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Identification of Glycoinositol Phospholipid Linked and Truncated Forms of the Scrapie Prion Protein†

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ABSTRACT: Analysis of carboxy-terminal peptides derived from endoproteinase Lys-C digests of the scrapie isoform of the hamster prion protein revealed that the majority of the molecules are glycoinositol phospholipid linked through ethanolamine attached at serine-231. However, ~15% of PrP^{Sc} had a carboxy-terminal peptide that ends at glycine-228. It is intriguing that this glycine is part of the PrP sequence Gly-Arg-Arg, which is an established target sequence for the proteolysis and release of bioactive peptides from larger precursors. The mechanism of formation, as well as the role of the truncated carboxy terminus in the dissemination and neuropathology of scrapie, remains to be determined.

The neurodegenerative disease scrapie is transmitted by an infectious agent called a prion because of its unusual properties (Prusiner, 1982, 1988). There is no compelling evidence that requires a nucleic acid component of the prion, despite extensive investigation (Alper et al., 1967; Bellinger-Kawahara et al., 1987a,b; Diener et al., 1982; Gabizon et al., 1987; Hunter, 1979; McKinley et al., 1983b). The only identified component of the scrapie prion is a host-encoded protein called PrP^{Sc} (Bolton et al., 1982, 1984; Diringer et al., 1983; McKinley et al., 1983a; Prusiner et al., 1982, 1983), which is a sialoglycoprotein with a glycoinositol phospholipid (GPI)[1] attached at its carboxyl terminus (Stahl et al., 1987). This protein appears to have the same amino acid sequence as that of a normal host protein of unknown function called PrP^{C} (Oesch et al., 1985; Turk et al., 1988), yet the two isoforms have different physical properties. While PrP^{C} is sensitive to protease digestion and is soluble in the presence of detergents (Meyer et al., 1986; Oesch et al., 1985), PrP^{Sc} loses an amino-terminal peptide upon proteinase K digestion to give PrP 27–30 (Prusiner et al., 1984), which aggregates in the presence of detergents to form amyloid rods (Prusiner et al., 1983; McKinley et al., 1988). It is likely that PrP^{Sc} is derived from PrP^{C} or a closely related precursor through a posttranslational event, such as a chemical or conformational modification, or tight association with another molecule (Basler et al., 1986; Borchelt et al., 1990; Oesch et al., 1985). Metabolic pulse–chase radiolabeling experiments in scrapie-infected cultures of neuroblastoma cells indicate that the protease resistance of PrP^{Sc} is acquired with a half-time of 15 h during a nonradioactive chase (Borchelt et al., 1990). We are cataloging all of the posttranslational chemical modifications of PrP 27–30 to identify candidates that may account for the altered physical properties, as well as to provide clues to the mechanism of prion replication and its effect on cellular functions.

GPI modification of nascent proteins probably occurs by transamidation: a <50-residue carboxy-terminal peptide is lost with concomitant amide bond formation between the α-carboxyl group at the cleavage site and the ethanolamine (Ea) of a putative GPI precursor (Ferguson & Williams, 1988;

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[1] Abbreviations: GPI, glycoinositol phospholipid; LSIMS, liquid secondary ion mass spectrometry; PAM, peptidyl-glycine α-amidating monooxygenase; ChAT, choline acetyltransferase; ARIA, acetylcholine receptor inducing activity; Ea, ethanolamine.

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Menon et al., 1988; Masterson et al., 1989; Doering et al., 1990). The exact amino acid that becomes GPI-linked varies and is known for only a few proteins (Ferguson & Williams, 1988; Boothroyd, 1985; Seki et al., 1985; Micanovic et al., 1988; Ogata et al., 1988; Tse et al., 1985; Gibney et al., 1988; Haas et al., 1986). Assignment of this position by inspection of the protein sequence is more intuitive than rule-based; serine-231 was predicted as the site of GPI modification for PrP (Ferguson & Williams, 1988).

In this paper, we describe two alternative carboxy termini of PrP^{Sc}. The majority of the molecules are GPI-linked at Ser_{231} as predicted, while ~15% are truncated, ending at Gly_{228}.

