Reprinted from Biochemistry, 1993, 32.

Processing of a Cellular Prion Protein: Identification of N- and C-Terminal Cleavage Sites†

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Received May 27, 1992; Revised Manuscript Received November 18, 1992

ABSTRACT: ChPrP is the chicken homologue of PrP^C, the cellular isoform of the mammalian prion protein. We have used sequence-specific antibodies to immunoprecipitate and immunoblot chPrP derived from stably transfected cultures of neuroblastoma cells, as well as from chicken brain and cerebrospinal fluid. We have also used mass spectrometry to characterize fragments of the protein purified from conditioned medium. The majority of chPrP protein present in neuroblastoma cells and on isolated brain membranes can be released by incubation with phosphatidylinositol-specific phospholipase C, indicating that these molecules are attached to the cell surface by a glycosylphosphatidylinositol anchor. Surprisingly, most of the surface-anchored molecules are truncated at their N-terminus distal to the proline/glycine-rich repeats. The corresponding N-terminal fragments are found in medium conditioned by neuroblastoma cells, as well as in cerebrospinal fluid and a postmicrosomal supernatant of brain. One of these fragments extends from Lys^{25} to Phe^{116}. 35–45-kDa forms of chPrP that can be metabolically labeled with [^{3}H]ethanolamine can also be found in extracellular media. We propose that the chPrP molecule undergoes at least two cleavages as part of its normal metabolism: one within the glycosylphosphatidylinositol anchor and one within or just N-terminal to the central hydrophobic domain. The second cleavage lies within a region of 24 amino acids that is identical in chPrP and mammalian PrP, and represents a major processing event that may have physiological as well as pathological significance.

The spongiform encephalopathies are a group of transmissible, degenerative diseases of the central nervous system including Creutzfeldt–Jakob disease, Gerstmann–Sträussler syndrome, and kuru in man, as well as scrapie and bovine spongiform encephalopathy in animals [reviewed by Gajdusek (1990)]. The infectious agent thought to be responsible for these diseases has been called a *prion*, to distinguish it from a conventional virus (Prusiner, 1982). The major and possibly only constituent of prions is a glycoprotein of molecular mass 33–35 kDa, referred to as PrP^{Sc},[1] which is a posttranslationally modified isoform of the normal cell-surface protein PrP^C [1] [reviewed by Prusiner (1989, 1991)]. The nature of the modification that distinguishes the two isoforms has remained elusive.

The normal function of PrP^C is unknown, but the protein is likely to be important since it is widely expressed in the central nervous system beginning early in embryonic development (Manson et al., 1992; Harris et al., 1993), and portions of its sequence have been highly conserved during evolution (Gabriel et al., 1992). A role for PrP^C in lymphocyte activation, neural development and differentiation, and cell adhesion has been suggested (Cashman et al., 1990; Manson et al., 1992; Mobley et al., 1988). Mice in which the PrP gene has been deleted do not exhibit obvious phenotypic abnormalities, indicating that loss of this protein produces effects that are subtle or that other proteins can substitute for PrP^C in these animals (Büeler et al., 1992).

To gain further insight into the physiological function of PrP^C, we have been studying the posttranslational processing and cellular trafficking of chPrP,[1] the chicken homologue of mammalian PrP^C. Originally identified in preparations of an acetylcholine receptor-inducing activity from brain, chPrP is identical to mouse PrP at 33% of its 267 amino acid positions, including an uninterrupted stretch of 24 identical residues (Harris et al., 1991). ChPrP and mammalian PrP also share the same primary sequence domains, including a series of proline- and glycine-rich peptide repeats and two hydrophobic segments as well as cysteine residues and potential N-glycosylation sites that occur in similar locations. The C-terminal hydrophobic segment serves as a signal for addition of a glycosylphosphatidylinositol (GPI)[1] anchor to both proteins (Stahl et al., 1990a; Harris et al., 1991). Finally, the chicken and mammalian PrP genes have a similar intron–exon structure (Harris, unpublished data; Gabriel et al., 1992).

We present here a study of the metabolic processing of chPrP. Our results suggest that posttranslational processing of the chPrP molecule involves at least two separate cleavage events. One is a major proteolytic cleavage within the uninterrupted stretch of 24 amino acids that is identical in the chicken and mammalian proteins. Most of the cell-surface chPrP molecules have been truncated at this site, and the

† This work was supported by grants to D.A.H. from the NIH (Clinical Investigator Development Award and NS30137) as well as the Alzheimer's Disease Research Center and the McDonnell Center for Cellular and Molecular Neurobiology at Washington University. D.A.H. is the recipient of a fellowship award from the Esther A. and Joseph Klingenstein Fund. B.T.C. and R.W. are supported by NIH Grants RR00862 and GM38274.

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[1] Abbreviations: PrP, prion protein; PrP^{Sc}, scrapie isoform of PrP; PrP^C, cellular isoform of PrP; chPrP, chicken prion protein [referred to as ch-PrLP in Harris et al. (1991)]; GPI, glycosylphosphatidylinositol; PIPLC, bacterial phosphatidylinositol-specific phospholipase C; CSF, cerebrospinal fluid; HPLC, high-performance liquid chromatography; MALDMS, matrix-assisted laser desorption mass spectrometry; TFA, trifluoroacetic acid.

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resulting N-terminal fragments are found in substantial amounts in the extracellular medium. A second cleavage occurs within the GPI anchor that attaches the protein to the plasma membrane. These two cleavages may be important in the generation of soluble and membrane-bound forms of the protein that are biologically active.

The results presented here have been reported in abstract form.[2]

EXPERIMENTAL PROCEDURES

Antisera. Figure 1 illustrates the immunogens used to raise rabbit polyclonal antisera specific for chPrP. Peptides were conjugated to keyhole limpet hemacyanin (P25–40 and P92–103) or soybean trypsin inhibitor (P47–65) using sulfo-*m*-maleimidobenzoyl-*N*-hydroxysuccinimide (Harlow & Lane, 1988). Fusion proteins were constructed by cloning appropriate cDNA fragments generated by PCR amplification or restriction digestion of p65-21 (Harris et al., 1991) into the *Bam*HI site of the vector pET-3xa (Studier et al., 1990) which fuses amino acids 1–260 of the gene 10 protein of phage T7 to the N-terminus of the inserted protein. Fusion proteins were purified by SDS–PAGE, and the appropriate region of the gel was excised and pulverized. Initial immunizations included complete Freund's adjuvant and subsequent boosts incomplete adjuvant.

