{C}) into its pathogenic isoform PrP scrapie (PrP^{Sc}). However, the speculation that GPI anchoring might contribute to the pathogenicity of PrP is controversial.[10] As the isolation of homogeneous GPI-anchored PrP has not yet been possible, the majority of in vitro studies on the function, structure, folding, and stability of PrP have been carried out with recombinant protein lacking the GPI anchor, simple GPI-anchor mimics, or heterogeneous protein preparations from mammalian cell lines.[11] Thus, the exact function of the GPI anchor could not be assessed directly. Homogenous GPI-anchored proteins would be ideally accessed by chemical synthesis. Herein we report the development of a general strategy for the synthesis of homogeneous GPI-anchored proteins, with a particular focus on the prion protein.

We envisioned a general solution based on EPL to the construction of defined GPI-anchored proteins. We anticipated that the synthetic GPI anchor **2**, with a cysteine residue on the 2-aminoethyl phosphate moiety, would undergo ligation with peptides and proteins with a C-terminal C^{α} thioester to give GPI-anchored proteins **1** (Scheme 1). Several synthetic approaches can be proposed for the construction of a cysteine-containing GPI anchor of this type. The direct coupling of cysteine to a native GPI anchor through an amide linkage is plausible. However, the difficulties in handling native GPI anchors, as well as the instability of lipid esters under basic conditions, render this approach less appealing. A more realistic solution is the installation of a protected cysteine residue on the GPI anchor prior to global deprotection. The cysteine ethanolamine phosphate residue will be incorporated into the glycan backbone at the final stage of the chemical synthesis of GPI. The thiol and amino groups of the cysteine residue would be protected with acid-labile groups, such as *tert*-butyl and *tert*-butoxycarbonyl (Boc) groups (e.g. in **3**). The benzyl groups would be removed by hydrogenolysis, and treatment with acid would then liberate the cysteine residue to furnish the cysteine-tagged GPI anchor **2**.

Two key transformations had to be studied carefully prior to executing the synthesis. The incorporation of the phosphate diester relies on the H-phosphonate method, which requires the oxidation of phosphorus(III) to phosphorus(V) with iodine in pyridine and water.[12] It was unclear whether the thioether would also be oxidized. Moreover, the cleavage of benzyl ethers by hydrogenolysis requires the use of the heterogeneous catalyst Pd/C, and thioethers, although less troublesome than thiols, can poison heterogeneous catalysts.[13] To address these concerns, the two transformations were evaluated with a model compound (Scheme 2).

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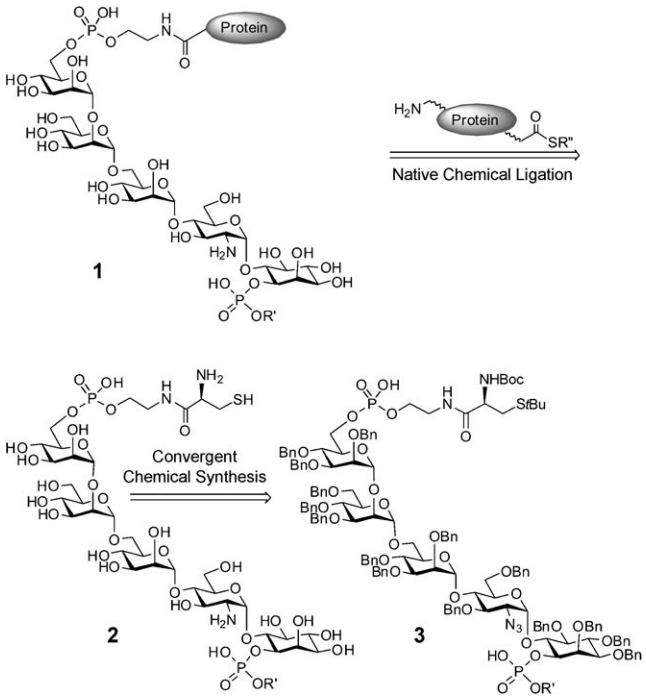
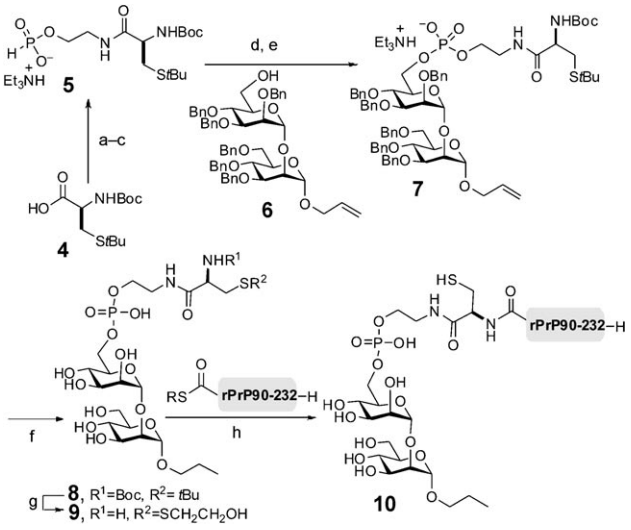