Materials and Methods

Protein Purification. PrP^{Sc} was purified by a modification of the large-scale PrP 27–30 preparation (Prusiner et al., 1983; Turk et al., 1988). The two procedures were identical until the P3 pellet was obtained except for omission of the proteinase K digestion. The P3 pellet was then taken through three cycles of resuspension in 0.2 M sodium chloride and 5% Sarkosyl, probe sonication with eight bursts of 7-s duration, and recovery of the pellet by centrifugation at 300000*g* for 30 min. PrP 27–30 was purified from hamster brain and precipitated from sucrose gradient fractions as previously described (Stahl et al., 1987).

The ethanol-precipitated protein pellets were solubilized at a concentration ≤0.5 mg/mL in 6 M guanidine hydrochloride, 50 mM Tris·HCl (pH 8.2–8.45), and 5 mM EDTA. The protein was reduced with 2 mM dithiothreitol at room temperature for 1 h, alkylated with 6 mM iodoacetic acid for 1.5 h at 4 °C, and precipitated with 10–15 volumes of ethanol overnight at −20 °C. The pellet was collected by centrifugation at 6000*g* for 30 min and then washed with 1 mL of cold ethanol to remove the last traces of guanidine hydrochloride. The protein pellet was resuspended at ≤0.25 mg/mL in 0.1% SDS, 50 mM Tris·HCl (pH 8.45), and 1 mM EDTA and boiled for 1 min. This preparation was digested overnight at 37 °C with 5–10 μg of endoproteinase Lys-C (sequencing grade, Boehringer Mannheim). After the addition of 1 mM PMSF, the sample was incubated with 0.3 unit of *Bacillus thuringiensis* PIPLC (a gift from Martin Low) for 3–6 h at 37 °C. Since SDS interferes with reverse-phase chromatography, dodecyl sulfate was precipitated from the sample by addition of 1 M guanidine hydrochloride, incubation at 4 °C for ~15 min, and centrifugation in a microfuge for 5 min (Shively, 1986).

Chromatography was performed on a two-pump system (Gilson) and a reverse-phase C-18 column (Vydac; 4.1 × 100 mm, 300-Å pore size). Peptides were chromatographed with gradients from 0.06% trifluoroacetic acid in water (buffer A) to 0.052% trifluoroacetic acid in 80:20 acetonitrile–water (buffer B) pumped at 0.75 mL/min, except for the chromatograph in Figure 2A where the flow rate was 0.5 mL/min. The eluate absorbance was monitored at both 214 and 280 nm on the Gilson 116 detector. Fractions were collected in 1.5-mL polyethylene tubes with a Gilson Model 203 operating in the peak detection mode from the absorbance at 214 nm.

Amino Acid Analysis. HPLC fractions containing peptides were dried in 6 × 50 mm glass tubes (Corning 9820) and hydrolyzed at 110 °C overnight under vacuum in 6 M HCl (Pierce) and phenol with the vapor method described by Tarr (1986). Following hydrolysis, residual HCl was evaporated under vacuum, and the hydrolysate was dissolved in 10 μL of water. The 6 × 50 mm tubes were loaded directly into the Gilson 231 autosampler (code 2 rack), which controlled the derivatization of the sample with 10 μL of an *o*-phthalaldehyde solution (Fluoraldehyde, Pierce) for 1 min, followed by dilution with 150 μL of 50 mM acetic acid before injection. The OPA-amino acids were separated at a flow rate of 1.5 mL/min on a Beckman C-18 column (3-μm silica, 4.6 × 75 mm) where solvent A was 46 mM sodium acetate and 4 mM acetic acid and solvent B was methanol. The separation gradient of 0–10% at 2 min, 12% at 5 min, 17% at 7 min, 20% at 9 min, 22% at 11 min, 25% at 17 min, 40% at 23 min, 42% at 26 min, 50% at 30 min, 50% at 31 min, 65% at 35 min, 67% at 37 min, 82% at 44 min, and 100% at 48 min gave excellent resolution of all peaks, including glycine and threonine. Fluorescence of the eluate was measured with a Gilson 121 set at a range of 0.02. Amino acids were quantitated by comparison to 125- and 25-pmol samples of amino acid hydrolysate standard H (Pierce).