Antisera F35–96 and F144–220 were affinity-purified by passage through a column of CNBr-activated Sepharose (Pharmacia) to which the corresponding fusion protein had been coupled (Harlow & Lane, 1988). Antibodies to the phage part of the fusion protein were removed by passage through a column prepared from lysates of bacteria carrying the pET-3xa vector alone.

Transfected Cell Lines. N2a mouse neuroblastoma cells (ATCC CCL131) were grown in minimal essential medium (MEM) containing 10% fetal calf serum, nonessential amino acids, and penicillin/streptomycin in an atmosphere of 5% CO_2/95% air. The expression construct, used previously, places the chPrP coding region (nucleotides −45 to +814) under control of a human cytomegalovirus promoter/enhancer in the vector pBC12/CMV (Cullen, 1986; Harris et al., 1991). Cells were transfected with a 5:1 or 10:1 mixture of this DNA and pRSVneo (Ulrich & Ley, 1990) using Lipofectin (BRL) according to the manufacturer's directions. Antibiotic-resistance clones were selected in 600 μg/mL G418, expanded, and then maintained in 300 μg/mL G418. The line we have designated A26 was used for all of the experiments reported here.

Purification of ChPrP Fragments. Opti-MEM medium (Gibco) conditioned for 2–3 days by A26 cells was collected, protease inhibitors (peptstatin and leupeptin, 1 μg/mL; phenylmethanesulfonyl fluoride, 0.5 mM; EDTA, 2 mM) and Tris-HCl (pH 7.5, final concentration 50 mM) were added, and the medium was clarified at 16000*g* for 30 min. The pH was adjusted to 5.0 with glacial acetic acid, and 150 mL was rotated overnight at 4 °C with 15 mL of (carboxymethyl)-cellulose cation-exchange resin (CM-52, Whatman). The slurry was then poured into a column, washed with 150 mL of 0.2 M NaCl/50 mM sodium acetate (pH 5.0), and eluted with 30 mL of 0.5 M NaCl/50 mM sodium acetate (pH 5.0). The eluate was adjusted to a pH of 2.5 using TFA,[1] and 15 mL was applied to a Vydac C_{18} semianalytical column (10 × 25 mm) attached to a Gilson Model 715 automated HPLC[1] apparatus. The column was washed with 0.1% TFA until the absorbance of the eluate returned to base line, and then a linear gradient of acetonitrile (0–40%, 120 mL) in 0.1% TFA was applied at a flow rate of 3 mL/min, collecting 1.5-mL fractions. The overall recovery of N-terminal fragments from conditioned medium using this two-step purification was 30–50%, as determined by immunoblotting.

Characterization of ChPrP Fragments. Edman degradation of selected HPLC fractions was performed on an Applied Biosystems Model 477 automated protein sequenator at the Washington University Protein Chemistry Laboratory. PTH amino acids were identified on an Applied Biosystems Model 120A on-line amino acid analyzer using UV detection.

Matrix-assisted laser desorption mass spectrometry (MALDMS)[1] was carried out on a laser desorption time-of-flight instrument constructed at Rockefeller University (Beavis & Chait, 1989, 1990). One microliter of concentrated HPLC fraction was mixed with 1 μL of 3,5-dimethoxy-4-hydroxycinnamic acid [sinapinic acid, 5 g/L, in 0.1% TFA/acetonitrile (2:1, v/v)], and 1 μL of this mixture was applied to the sample probe. A Nd(YAG) laser with pulsed 355-nm wavelength light of 10-ns duration was used as the ionization source. Spectra were recorded in the LeCroy Model 8828D transient digitizer. A total of 200 single-shot spectra were averaged to give improved statistics. Spectra were internally calibrated using the $[M+H]^+$ and $[M+2H]^{2+}$ peaks of cytochrome *c* from horse heart. Computerized mass searches were performed using the program PROCOMP, written by P. C. Andrews (University of Michigan).

Chicken Brain and Cerebrospinal Fluid (CSF).[1] Adult chicken brains were homogenized using a Teflon–glass apparatus (5 strokes at 1000 rpm) in ice-cold 250 mM sucrose/50 mM Tris-HCl (pH 7.5) containing protease inhibitors. The supernatant obtained after centrifugation at 1000*g* for 10 min was recentrifuged at 100000*g* for 1 h to yield a postmicrosomal supernatant and a pellet, which was resuspended by homogenization in 150 mM NaCl/50 mM Tris-HCl (pH 7.5) containing protease inhibitors. We refer to the pellet as a microsomal fraction for convenience, although it undoubtedly contains mitochondrial and synaptic membranes. The use of fresh as compared to frozen brains (from Pel-Freez) or the inclusion of two additional protease inhibitors (aprotinin, 1 μg/mL, and α_2-macroglobulin, 1 unit/mL) did not affect the reported results.

CSF was collected by inserting a 25-gauge needle into the cisterna magna of anesthetized adult chickens. After addition of protease inhibitors, CSF was centrifuged at 16000*g* for 10 min at 4 °C to remove cellular elements.

ChPrP was affinity-purified from CSF and postmicrosomal supernatants on an antibody column. The column was prepared by incubating protein A–Sepharose CL-4B (Sigma) with antiserum F1–267 (200 μL/mL of packed Sepharose) and covalently coupling the bound antibodies using dimethyl pimelimidate (Harlow & Lane, 1988). Prior to purification, samples were made 0.5% in SDS, heated at 95 °C for 10 min, and diluted with 4 volumes of 0.5% Triton X-100/50 mM Tris-HCl (pH 7.5) containing protease inhibitors. After overnight incubation of 0.1 mL of affinity resin with 5 mL of sample at 4 °C, the resin was washed 5 times in RIPA buffer [150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris-HCl (pH 7.5)] and then twice in 10 mM Tris-HCl (pH 7.5). Proteins were eluted in 0.1% TFA,[1] dried, and resuspended in SDS–PAGE sample buffer.