Scheme 1. General strategy for the semisynthesis of a GPI-anchored protein by native chemical ligation (R′ = lipid chain, R″ = alkyl or aryl group). Bn = benzyl, Boc = *tert*-butoxycarbonyl.

The synthesis commenced with the preparation of the H-phosphonate **5** (Scheme 2). Commercially available *N*-Boc-*S*-*t*Bu-L-cysteine (**4**) was activated with succinimide and treated with ethanolamine at 0 °C. Subsequent conversion into the H-phosphonate monoester **5** was mediated by phosphonic acid in the presence of pivaloyl chloride.[14] We chose the α(1→2) dimannoside **6**, which resembles a portion of the GPI glycan, as a model to evaluate the phosphorylation and global deprotection strategy (Scheme 2). The phosphonylation of the C6 hydroxy group of **6** was mediated by pivaloyl chloride. The selective in situ oxidation to phosphorus(V) with iodine at 0 °C was complete within 1 h, and no product of oxidation at the sulfur atom was observed. We carefully surveyed conditions for hydrogenolysis in the presence of thioethers.[15] A solution of formic acid in methanol in combination with Pd/C and hydrogen proved effective for the rapid cleavage of all benzyl ether groups present in **7** within 2 h to furnish **8** in quantitative yield (Scheme 2). The *N*-Boc and *S*-*t*Bu protecting groups were removed with $Hg(OTFA)_2$ in trifluoroacetic acid (TFA), and excess mercury salts were precipitated by treatment with 2-sulfanylethanol.[13b] Purification with a sephadex G-25 column gave **9** as a mixed disulfide with a 2-mercaptoethanol group.

The dimannoside **9** was tested as a substrate for NCL with recombinant PrP (rPrP) containing a C-terminal MESNa thioester functionality (MESNa = 2-mercaptoethane sulfonate).[11f] The treatment of the rPrP thioester with **9** (1.5 equiv) in the presence of thiophenol produced the glycan–rPrP conjugate **10** in 80 % yield. The ligation was complete within 12 h at room temperature, as indicated by reversed-phase (RP) HPLC (Figure 1 A). The homogeneity of the product was assessed by electrospray ionization mass spectrometry (ESIMS), and the observed mass of 16929.5 Da agrees well with the calculated mass of **10** (16927 Da; Figure 1 B).

On the basis of these results, we proceeded to assemble a complete GPI anchor. Native prion GPI anchors contain a core pseudopentasaccharide glycan common to mammalian GPI anchors; this core glycan is amended with an oligosaccharide branch up to the trisaccharide Neu-Gal-GalNAc.[16]



Scheme 2. Evaluation of the synthetic strategy. Synthesis of a GPI dimannoside fragment: a) *N*-hydroxysuccinimide, DIPC, THF, room temperature; b) ethanolamine, THF, DMF, 90 % (2 steps); c) H_3PO_3, PivCl, pyridine, room temperature, 75 %; d) **5**, PivCl, pyridine; e) I_2, pyridine/H_2O, 0 °C, 94 % (2 steps); f) Pd/C, H_2, 4 % HCOOH in MeOH, quantitative; g) $Hg(OTFA)_2$, TFA/anisole, 0 °C; then $HSCH_2CH_2OH$, AcOH, H_2O, room temperature, 93 %; h) PhSH (1 %, v/v), pH 7.8 (R = $(CH_2)_2SO_3^-$). DIPC = diisopropylcarbodiimide, DMF = *N*,*N*-dimethylformamide, Piv = pivaloyl.