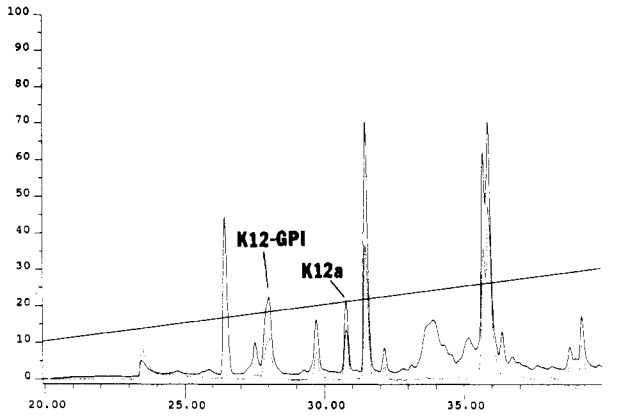

FIGURE 1: HPLC of endoproteinase Lys-C-digested PrP 27–30. The solid and dotted lines indicate the absorbance at 214 and 280 nm, respectively. The absorbance at 280 nm is plotted on a scale 5-fold more sensitive than the 214-nm trace, and the acetonitrile (percent buffer B) gradient is indicated by the diagonal line. The vertical scale indicates either the percentage of buffer B or the absorbance, where 100% equals 1.4 absorbance units for the solid line (214 nm) and 0.28 absorbance unit for the dotted line (280 nm). The recovery of K12-GPI (28 min) was relatively low in this particular experiment, probably from an incomplete reaction by PIPLC (compare ratio of peak areas at 26 and 28 min to Figure 4).

Mass Spectrometry. Liquid secondary ion mass spectrometry (LSIMS) was carried out in the positive ion mode on a Kratos MS50 mass spectrometer at 6-kV accelerating voltage and 2500-kV resolving power, equipped with a cesium ion LSIMS source and postacceleration detector (10 kV). Mass calibration was carried out with Ultramark 1621 (PCR). Samples were introduced onto the target stage in aqueous solution (1 μL) and a glycerol–thioglycerol matrix was added (1–2 μL). Spectra were obtained by analog recording with magnet scans at 300 s/decade and a bandwidth of 30 Hz.

Results and Discussion

Our strategy for determining the GPI attachment site of PrP 27–30 involved the characterization of peptides generated by digestion with endoproteinase Lys-C. On the basis of the PrP genomic (Basler et al., 1986) and cDNA (Oesch et al., 1985) sequences, the lysine nearest the carboxy terminus in the protein molecule occurs at position 220, well before the predicted attachment site at serine-231. A portion of the reverse-phase HPLC chromatogram of endoproteinase Lys-C and PIPLC-digested PrP 27–30 is shown in Figure 1. Amino acid analysis of each peak allowed tentative assignment of PrP peptides (denoted K1–K12) by reference to the gene sequence. The peptide eluting at 28 min contained 1.5 equiv of Ea, a GPI anchor component, and had an amino acid composition con-

Table I: Amino Acid Composition of C-Terminal PrP Peptides[a]

	K12-GPI			K12a (PrP 27–30)	
	predicted	obsd	K12-Ea		K12a (PrP^{Sc})
D	1	1.0	1.0	1.0	1.0
E	2	1.7	2.0	1.7	1.8
S	2	1.7	1.8	0.7 (1)	0.9 (1)
G	1	1.2	1.2	1.2	1.3
R	2	1.5	1.8	0.1 (0)	0.2 (0)
A	1	1.0	1.1	0.8	0.8
Y	2	1.6	2.1	1.3	1.4
Ea	>1	1.5	0.9 (1)	<0.05 (0)	<0.05 (0)