PIPLC[1] Digestion. Cultures of neuroblastoma cells were incubated at 37 °C for 1 h in Opti-MEM containing PIPLC

[2] Harris (1991, 1992).

from *Bacillus thuringiensis* (the kind gift of Martin Low) at 1 unit/mL. After addition of protease inhibitors and Tris-HCl (pH 7.5, final concentration 50 mM), samples were clarified by centrifugation at 16000*g* for 10 min, and proteins were precipitated with 4 volumes of ice-cold methanol.

Microsomal membrane fractions of brain were incubated at 37 °C in 150 mM NaCl/50 mM Tris-HCl (pH 7.5) containing protease inhibitors and PIPLC at 0.5–1.0 unit/mL. To facilitate access of the enzyme to closed vesicles, samples were frozen and thawed after 1 h of incubation, an aliquot of PIPLC equal to the initial amount was added, and incubation was continued for an additional hour. After a final freeze/thaw cycle, samples were centrifuged at 100000*g*, and the supernatants and pellets were analyzed.

N-Glycosidase F Digestion. Samples were made 0.5% in SDS and 50 mM in β-mercaptoethanol, heated to 95 °C for 5 min, and then diluted 3-fold into 50 mM Tris-HCl (pH 7.5) containing 1.88% NP-40 and protease inhibitors. Samples were then incubated overnight at 37 °C with 0.01–0.02 unit/mL *N*-glycosidase F (Boehringer Mannheim), dried, resuspended in 2× SDS–PAGE sample buffer, and immunoblotted.

Immunoblot Analysis. Cells were lysed in 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, and 50 mM Tris-HCl (pH 7.5) containing protease inhibitors. Proteins were concentrated from cell lysates and medium samples by precipitation with 4 volumes of ice-cold methanol prior to resuspension in SDS–PAGE sample buffer. Proteins were separated using SDS–PAGE performed by the method of Laemmli (1970), or of Schägger and von Jagow (1987) to resolve low molecular mass fragments. Gels were blotted electrophoretically onto Immobilon-P (Millipore) in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid containing 10% methanol at pH 11. Blots were blocked for 1 h at 37 °C with 5% nonfat dry milk in Tris-buffered saline (TBS) and then incubated with antisera (1:100 to 1:1000) or affinity-purified antibodies in blocking solution for 1 h at room temperature. After being washed in TBS containing 0.1% Tween-20, blots were incubated with ^{125}I-protein A (ICN) in TBS/Tween for 1 h at room temperature, washed, and exposed to X-ray film at −70 °C with an intensifying screen.

Metabolic Labeling and Immunoprecipitation. Confluent cultures of neuroblastoma cells were incubated overnight in Opti-MEM and then labeled with L-[^{35}S]methionine (ICN Tran^{35}S-Label, 250 μCi/mL, 1000 Ci/mmol), L-[2,3,4,5-^{3}H]-proline (Amersham, 130–250 μCi/mL, 90 Ci/mmol), or L-[3-^{3}H]threonine (Amersham, 250 μCi/mL, 15.9 Ci/mmol) in serum-free medium lacking the corresponding amino acid. Labeling with [1-^{3}H]ethan-1-ol-2-amine hydrocholoride (Amersham, 150 μCi/mL, 29 Ci/mmol) was performed in Opti-MEM. Cultures were lysed in 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, and 50 mM Tris-HCl (pH 7.5) containing protease inhibitors, or they were chase-incubated in Opti-MEM. Protease inhibitors and Tris-HCl (pH 7.5, final concentration 50 mM) were then added to the chase medium, which was centrifuged at 16000*g* for 10 min at 4 °C.

Prior to immunoprecipitation, samples were made 0.5% in SDS and heated at 95 °C for 10 min to denature proteins and improve reactivity with anti-chPrP antibodies. After dilution with 4 volumes of 0.5% Triton X-100/50 mM Tris-HCl (pH 7.5) containing protease inhibitors, samples were precleared with formalin-fixed Staph A (Immunoprecipitin, BRL), and BSA was added to a final concentration of 0.5%. Samples were than incubated for 1–16 h at 4 °C with 1–5 μL of antiserum, and immune complexes were collected with Staph A. Staph A pellets were washed 3 times in RIPA buffer containing 0.5% BSA, and once in 150 mM NaCl/50 mM Tris-HCl (pH 7.5). Proteins were eluted in sample buffer at 95 °C for 10 min and analyzed by SDS–PAGE (Laemmli, 1970) followed by fluorography using PPO.

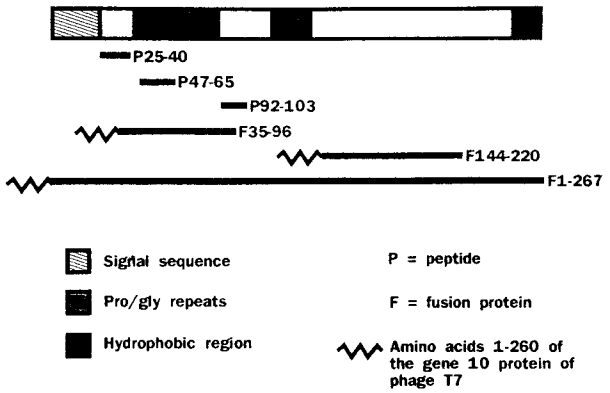

FIGURE 1: Immunogens used to raise rabbit polyclonal antisera specific for chPrP. Synthetic peptide (P) and bacterial fusion protein (F) immunogens are shown aligned with a schematic of the structure of chPrP. The numbering of each immunogen refers to amino acid positions in the protein [see Harris et al. (1991)].

