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A prion-like protein from chicken brain copurifies with an acetylcholine receptor-inducing activity

(neuromuscular junction/synaptogenesis/neurodegenerative diseases/chemoreceptor/trophic)

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ABSTRACT **The mammalian prion protein (PrP^C) is a cellular protein of unknown function, an altered isoform of which (PrP^{Sc}) is a component of the infectious particle (prion) thought to be responsible for spongiform encephalopathies in humans and animals. We report here the isolation of a cDNA that encodes a chicken protein that is homologous to PrP^C. This chicken prion-like protein (ch-PrLP) is identical to the mouse PrP at 33% of its amino acid positions, including an uninterrupted stretch of 24 identical residues, and it displays the same structural domains. In addition, ch-PrLP, like its mammalian counterpart, is attached to the cell surface by a glycosylphosphatidylinositol anchor. We find that ch-PrLP is the major protein in preparations of an acetylcholine receptor-inducing activity that has been purified $>10^6$-fold from brain on the basis of its ability to stimulate synthesis of nicotinic receptors by cultured myotubes. The ch-PrLP gene is expressed in the spinal cord and brain as early as embryonic day 6; and in the spinal cord, the protein appears to be concentrated in motor neurons. Our results therefore raise the possibility that prion proteins serve normally to regulate the chemoreceptor number at the neuromuscular junction and perhaps in the central nervous system as well.**

Prions, proteinaceous infectious particles, have been implicated in the pathogenesis of scrapie in sheep, goats, and cattle, and in certain neurodegenerative disorders in humans, including kuru, Creutzfeldt–Jakob disease, and Gerstmann–Sträussler syndrome (1, 2). The principal prion protein (PrP) as first purified from scrapie-infected hamster brains by detergent extraction, differential centrifugation, and digestion with proteinase K, migrates in SDS gels with a molecular mass of 27–30 kDa and has been designated PrP 27–30 (3). Isolation of cDNA and genomic clones showed that PrP 27–30 is a fragment of a larger, 254-amino acid protein named PrP^{Sc} (4, 5). In these studies, evidence was presented that the gene present in infected brains is also present in uninfected brains where it encodes a cellular protein called PrP^C. Unlike PrP^{Sc}, PrP^C is completely digested by proteinase K. The entire PrP open reading frame is contained within a single exon, so the difference between PrP^{Sc} and PrP^C probably resides in a posttranslational modification (5).

The PrP sequence is highly conserved (>90% at the amino acid level) among mammals (6). Although this argues that the molecule is important, no role for the normal cellular protein and no mechanism for the encephalopathy caused by the altered isoform have been demonstrated. We report here the complete amino acid sequence, predicted from a cDNA, of a protein from chicken brain that is homologous to the mammalian PrPs and that may shed light on their action in the brain.§

In our studies, the chicken prion-like protein (ch-PrLP) emerged as the major protein and the only sequenceable protein in purified preparations of ARIA (7), an acetylcholine receptor-inducing activity isolated from chicken brain on the basis of its ability to stimulate the synthesis of acetylcholine receptors (AChRs) in cultured chicken myotubes (8, 9). ARIA copurifies through a number of chromatographic procedures with ch-PrLP, which migrates in SDS gels as a broad band that extends between 35 and 45 kDa (7). Thus, it is possible that ch-PrLP and, by implication, mammalian PrP acts as "trophic" agents in the brain, perhaps by regulating the number of chemoreceptors and hence ongoing synaptic activity.

MATERIALS AND METHODS

ARIA Purification and Amino Acid Sequence Analysis. ARIA was purified as described (7, 8). In summary, the seven-step protocol includes the following: (*i*) acetone/ether extraction of 1000 frozen, adult chicken brains (Pel-Freez Biologicals); (*ii*) extraction of the residue with trifluoroacetic acid, formic acid, hydrochloric acid, sodium chloride, and protease inhibitors, followed by adsorption on Vydac C18 and batch elution with acetonitrile to desalt and concentrate; (*iii*) ion-exchange chromatography on CM-Sepharose eluted with a linear salt gradient; (*iv*) reverse-phase chromatography on a 1-cm Vydac C4 column eluted with a gradient of isopropanol in trifluoroacetic acid; (*v*) reverse-phase chromatography on the same C4 column with an isopropanol gradient in heptafluorobutyric acid; (*vi*) gel filtration through a TSK-3000 column; (*vii*) analytical reverse-phase chromatography on a microbore C18 column eluted with an acetonitrile gradient in trifluoroacetic acid.

N-terminal sequence analysis was performed on an Applied Biosystems model 470A gas-phase sequenator.