[a] K12-GPI is the carboxy-terminal peptide generated by endoproteinase Lys-C that contains the GPI anchor; K12-Ea is the peptide ending in Ea that is generated from K12-GPI by incubation in aqueous HF; and K12a is the truncated C-terminal peptide ending at Gly_{228} purified from endoproteinase Lys-C digests of either PrP 27–30 or PrP^{Sc}. The values represent the ratio of each amino acid to the amount of aspartic acid. The predicted values for K12-GPI are listed; if different, the values predicted for other peptides are indicated in parentheses. These numbers reflect the fact that glutamine (Q) is converted to glutamate (E) during acid hydrolysis. The values reported for Ea have been increased by a factor of 1.4 on the basis of the loss observed during hydrolysis of an Ea standard.

sistent with the sequence ESQAYYDGRRS–GPI (K12-GPI, Table I). Gas-phase Edman sequencing of this peptide confirmed the amino acid sequence through serine-231 (N. Stahl, D. Teplow, and L. Hood, unpublished data) and will be presented elsewhere with the complete analysis of the remaining PrP peptides.

In order to verify this attachment site by mass spectrometry, the GPI glycan portion of purified K12-GPI was removed by incubation with 50% aqueous HF at 4 °C, which cleaves phosphodiester bonds and leaves glycosidic bonds largely intact (Ferguson et al., 1988). Rechromatography of the peptide following this treatment gave a sharp absorbance peak with a longer retention time (Figure 2A). The amino acid composition of the major peak matched that of the original, except that only one Ea residue remained (K12-Ea, Table I). The LSIMS (Aberth et al., 1986; Falick et al., 1986) spectrum of this peptide gave a protonated molecular ion at m/z = 1374, consistent with the sequence ESQAYYDGRRS-Ea (Figure 3A). The glycan portion of the GPI anchor can be recovered from the unretained fraction upon rechromatography following aqueous HF.

We have consistently observed the small peak eluting at 32.3 min (at 0.5 mL/min flow rate) upon chromatography of aqueous HF-treated K12-GPI (Figure 2A); amino acid analysis showed a similar composition for this peptide and the main product K12-Ea. Unfortunately, no peaks were observed upon LSIMS analysis of this small peak. Rechromatography of untreated K12-GPI also gave a small peak eluting in a corresponding position (see Figure 2C). Structural characterization of this small component will require further investigation.

Other results corroborate serine-231 as the GPI attachment site in PrP 27–30. (1) Although Pronase digestion of PrP 27–30 followed by purification of the lipid-containing GPI by C-4 reverse-phase chromatography gave a low yield, the product contained only serine and Ea upon amino acid analysis (N. Stahl, unpublished data). (2) Trypsin cleavage of K12-GPI gave a product (29.8 min; Figure 2B) with the amino acid composition ESQAYYDGR and a protonated molecular ion of m/z 1088 in the LSIMS spectrum (data not shown), consistent with attachment of the GPI anchor at Ser_{231} and not internally at Ser_{222}.

Since the size of the hydrophobic stretch and the number of amino acids removed from the carboxy terminus vary for