RESULTS

Antisera. Figure 1 shows the synthetic peptide (P) and fusion protein (F) immunogens used to raise rabbit polyclonal antisera specific for chPrP. Antibodies to fusion proteins F35–96 and F144–220 were affinity-purified before use. The reactivity of each antibody in immunoblot and immunoprecipitation experiments was specifically blocked by preincubation with the immunogen, and nonimmune or preimmune serum was unreactive (see Figures 3–5). In addition, the reactivity of the serum raised against F1–267, a fusion protein containing the entire coding region of chPrP, could be blocked almost completely with peptide P47–65, indicating that this region of the proline/glycine-rich repeats represents an immunodominant epitope (data not shown). None of the sera cross-reacted with mouse PrP (not shown).

GPI-Anchored and Soluble Forms of ChPrP. N2a mouse neuroblastoma cells have been used extensively to study the metabolism of mammalian PrP^{C} and PrP^{Sc} (Caughey et al., 1989, 1991; Taraboulos et al., 1990, 1992; Borchelt et al., 1990, 1992). We have previously described the expression of chPrP by N2a cells that were transiently transfected with a vector that places the chPrP coding region under control of a human cytomegalovirus promoter (Harris et al., 1991). We have now prepared stable lines of N2a cells by cotransfecting the chPrP coding region (in the same vector) along with the gene for aminoglycoside phosphotransferase, and selecting transfectants in the presence of the antibiotic G418. The experiments presented here were performed with one of these lines (designated A26), but similar results have been obtained with other lines expressing several different levels of chPrP.

ChPrP can be released from intact A26 cells by incubation at 37 °C for 1 h with bacterial phosphatidylinositol-specific phospholipase C (PIPLC),[1] suggesting that the protein is attached to the cell surface by a GPI anchor (Figure 2B). Approximately 70–80% of the protein is released under these conditions, and the released material migrates as a broad band between 35 and 45 kDa. Two major fragments of 35–40 and 11.5 kDa are also present in medium conditioned for 24 h in the absence of added enzyme (Figure 2A). The presence of

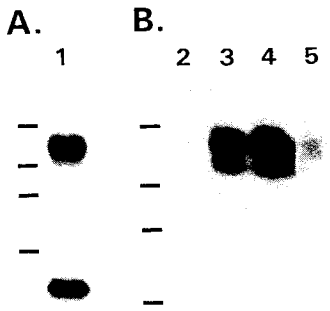

FIGURE 2: Soluble and GPI-anchored forms of chPrP detected by immunoblotting. Panel A: Opti-MEM medium conditioned for 18 h by A26 cells (equivalent to half of a 35-mm plate) was precipitated with methanol and immunoblotted with antiserum F35–96. Major fragments of 35–40 and 11.5 kDa are visible. Panel B: A26 cells were incubated in Opti-MEM at 37 °C for 1 h with (lanes 3 and 5) or without (lanes 2 and 4) PIPLC (1 unit/mL). The incubation medium (lanes 2 and 3, half of a 35-mm dish) and cell lysates (lanes 4 and 5, one-fourth of a 35-mm dish) were precipitated with methanol and immunoblotted with antiserum F35–96. Approximately 70–80% of the chPrP is released by incubation with PIPLC, while none is released in the absence of enzyme. Molecular mass markers in all panels are (top to bottom) 49.5, 32.5, 27.5, and 18.5 kDa.

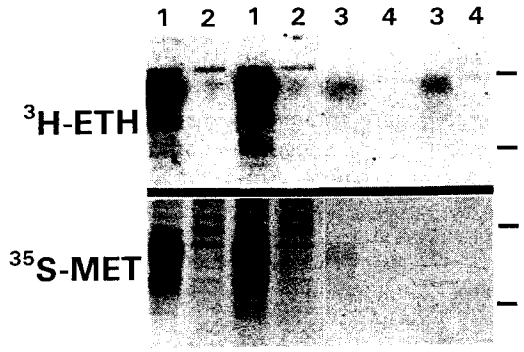

FIGURE 3: Immunoprecipitation of chPrP from conditioned medium and cell lysates after metabolic labeling with either [^{3}H]ethanolamine or [^{35}S]methionine. A26 cells were labeled for 25 h with [^{3}H]-ethanolamine, or for 3 h with [^{35}S]methionine. Cell lysates were then prepared from one set of cultures, while the other set of cultures was chased for 24 h. ChPrP was immunoprecipitated from cell lysates (lanes 1 and 2) and chase medium (lanes 3 and 4), using either antiserum F35–96 (lanes 1 and 3) or preimmune serum (lanes 2 and 4). Lanes marked with the same number represent duplicate cultures. ChPrP labeled with both precursors is specifically immunoprecipitated from both cell lysates and conditioned medium. Autoradiographic exposure times were 77 days ([^{3}H]ethanolamine, top panel) and 16 h ([^{35}S]methionine, bottom panel). Molecular mass markers are 49.5 and 32.5 kDa.

these soluble fragments suggests that membrane-bound chPrP molecules are cleaved in at least two locations as part of their normal processing.

Characterization of a C-Terminal Cleavage Site. The presence of a 35–40-kDa fragment in conditioned medium suggests that some chPrP molecules are cleaved near the C-terminus to release them from their GPI anchor. To test whether these molecules retain any of the anchor structure, A26 cells were metabolically labeled with [^{3}H]ethanolamine, an anchor precursor, or with [^{35}S]methionine. ChPrP immunoprecipitated from conditioned medium as well as from cell lysates was labeled with both precursors (Figure 3). We quantitated the amount of immunoprecipitated radioactivity by scintillation counting, and found that the ratio of ^{3}H/^{35}S was 6.25 for conditioned medium and 6.44 for cell lysates (average of duplicate plates; nonspecific precipitation subtracted). Since most of the cell-associated chPrP is GPI-anchored (see Figure 2B), these data indicate that the bulk