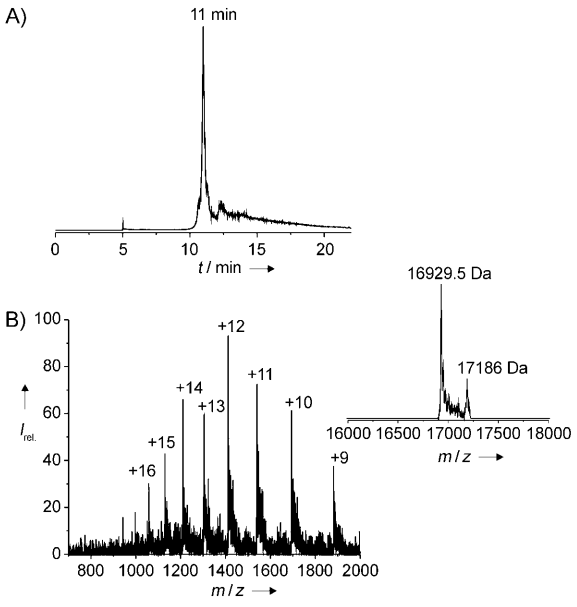


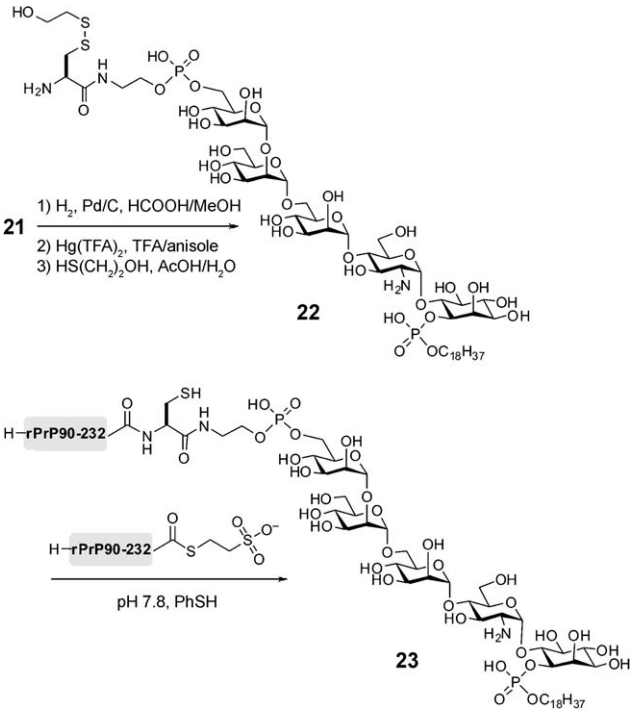
Figure 1. A) RP-HPLC chromatogram for the NCL reaction of the rPrP thioester with **9** after 12 h. B) ESI mass spectrum of material from the fraction with the HPLC peak in (A).

Six glycoforms of prion protein GPI have been reported; however, in all cases, the exact linkage position and anomeric configuration of the oligosaccharide branch were not defined.[17] No details of the lipid composition of prion protein GPIs are known. In view of the structural uncertainty associated with the prion protein GPI glycan, we selected the core GPI pseudopentasaccharide **2** as the initial synthetic target.