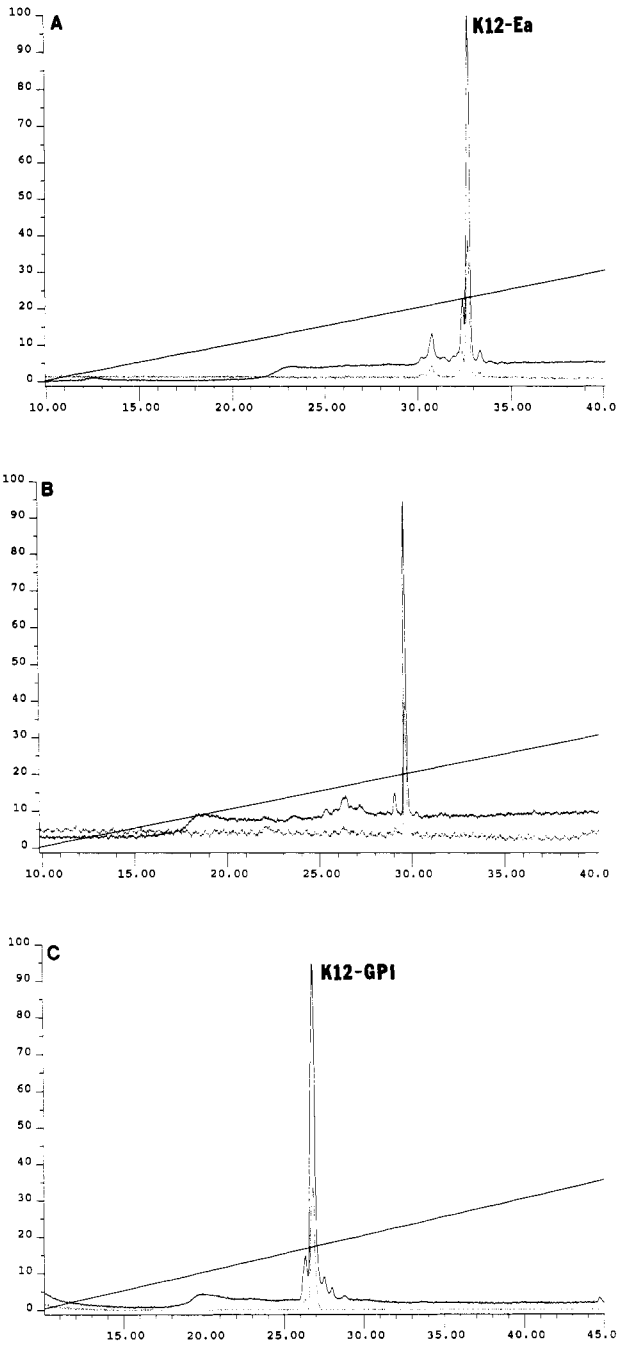

FIGURE 2: Chemical and enzymatic digestion of K12-GPI. (A) Rechromatography of K12-GPI after incubation with 50% aqueous HF. Fractions containing K12-GPI peptide were dried by vacuum centrifugation in 0.5-mL polyethylene tubes and incubated at 4 °C for 16–36 h in 20 μL of 50% aqueous HF (Aldrich) as described (Ferguson et al., 1988). The aqueous HF was removed in a SpeedVac, and the sample was solubilized in 0.06% trifluoroacetic acid before rechromatography at 0.5 mL/min. The main peak elutes at 30.3 min when chromatographed at 0.75 mL/min. (B) Rechromatography of K12-GPI after digestion with trypsin. The vertical scale equals 0.1 absorbance unit (solid line) or 0.02 absorbance unit (dotted line). HPLC fractions containing K12-GPI were brought to pH ~8 with ammonium hydroxide and then incubated with 0.66 μg/mL trypsin for 4 h at 37 °C before repurification as in Figure 1. (C) Redigestion of purified K12-GPI with endoproteinase Lys-C. HPLC fractions containing K12-GPI were dried to a small volume in a SpeedVac, resuspended in the endoproteinase Lys-C digestion buffer described in the legend to Figure 1, and incubated overnight at 37 °C with 1 μg of endoproteinase Lys-C. The sample was rechromatographed following precipitation of dodecyl sulfate with 1 M guanidine hydrochloride. The vertical scale equals 0.2 absorbance unit (solid line, 214 nm) or 0.04 absorbance unit (dotted line, 280 nm).