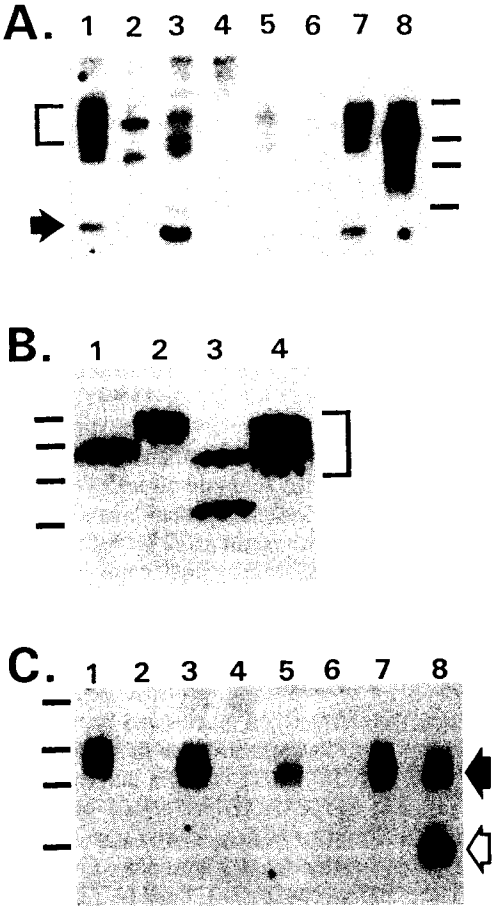

FIGURE 4: Antibody reactivity of soluble and GPI-anchored fragments of chPrP. Panel A: Medium conditioned by A26 cells for 3 days was precipitated with methanol and immunoblotted using antisera P24–40 (lanes 1 and 2), P92–103 (lanes 3 and 4), P47–65 (lanes 5 and 6), affinity-purified F35–96 (lane 7), and affinity-purified F144–220 (lane 8). Sera used for lanes 2, 4, and 6 were preincubated with the corresponding peptide immunogen (0.1 mg/mL). The 11.5-kDa fragment (solid arrow) reacts with all sera except F144–220; the 35–40-kDa fragment (bracket) reacts with all sera. The circular spot in lane 8 is an artifact. Panel B: Protein released from A26 cells by PIPLC was precipitated with methanol and incubated overnight with (lanes 1 and 3) or without (lanes 2 and 4) *N*-glycosidase F. Samples were then immunoblotted using antiserum F35–96 (lanes 1 and 2) or affinity-purified antiserum F144–220 (lanes 3 and 4). A broad band of immunoreactive material is seen with the C-terminal antiserum (lane 4, bracket), which can be resolved into two species of 30 and 20 kDa after glycosidase treatment (lane 3); the 20-kDa fragment does not react with the N-terminal antiserum (lane 1). Panel C: Protein released from A26 cells by PIPLC was precipitated with methanol, incubated overnight with *N*-glycosidase F, and immunoblotted with antisera P24–40 (lanes 1 and 2), P92–103 (lanes 3 and 4), P47–65 (lanes 5 and 6), affinity-purified F35–96 (lane 7), and affinity-purified F144–220 (lane 8). Sera used for lanes 2, 4, and 6 were preincubated with the corresponding peptide immunogen (0.1 mg/mL). The 20-kDa fragment (open arrow) reacts only with antiserum F144–220, while the 30 kDa-fragment (filled arrow) reacts with all sera. Molecular mass markers in all panels are (top to bottom) 49.5, 32.5, 27.5, and 18.5 kDa.

of the 35–40-kDa protein in conditioned medium contains an ethanolamine residue derived from the GPI anchor.

Characterization of an N-Terminal Cleavage Site. The presence of an 11.5-kDa fragment in conditioned medium (Figure 2A) suggests that chPrP molecules are also being cleaved at a second site. Immunoblots of conditioned medium demonstrate that the 11.5-kDa fragment reacts with antisera P25–40, P47–65, P92–103, and F35–96, but not F144–220 (Figure 4A, arrow). These results indicate that this fragment is likely to extend from near Lys^{25}, which immediately follows

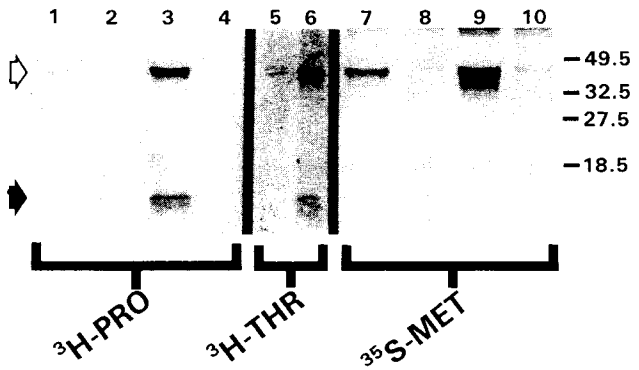

FIGURE 5: Immunoprecipitation of chPrP from conditioned medium after metabolic labeling with either [^{3}H]proline, [^{3}H]threonine, or [^{35}S]methionine. A26 cells were labeled for 6 h with the respective radioactive amino acids and then chased for 22 h. Chase medium was then immunoprecipitated with P47–65 serum (lanes 1 and 7), P47–65 serum preincubated with P47–65 peptide (lanes 2 and 8), F1–267 serum (lanes 3 and 9), F35–96 serum (lane 6), or nonimmune serum (lanes 4, 5, and 10). Although 35–45-kDa protein (open arrow) is detected in all three sets of cultures, the 11.5-kDa fragment (filled arrow) is visualized only in cultures labeled with [^{3}H]proline or [^{3}H]-threonine. Autoradiographic exposure times were 48 days ([^{3}H]-proline), 44 days ([^{3}H]threonine), and 60 h ([^{35}S]methionine).

the predicted signal sequence cleavage site (Harris et al., 1991), through a point that lies roughly between Tyr^{103} and Met^{144}.