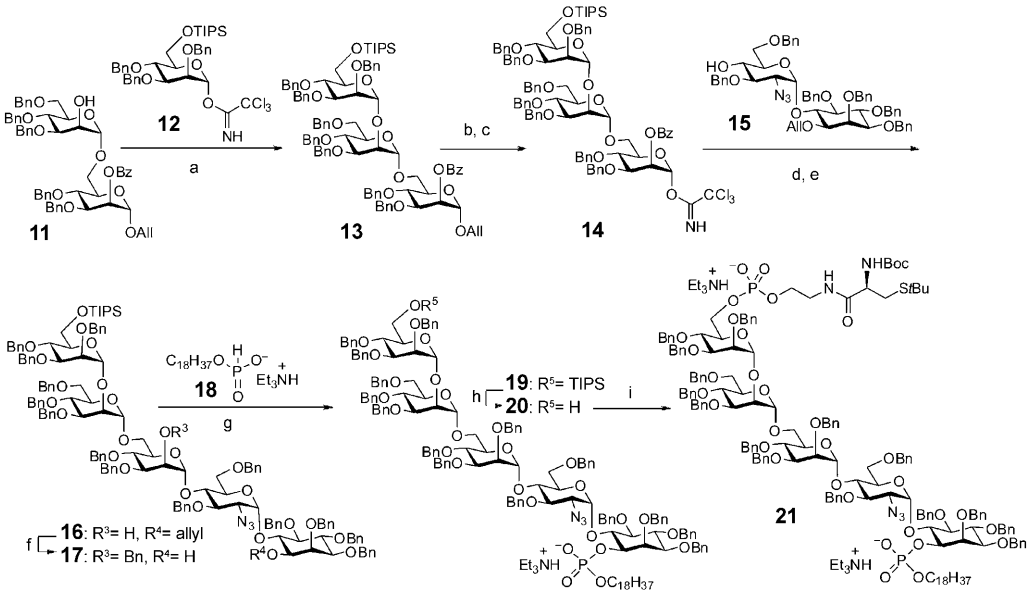
The convergent assembly of **2** relied on a [3+2] glycosylation strategy to connect a trimannoside with a glucosamine–inositol pseudodisaccharide (Scheme 3). The selective coupling of the mannosyl trichloroacetimidate **12** and the known dimannoside **11**[18] was mediated by TMSOTf in diethyl ether. The allyl group at the reducing terminus of **13** was removed with $PdCl_2$ in acetate buffer. Treatment with trichloroacetonitrile and DBU then furnished the trimannosyl trichloroacetimidate **14**. The union of **14** and pseudodisaccharide **15** in the presence of a catalytic amount of TMSOTf led to the differentially protected pseudopentasaccharide, which was treated with sodium methoxide to give **16** in 86% yield over two steps. Benzylation of the central mannose unit and removal of the allyl protecting group on the inositol residue furnished **17**. Subsequent phosphorylation was carried out in a one-pot, two-step sequence by using the H-phosphonate **18** as a diacyl glycerol phosphate surrogate to give **19**.[19] The C6 silyl ether of the terminal mannose residue was removed with HCl generated in situ in methanol. Further phosphorylation with **5** gave the fully protected lipidated GPI anchor **21**.

The stepwise final deprotection was carried out under the conditions described for the model dimannoside **9** (Scheme 4). Hydrogenolysis with Pd/C in formic acid and methanol was followed by removal of the acid-labile Boc and *tert*-butyl groups in TFA with $Hg(OTFA)_2$. Excess mercury salts were precipitated with 2-sulfanylethanol to give the cysteine-tagged GPI anchor **22** as a heterodisulfide, as identified by ESIMS (see the Supporting Information).

The crude GPI anchor **22** was subjected directly to NCL with recombinant PrP (rPrP) containing a C-terminal MESNa



Scheme 4. Completion of the synthesis of the cysteine-tagged GPI pseudopentasaccharide and native chemical ligation with the rPrP thioester.



Scheme 3. Assembly of the protected GPI pseudopentasaccharide **21** with a 2-(cysteinylamino)phosphate moiety: a) TMSOTf, CH_2Cl_2, 95%; b) $PdCl_2$, AcONa, AcOH, AcOEt, room temperature; c) Cl_3CCN, DBU, CH_2Cl_2, 68% (2 steps); d) TMSOTf, CH_2Cl_2, 0°C; e) NaOMe, MeOH, 50°C, 86% (2 steps); f) BnBr, NaH, DMF, 0°C→RT; then $PdCl_2$, AcONa, AcOH, room temperature, 76% (2 steps); g) 1) **18**, PivCl, pyridine, room temperature; 2) I_2, pyridine/water, quantitative (2 steps); h) HCl, MeOH, 0°C→RT, 88%; i) 1) **5**, PivCl, pyridine, room temperature; 2) I_2, pyridine/water, quantitative (2 steps). All = allyl, Bz = benzoyl, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, TIPS = triisopropylsilyl, TMSOTf = trimethylsilyl trifluoromethanesulfonate.