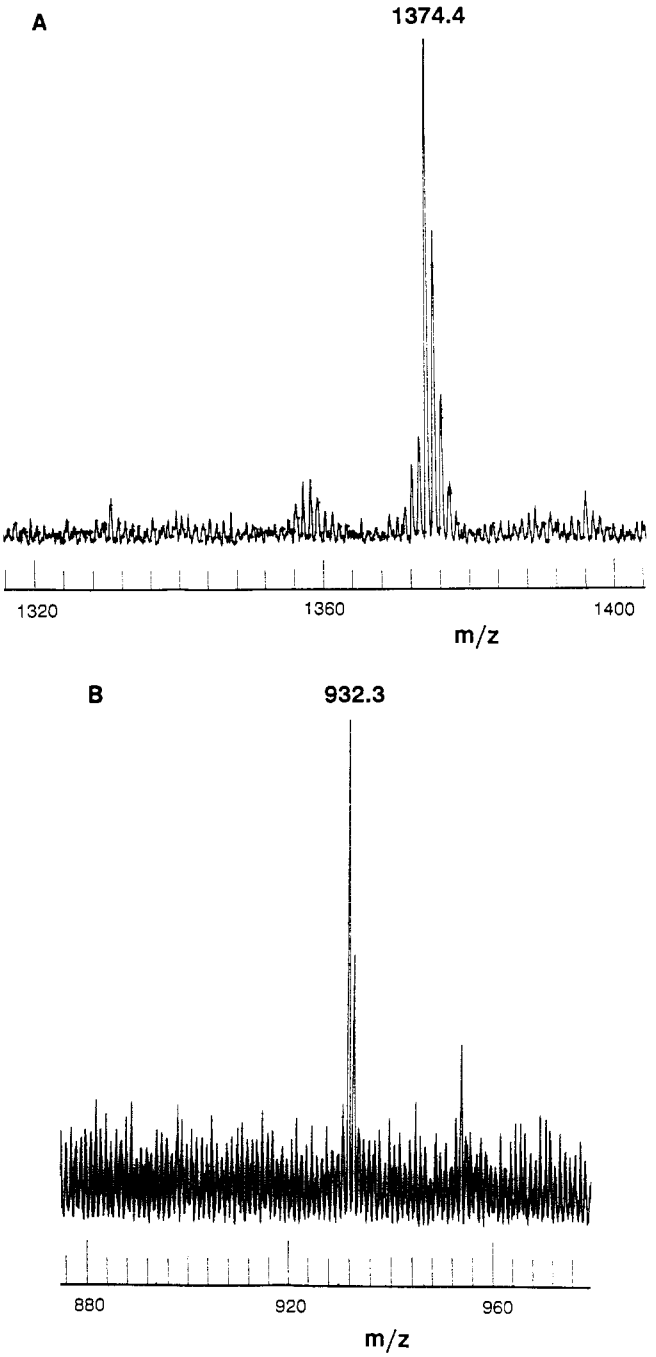

FIGURE 3: Liquid secondary ion mass spectrometry (LSIMS) of carboxy-terminal peptides from PrP 27–30. (A) LSIMS spectrum of K12-Ea. (B) LSIMS spectrum of K12a. In both spectra, the small peaks located 22 mass units above the main peak are the M + Na^+ species.

different proteins, it was suggested that GPI attachment generally occurs 10–12 residues before the carboxy-terminal hydrophobic sequence (Ferguson & Williams, 1988). It will therefore be interesting to determine the site of GPI attachment for murine PrP, which differs from the hamster sequence by the insertion of an extra serine adjacent to residue 231, giving three sequential serines (Westaway et al., 1987). If the GPI modification machinery measures strictly from the carboxy-terminal hydrophobic sequence, one might expect attachment to the middle serine. However, if the sequence of the target site is also important for alignment, GPI attachment may still occur at the first serine. There is evidence for some sequence requirements at the target site from experiments with chimeric and mutant proteins (Caras et al., 1989; Hibbs et al., 1989; Lanier et al., 1989; Kurosaki & Ravetch, 1989).

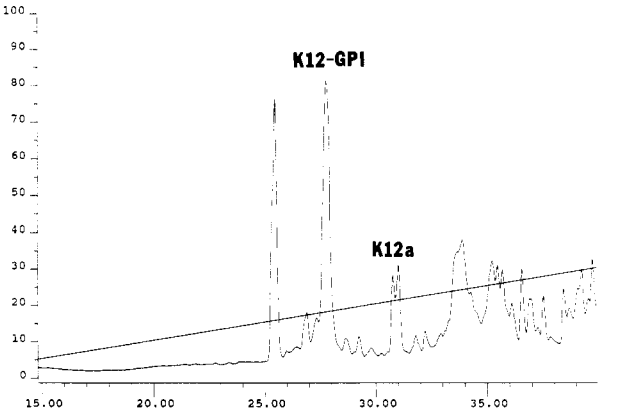

FIGURE 4: HPLC of endoproteinase Lys-C-digested PrP^{Sc}. The absorbance trace at 214 nm is shown plotted on a vertical scale equal to 0.3 absorbance unit.