A corresponding C-terminal fragment would presumably remain attached to the membrane. To detect this fragment, PIPLC-released protein was immunoblotted using antiserum F144–220. A band of immunoreactive protein was observed that was much broader than the band produced with antiserum F35–96 (Figure 4B, compare lanes 2 and 4). To better resolve the individual proteins in this band, samples were treated with *N*-glycosidase F to remove N-linked oligosaccharides (Figure 4B, lanes 1 and 3). Discrete bands of 30 and 20 kDa were then detected with antiserum F144–220. Only the larger band was visualized with antiserum F35–96, as well as with a variety of other N-terminally directed antisera (Figure 4C). The 20-kDa band therefore represents a C-terminal fragment that does not extend beyond approximately Tyr^{103}. The size difference between the 20- and 30-kDa forms is consistent with the molecular mass of the N-terminal fragment seen in conditioned medium (11.5 kDa). More than half of the cell-surface chPrP appears to be N-terminally-truncated (compare the intensity of the two bands in lane 3 of Figure 4B and in lane 8 of Figure 4C). A significant amount of the 35–40-kDa protein in conditioned medium also appears to be truncated (compare lanes 7 and 8 in Figure 4A).

To further localize the N-terminal cleavage site, we compared A26 cells labeled with three different amino acids: [^{3}H]proline, [^{3}H]threonine, and [^{35}S]methionine (Figure 5). There are 16 proline residues within the hexapeptide repeat region of chPrP (residues 42–89). The first threonine residue following the signal peptide occurs at position 114, and the first methionine residue at position 137. We found that when cells were labeled with [^{3}H]proline or [^{3}H]threonine, both the 35–40- and 11.5-kDa forms of chPrP could be observed in conditioned medium. In contrast, only the larger form was detectable after labeling with [^{35}S]methionine. This result indicates that the C-terminus of the 11.5-kDa fragment lies between Thr^{114} and Met^{137}. By quantitating the autoradiographic signal produced by the 35–40-kDa form, which contains five methionine residues, we determined that it would have been possible to detect the 11.5-kDa fragment after ^{35}S-labeling had it contained a single methionine residue.

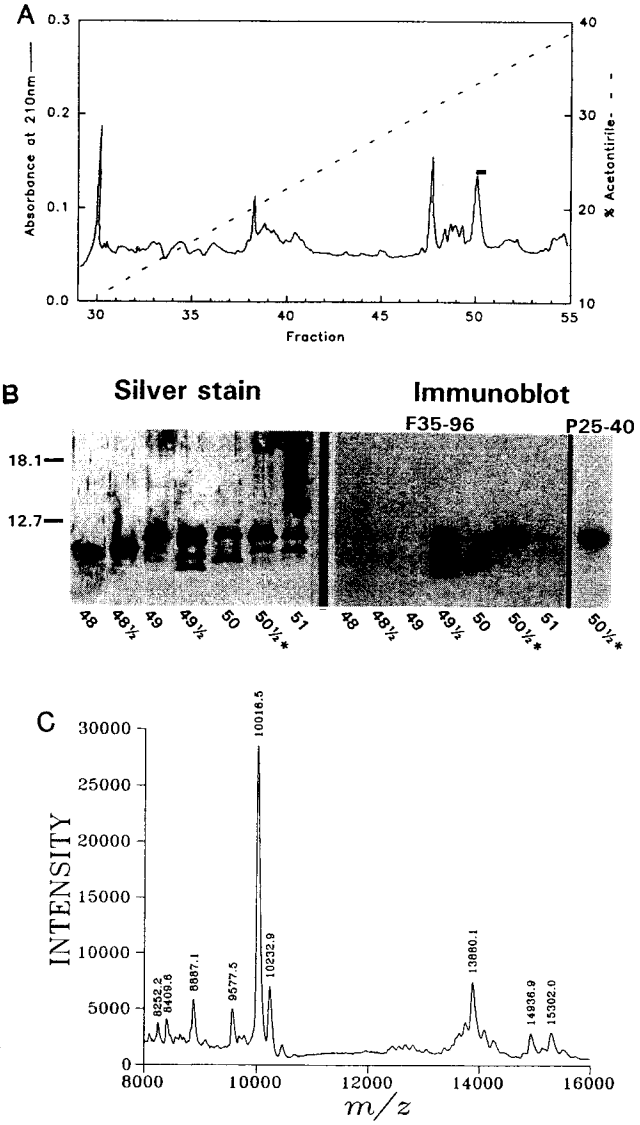

FIGURE 6: Purification and analysis of N-terminal fragments of chPrP in conditioned medium. Panel A: Medium conditioned by A26 cells was chromatographed on a CM-52 cation-exchange column, and the eluate was applied to a Vydac C_{18} HPLC column. Shown here is the elution profile of the HPLC column, which was developed with a gradient of acetonitrile in 0.1% TFA. The position of fraction 50.5, which was subjected to Edman degradation and MALDMS analysis, is indicated by the solid bar over the absorbance trace. Panel B: Silver stain and immunoblot of fractions from the Vydac C_{18} column after separation on Tris/tricine gels. 150 μL (silver strain) or 75 μL (immunoblot) of each fraction was analyzed. Fractions 48–51 were reacted with antiserum F35–96, and fraction 50.5 (asterisk) was also reacted with antiserum P25–40. Panel C: MALDMS analysis of HPLC fraction 50.5. Four percent of the fraction was applied. The $[M+H]^{+1}$ region of the spectrum is shown.

We have also defined the cleavage site by mass spectrometric analysis of N-terminal fragments of chPrP purified from conditioned medium by ion-exchange chromatography and reverse-phase HPLC (Figure 6A). At least six different fragments that reacted with antiserum F35–96 were resolved on Tris/tricine gels of sequential HPLC fractions, ranging in size from 8.5 to 11.5 kDa (Figure 6B). These fragments comigrate on the lower percentage SDS–PAGE gels shown in Figures 2A, 4A, and 5.

Fraction 50.5 contained a single, major immunoreactive fragment that migrated at 11.0 kDa on Tris/tricine gels; this fragment represented more than 90% of the total protein in the fraction. The 11.0-kDa fragment reacted strongly with

antiserum P25–40 (Figure 6B), and Edman degradation through eight cycles yielded a sequence consistent with an N-terminus at Lys^{25}. Analysis of this same fraction by MALDMS revealed a major peak at m/z = 10 016.5 Da (corresponding to $[M+H]^+$), as well as several minor peaks (Figure 6C). Computerized mass searching between residues 25 and 136 revealed several sequences with protonated molecular masses close to that of the major peak, but only one was also consistent with the Edman degradation and immunoblot results: Lys^{25}–Phe^{116} (calculated m/z = 10 011.93). This analysis assumes the absence of posttranslational modifications.