thioester.[11f] The ligation of the rPrP thioester with **22** (1.5 equiv) proceeded in 6 M guanidine hydrochloride at pH 7.8 in the presence of 1% (v/v) thiophenol to produce **23** in 50% yield (Scheme 4). The GPI-anchored rPrP **23** was purified by RP HPLC and analyzed by MS (calculated mass: 17705 Da; Figure 2A,B).[20] Excess GPI anchor (derived from **22**) without the 2-mercaptoethanol protecting group was recovered and recycled to improve the overall efficiency of the synthesis. No addition of detergents or lipids was required to solubilize **22** or **23** during the ligation, in contrast to described ligations of lipidated peptides.[7b,16,21]

The GPI anchor **22** confers lipophilic properties to proteins; at the same time, the large hydrophilic head group ensures solubility in water. The folding of **23** into its native form was promoted by rapid 10-fold dilution with a buffer containing 20 mM NaOAc at pH 5.5, followed by gel filtration.[22] The folded rPrP–GPI **23** was obtained in 90% yield. SDS-PAGE analysis confirmed the homogeneity of **23**, with the observation of a sharp band at approximately 17 kDa (Figure 2C). CD spectroscopy indicated an α-helical secondary structure, which is characteristic of PrP^C. The observed molar ellipticities for folded **23** are comparable to those measured for PrP^C obtained from expression in bacterial and eukaryotic systems and indistinguishable from those of folded rPrP without a GPI anchor (Figure 3A).[11b,e,f] Folded **23** was soluble in aqueous buffers without the addition of detergents or lipids.

The influence of GPI anchoring on the membrane association of $rPrP^C$ was studied by incorporating **23** into

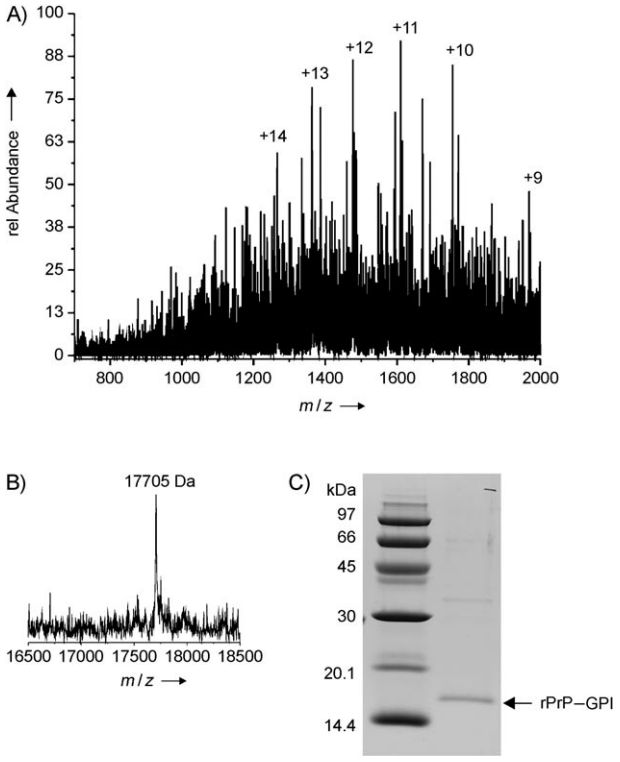


Figure 2. A) ESI mass spectrum of **23**. B) Deconvoluted mass spectrum of **23** (MW_{calcd}: 17704 Da). C) SDS-PAGE of purified **23** (right lane); left lane: molecular-weight marker.