The method we used for GPI anchor purification has the advantage of allowing recovery of both the carboxy-terminal peptide and the GPI glycan for structural characterization in a solvent compatible with analytical methods such as mass spectrometry. Following permethylation, tandem mass spectrometry of the GPI glycan can reveal structural information on subnanomole quantities of carbohydrate (Baldwin et al., 1990). Poor recovery of the GPI anchor by reverse-phase HPLC on C-18 or C-4 columns was observed without PIPLC cleavage, even if the peptide was first removed by Pronase digestion (Stahl et al., 1990, and unpublished data). However, reverse-phase HPLC purification following digestion with a specific protease and removal of diradylglycerol by PIPLC gave nearly quantitative recovery of the GPI-linked peptide, which can be identified by the presence of Ea upon amino acid analysis. A similar strategy applied to carcinoembryonic antigen employed anhydrous HF treatment of the intact protein, which cleaves glycosidic bonds, resulting in destruction of the carbohydrate and the GPI anchor (Hefta et al., 1988).

Non-GPI-Linked Carboxy Terminus. Amino acid analysis of the peak eluting at 30.8 min in the chromatogram shown in Figure 1 gave a composition consistent with a truncated carboxy-terminal peptide of the sequence ESQAYYDG (K12a, Table I). LSIMS analysis confirmed this assignment, giving a protonated molecular ion with m/z 932 (Figure 3B). This peptide corresponds to the loss of three amino acids and the GPI anchor from the carboxy terminus.

Two potential artifactual sources of this truncated peptide were addressed in the following control experiments. First is the possibility that endoproteinase Lys-C might create this peptide by cleavage after the glycine. This was ruled out by reincubation of purified K12-GPI with endoproteinase Lys-C under conditions identical with those of the original digest. Rechromatography of the products indicated no formation of K12a eluting at 30.8 min (Figure 2C). A similar experiment carried out on a synthetic peptide that spans the PrP residues from Asp_{202} to Val_{234} gave the peptides expected from digestion with endoproteinase Lys-C, but not the product corresponding to cleavage after glycine-228 (data not shown).

Another possibility is that the truncated carboxy terminus is generated upon purification, during which PrP 27–30 is formed by limited proteolysis of PrP^{Sc} with proteinase K. We therefore examined preparations of PrP^{Sc} purified in the absence of proteases. Endoproteinase Lys-C digests of these preparations contained at least two absorbance peaks eluting with K12a near 30.8 min (Figure 4). Rechromatography of

these two peaks on a more gradual acetonitrile gradient gave three peaks, one of which had an amino acid composition consistent with the sequence ESQAYYDG (Table I). LSIMS analysis of this fraction confirmed the presence of a weak protonated molecular ion with m/z 932.

The truncated peptide K12a was present in two preparations each of PrP 27–30 and PrP^{Sc} at 10–20% relative to the amounts of other PrP peptides. The presence of a truncated carboxy-terminal peptide implies that ~15% of the PrP 27–30 or PrP^{Sc} molecules end at Gly_{228}. It is unlikely that the carboxy terminus ending at Gly_{228} arises from *nonspecific* proteolysis during purification of PrP^{Sc} since the amino terminus is found intact, even though it is protease-sensitive. It is also unlikely that the truncated protein is synthesized from an alternative mRNA since the PrP gene is single copy (Oesch et al., 1985), the open reading frame is contained within a single exon (Basler et al., 1986), only one form of mRNA is detected on Northern blots (Oesch et al., 1985), and a single cDNA sequence has been observed (Oesch et al., 1985; Basler et al., 1986). More exotic explanations such as mRNA editing (Powell et al., 1987) or alternative translation products from a single message (Huang et al., 1988) have not been explored. Although we believe that the truncated PrP 27–30 is probably generated by posttranslational processing in vivo from a GPI-linked form, we cannot rule out the possibility that an artifactual cleavage occurs during purification. On the basis of experiments with protease inhibitors, it was suggested previously that both PIPLC and proteolytic cleavage of alkaline phosphatase were required to generate a GPI-related mediator after stimulation of cells with insulin (Romero et al., 1988).