Chicken Brain and Cerebrospinal Fluid. To determine whether GPI-anchored and soluble forms of chPrP are also found in chicken brain, we immunoblotted crude membrane and soluble fractions prepared from brain homogenates, as well as CSF.

We found that immunoreactive chPrP (35–45 kDa) is present in microsomal brain membranes and that most of the protein can be released by incubation with bacterial PIPLC or the detergent sodium deoxycholate, but not by incubation with buffer alone (Figure 7A). ChPrP thus appears to be GPI-anchored in brain also. Most of these molecules are N-terminally truncated, as they are in A26 cells, since the majority of the PIPLC-released, deglycosylated protein migrates as a 20-kDa band that reacts only with antiserum F144–220 (Figure 7C; compare with Figure 4B).

ChPrP could also be detected in a postmicrosomal supernatant fraction of brain, and in CSF (Figure 7B). For these experiments, the protein was affinity-purified using F1–267 antiserum prior to fractionation on a Tris/tricine gel and immunoblotting. Both sources contained immunoreactive chPrP that migrated between 35 and 45 kDa, as well as a major band that migrated at approximately 8.5 kDa. In addition, the postmicrosomal supernatant contained smaller amounts of material that migrated as a series of poorly resolved bands between 8.5 and 11.5 kDa. Reactivity of all bands was blocked by preincubation of the antiserum with peptide P47–65 (not shown). Thus, N-terminal fragments of chPrP are produced in brain too, and the array of fragment sizes is similar to A26 cells.

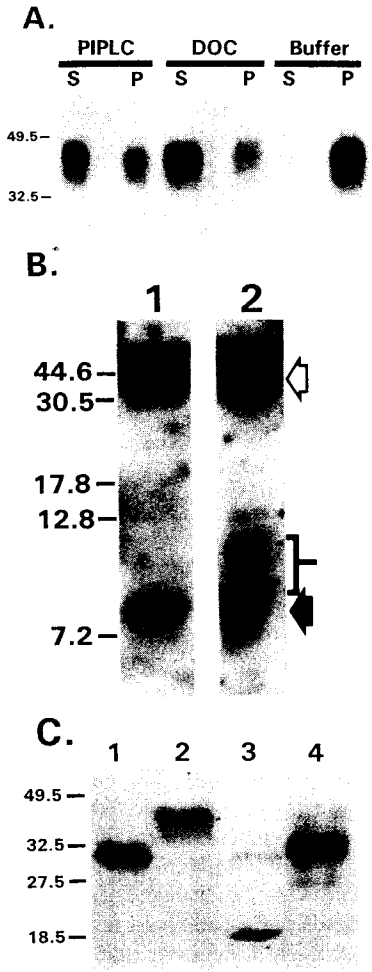

FIGURE 7: Immunoblot of chPrP in subcellular fractions of chicken brain and in CSF. Panel A: Microsomal membranes obtained from a 100000*g* centrifugation were incubated for 2 h with either PIPLC, 1% sodium deoxycholate (DOC), or buffer alone. After centrifugation at 100000*g*, equivalent amounts of supernatants (S) and pellets (P) were immunoblotted with antiserum F35–96. Most of the chPrP is released from membranes by PIPLC and deoxycholate, but not by buffer alone, suggesting that the majority of the molecules are GPI-anchored. Panel B: ChPrP was affinity-purified from CSF (lane 1) and a postmicrosomal (100000*g*) supernatant of brain (lane 2) by incubation with protein A–Sepharose containing covalently coupled F1–267 antibodies. Protein eluted from the affinity matrix was then immunoblotted with F1–267 antiserum. The open arrow indicates the position of immunoreactive material in the 35–45-kDa range and the filled arrow an 8.5-kDa fragment. The bracket indicates protein in the postmicrosomal supernatant (lane 2) that migrates as a poorly resolved series of bands between 8.5 and 11.5 kDa. Panel C: ChPrP released from microsomal membranes by PIPLC was incubated overnight with (lanes 1 and 3) or without (lanes 2 and 4) *N*-glycosidase F. Samples were then immunoblotted using affinity-purified antiserum F35–96 (lanes 1 and 2) or affinity-purified antiserum F144–220 (lanes 3 and 4). 30- and 20-kDa species are seen after glycosidase treatment (lane 3); the 20-kDa fragment, present in largest amounts, does not react with the N-terminal antiserum (lane 1). This experiment is analogous to the one shown in Figure 4B which uses chPrP released from A26 cells.

DISCUSSION

The results reported here indicate that chPrP, the chicken homologue of mammalian PrP^C, undergoes at least two cleavages as part of its normal metabolism (Figure 8). These cleavages have been identified by immunoblotting and immunoprecipitation of chPrP from transfected neuroblastoma cells, brain, and CSF, as well as by direct chemical analysis of protein fragments purified from conditioned medium.

One cleavage (labeled A in Figure 8) releases the protein into the extracellular medium by cleavage within the GPI membrane anchor. Released molecules of 35–45 kDa can be detected in medium conditioned by A26 cells, as well as in a postmicrosomal supernatant of brain and in CSF. Since the molecules in conditioned medium can be labeled with [^{3}H]-ethanolamine, cleavage A is likely to be produced by a phosphodiesterase, glycosidase, or phospholipase acting within the anchor structure itself, rather than by a protease acting on the polypeptide chain. An ethanolamine moiety is linked to the C-terminal amino acid, and in some cases to one of the mannose residues, in other anchors that have been characterized (Cross, 1990; Stahl et al., 1992). ChPrP molecules in conditioned medium partition into the aqueous phase after extraction with Triton X114 (Bordier, 1981), suggesting that they lack the diacylglycerol portion of the anchor (unpublished data).