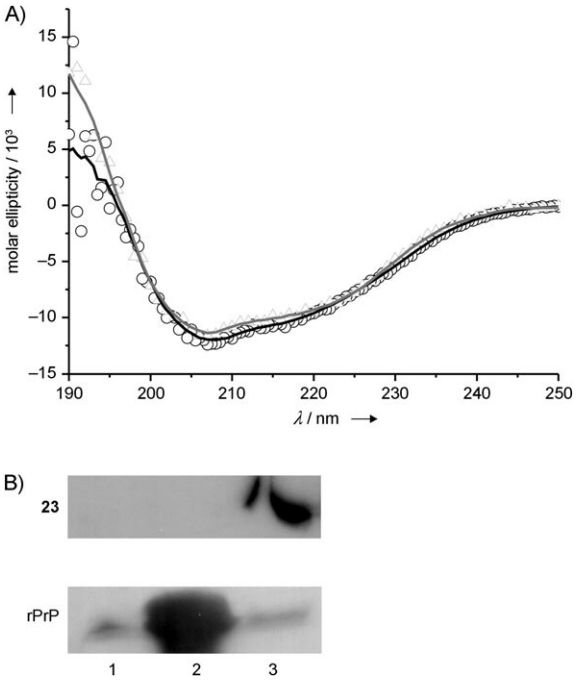


Figure 3. A) CD spectrum of folded **23** (○) and rPrP (△) at 0.2 mg mL^{-1} in NaOAc buffer at pH 5.5. B) Immunoblotting analysis of a vesicle pull-down assay of folded **23** (upper panel) and rPrP (lower panel) with the PrP-specific antibody A7. DOPC vesicles with an average diameter of 80 nm were mixed with **23**. Lane 1: pellet resulting from the low-spin centrifugation of **23** and rPrP solutions before addition to vesicles; lane 2: supernatant of high-spin centrifugation of vesicles incubated with PrP samples; lane 3: pellet resulting from the high-speed centrifugation of vesicles incubated with PrP samples.

small unilamellar 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) vesicles (SUVs) in a vesicle-spin-down assay. Low-spin centrifugation of **23** removed any aggregated protein prior to transfer into the vesicles (Figure 3B, lane 1). No precipitation of **23** was observed during this first centrifugation step. Fully soluble **23** was diluted 10-fold in a solution of SUVs at a concentration of 10 mg mL^{-1} and incubated for 30 min at room temperature. Subsequent high-speed centrifugation precipitated the SUVs and all vesicle-associated protein. Analysis of the pellets and the supernatant revealed that **23** was located exclusively in the pellet (Figure 3B, lanes 2 and 3). This observation indicates quantitative attachment of **23** to the vesicles, although the synthetic GPI anchor contains only one C_{18} lipid chain. In contrast, peptide-based GPI-anchor mimics require at least two lipid chains for efficient membrane attachment. rPrP without a lipid anchor remained in the supernatant under the same conditions (Figure 3B, lane 2). These observations emphasize the power of GPI anchors in the membrane association of proteins.

In summary, we have developed a general synthetic strategy for the preparation of homogeneous GPI-anchored proteins. Our approach is based on the native chemical ligation of a synthetic cysteine-tagged GPI anchor with a recombinant protein containing a C-terminal thioester. Access to the lipidated GPI anchor relies on the incorporation of the cysteine residue into the GPI backbone prior to global deprotection and on the judicious selection of protecting

groups. This strategy was applied to the semisynthesis of a GPI-anchored prion protein. Synthetic access to lipidated GPI anchors in milligram quantities for selective protein modification by native chemical ligation paves the way for detailed analysis of the influence of this complex posttranslational modification on protein structure and function. In vitro and in vivo experiments with this GPI-anchored PrP should be helpful in the elucidation of the influence of GPI-mediated membrane association on the conversion of PrP^{C} into pathogenic PrP^{Sc}.

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[19] The structure of the lipid moieties in prion GPI anchors has not been fully elucidated (see Refs. [16, 17]). It is presumed that they are derived from glycerol 1-phosphate; however, this presumption includes the possibility of diacyl or dialkyl glycerol lipids, 1-acyl or 1-alkyl 2-lysoglycerol lipids, or other glycerol lipids. Therefore, as a starting point for our study, we chose a single lipid chain (stearoyl phosphate), which should be long enough to interact with membranes.

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