An interesting possibility is that this processing occurs at the sequence Gly-Arg-Arg, which is an established target site for the proteolysis and maturation of bioactive peptides (Loh & Parish, 1987). The activities responsible for this processing are reported to be concentrated in secretory vesicles of various brain tissues (Loh & Parish, 1987) and consist of at least three enzymes. This processing involves (1) proteolysis between or after two basic residues, (2) removal of the remaining carboxy-terminal basic amino acids with a carboxypeptidase B like protease, and (3) cleavage of the glycine by peptidyl-glycine α-amidating monooxygenase (PAM) to leave an amide at the carboxy terminus. The truncated carboxy terminus of PrP corresponds to the product of the first two enzymes. After a careful search of the PrP endoproteinase Lys-C digests, we found no amidated peptide corresponding to $ESQAYYD_{NH_2}$. This reaction could be adversely affected by the aspartic acid preceding the glycine; PAM from one source prefers neutral amino acids in this position (Bradbury & Smyth, 1983). The Gly-Arg-Arg sequence is conserved in PrP from Armenian and Chinese hamsters (Lowenstein et al., 1990), mouse (Westaway et al., 1987), and rat (Liao et al., 1987), while human PrP has only a single arginine in this region of the protein (Kretzschmar et al., 1986). Although it is unknown whether human PrP is also found with a truncated carboxy terminus, there are other bioactive peptides, such as dynorphin B, that are cleaved on the amino-terminal side of a single arginine residue (Devi & Goldstein, 1985).

It is possible that truncated forms of PrP^{C} also exist, though this remains to be proven. PrP^{C} is spontaneously released from cultured cells (Caughey et al., 1988; Borchelt et al., 1990), as well as *Xenopus* oocytes injected with PrP mRNA synthesized in vitro (Hay et al., 1987). The spontaneous release of PrP^{C} from cultured cells occurs with a half-time of 15 h in pulse–chase metabolic radiolabeling experiments and accounts for 10% of the total immunoprecipitated PrP^{C} (Borchelt et al., 1990). The spontaneously released form partitions into the aqueous phase of Triton X-114, indicating the loss of at least the lipid portion of the GPI anchor (D. R. Borchelt, unpublished data). Two observations argue against the possibility that the spontaneous release is mediated by phospholipase C. PIPLC-released PrP^{C} derived from these cells, but not spontaneously released PrP^{C}, reacts with cross-reactive determinant antisera (D. R. Borchelt and S. B. Prusiner, unpublished data), which specifically binds an epitope created by PIPLC cleavage (Ferguson & Williams, 1988). Also, the spontaneously released form migrates faster on SDS–PAGE than PIPLC-released PrP (D. R. Borchelt and S. B. Prusiner, unpublished data). This difference of 2–3 kDa is close to that observed for PrP 27–30 upon removal of the GPI anchor by treatment with 50% aqueous HF (N. Stahl, unpublished data).

Recent experiments have identified an intriguing homology between PrP and ARIA—a protein purified from chicken brain that induces acetylcholine receptor expression in myotubes (Harris et al., 1989). Although ARIA is purified as a soluble factor, the preliminary report that the gene sequence predicts a hydrophobic carboxy-terminal peptide invites speculation that ARIA may also be released from a GPI-anchored precursor. Future experiments should reveal whether PrP is the mammalian homologue of chicken ARIA or perhaps carries out a distinct, but analogous, function. It is noteworthy that expression of PrP mRNA in the basal forebrain of neonatal hamsters during development parallels that of choline acetyltransferase (ChAT) activity, and injection of nerve growth factor coordinately increases PrP mRNA levels and ChAT activity in brain regions containing nerve growth factor responsive cholinergic neurons (Mobley et al., 1988).

Acknowledgments

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