ChPrP molecules undergo a second, proteolytic cleavage within or just N-terminal to the central hydrophobic region (bracket labeled B in Figure 8). This cleavage generates N-terminal fragments of 8.5–11.5 kDa that can be detected in conditioned medium, brain supernatant, and CSF. A corresponding C-terminal, GPI-anchored fragment that migrates at 20 kDa after deglycosylation is also observed in A26

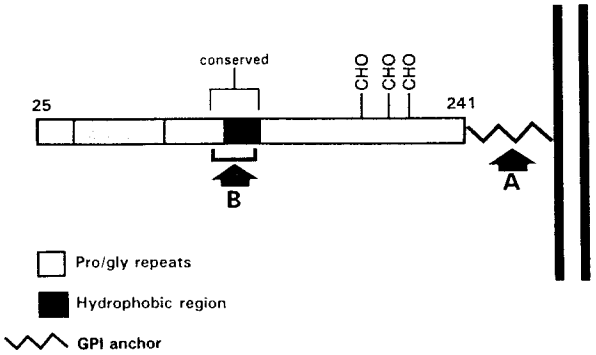

FIGURE 8: ChPrP undergoes at least two cleavages. Site A lies within the anchor structure itself, between the diacylglycerol moiety and the ethanolamine residue that is bonded to the C-terminal amino acid of the protein. This cleavage releases chPrP molecules into the extracellular medium. Cleavage at site B is proteolytic, and occurs between Thr^{114} and Met^{137} (lower bracket), within a region of 24 amino acids (upper bracket, labeled "conserved") that is identical in chPrP and mammalian PrPs. This cleavage generates a soluble N-terminal fragment, as well as a C-terminal fragment that remains anchored to the membrane via a GPI linkage. Further proteolytic trimming of the N-terminal fragment may generate the multiple species of 8.5–11.5 kDa observed in extracellular medium.

cells and brain. Quantitatively, cleavage B is a major processing event: the majority of the cell-surface chPrP molecules have been truncated at this site, and the resulting N-terminal fragments accumulate in substantial amounts in the extracellular medium.

The fact that the N-terminal fragments in conditioned medium can be metabolically labeled with [^{3}H]threonine but not [^{35}S]methionine indicates that a major cleavage site must lie between Thr^{114} and Met^{137}. MALDMS analysis indicates that one major fragment extends from Lys^{25} through Phe^{116}. However, multiple N-terminal fragments that migrate on Tris/tricine gels in the range from 8.5 to 11.5 kDa are observed in conditioned medium as well as in brain and CSF. The origin of these fragments is uncertain, but one possibility is that they result from further proteolytic trimming of a piece generated by cleavage at a single site within region B. The presence of a single C-terminal fragment of 20 kDa in both brain and A26 cells (Figures 4B and 7C) is consistent with this idea; the C-terminal fragment may be less susceptible to further trimming than the N-terminal piece.

The fact that a major portion of the chPrP present in both A26 cells and brain is N-terminally cleaved suggests that this processing event may have physiological significance. Although the normal function of the protein is unknown (Büeler et al., 1992), one possibility is that soluble N-terminal fragments play a role in intercellular communication, perhaps by binding to a target cell. A number of polypeptide growth factors are derived by cleavage of membrane-bound precursors (Massagué, 1990). Alternatively, generation of the GPI-anchored, C-terminal fragment might be physiologically important, if for example the protein functioned as a cell-surface receptor. Several receptors are known to be proteolytically cleaved as part of their normal metabolism (Mostov & Simister, 1985; Zupan & Johnson, 1991; Vu et al., 1991). We are currently investigating the subcellular compartment in which cleavage of chPrP occurs.

Mammalian PrP^C is likely to undergo cleavages that are analogous to those described here for chPrP. Mouse PrP^C is anchored to the surface of neuroblastoma cells by a GPI linkage, and some is also released spontaneously into the medium, presumably by cleavage near the C-terminus (Caughey et al., 1989; Borchelt et al., 1990). Whether this cleavage is proteolytic or occurs within the anchor structure itself is unknown. A C-terminally truncated form of PrP^{Sc} that lacks the GPI anchor has also been described (Stahl et al., 1990b).

A cleavage analogous to the one at site B has not been described for mammalian PrP^C, but it is very likely to occur since site B lies almost entirely within a segment of 24 amino acids (Pro^{112}–Tyr^{135}) that is identical in the chicken and mammalian proteins [see Figure 8 and Figure 3A of Harris et al. (1991)]. Mouse PrP^{Sc} undergoes N-terminal cleavage near the proteinase K-sensitive site (Trp^{88}) in scrapie-infected neuroblastoma cells, suggesting that this isoform is cleaved at a site that is distinct from the one cleaved in PrP^C (Caughey et al., 1991; Taraboulos et al., 1992). The significance of this difference remains to be determined.

Several considerations suggest that proteolytic processing of PrP may be important in the pathogenesis of prion diseases. Several human amyloidoses, including Alzheimer's disease, result from aberrant cleavage of a precursor protein (Levy et al., 1989; Yi et al., 1991; Selkoe, 1991), and recent evidence indicates that amyloid plaques in the brains of several patients with inherited Gerstmann–Sträussler syndrome are composed of N- and C-terminally truncated forms of PrP (Tagliavini et al., 1991; Kitamoto et al., 1991a,b). Moreover, PrP^{Sc} in extracts of brain membranes is found to form amyloid-like rods only if it is first digested with proteinase K in the presence of detergent (McKinley et al., 1991b). Finally, several observations implicate endosomes and lysosomes, known sites of proteolysis, in the metabolism of PrP (Caughey & Raymond, 1991; Caughey et al., 1991; McKinley et al., 1991a; Borchelt et al., 1992). It will be of interest to extend the studies of chPrP reported here to mammalian PrP, and to examine the role of proteolytic and other cleavages in the normal and pathologic function of this class of proteins.

ACKNOWLEDGMENT

We thank Douglas Falls and Gerald Fischbach for generously providing samples of antisera P47–65 and F1–267 and Philip Stahl and James Krause for the use of their HPLC equipment. We also thank Victoria Peters for help in preparing immunogens, Megan Morgan for assistance in constructing the transfected neuroblastoma cells, and Jonathan Cohen for comments on an earlier version of the manuscript.

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