PCR. The following nested pairs of oligonucleotide primers were used. Each primer contained an *Eco*RI site at its 5′ end (I, inosine). First pair: sense (amino acid residues 1–6 of the N-terminal sequence underlined), 5′-GCGGAATTCGA AAI AAI GGI AA(A,G) GG(A,T,G,C) AA-3′; anti-sense (residues 20–23 underlined), 5′-GGCGAATTCAT GG ITA I(G,C)-(A,T) (A,T,G,C)GG-3′. Second pair: sense (residues 1–7 underlined), 5′-GCGGAATTCGA AAI AAI GGI AA(A,G) GG(A,T,G,C) AA(A,G) CC-3′; anti-sense (residues 19–23 underlined), 5′-GGCGAATTCAT GG ITA I(G,C)(A,T) (A,T,G,C)GG (T,C)TG-3′.

Abbreviations: PrP, prion protein; ch-PrLP, chicken prion-like protein; AChR, acetylcholine receptor; ARIA, acetylcholine receptor-inducing activity; E18, etc., embryonic day 18, etc.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M61145).

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Poly(A)$^+$ RNA prepared from adult chicken brain (10) was used as a template for synthesis of cDNA with an oligo(dT) primer and avian myeloblastosis virus reverse transcriptase (11). cDNA (100 ng) was subjected to 30 cycles of PCR amplification using the first pair of primers with *Taq* polymerase under conditions recommended by the manufacturer (Perkin–Elmer/Cetus). In each cycle, denaturation was carried out for 1 min at 94°C, annealing for 2 min at 35°C, and extension for 3 min at 72°C. The amplified DNA was then used as template for 30 more cycles of amplification under the same conditions with the second pair of primers, which were longer at their 3′ ends.

DNA from the second round of amplification was digested with *Eco*RI and fractionated on a 10% polyacrylamide gel. The region of the gel containing DNA ≈82 nucleotides long (the predicted size of the amplified product, including primer sequence and 5′ overhangs) was cut out, and the electroeluted DNA was ligated into the *Eco*RI site of pBluescript II (Stratagene). DNA from 70 individual plasmid clones was digested with *Eco*RI and analyzed on a 10% acrylamide gel; 12 clones contained an insert of exactly 82 nucleotides, as determined by comparison with size markers. DNA sequencing confirmed that 3 of these clones encoded amino acids 8–18 of the chemically determined sequence.

Library Screening and Nucleotide Sequence Analysis. A synthetic oligonucleotide with the sequence 5′-GCGATGGCTCCCGGCGCCCCAACCCCCACCACTG-3′ (amino acids 18–8) was end-labeled with ^{32}P using polynucleotide kinase and hybridized to filter replicas of an embryonic day 18 (E18) chicken brain cDNA library in λgt10 (kindly provided by D. Fambrough, Johns Hopkins University) (11). Hybridization was at 50°C in 0.9 M NaCl/0.09 M Tris·HCl, pH 8/6 mM EDTA/0.1% SDS/5× Denhardt's solution/salmon sperm DNA (500 μg/ml). Washes were at 37°C or 42°C in 1× standard saline citrate (SSC)/0.1% SDS. Positive clones were rescreened once and subcloned into the *Eco*RI site of pBluescript II.

cDNAs were sequenced by the dideoxynucleotide chain-termination method using synthetic oligonucleotide primers and either Sequenase (United States Biochemical) or *Taq* polymerase (Promega) according to the manufacturer's directions. Both strands of the insert in p65-21 were sequenced in their entirety.

Northern Hybridization. Total cellular RNA was subjected to electrophoresis (10 μg per lane) in a 1% agarose/2.2 M formaldehyde gel and was transferred to GeneScreen (New England Nuclear). The entire spinal cord and brain were dissected free of surrounding tissues, except in adult animals where only the lumbar enlargement of the spinal cord was used. Blots were hybridized at 65°C in 100 mM NaCl/1 mM EDTA/5% SDS/20 mM Na_2HPO_4/50 mM Pipes, pH 7 with a 2.2-kilobase (kb) ^{32}P-labeled complementary RNA that was transcribed from *Bam*HI-cleaved p65-21 using T3 polymerase according to the directions of the manufacturer (Stratagene). Blots were washed at 65°C in 0.1× SSC/0.1% SDS, and then at 37°C in RNase A (20 μg/ml)/0.3 M NaCl/1 mM EDTA/10 mM Tris·HCl, pH 7.5, to reduce background.

Transfection and Phospholipase Digestion. An 858-nucleotide *Hin*fI/*Bsp*1286 fragment of p65-21 that contained the coding region (nucleotides −45 to +814; see Fig. 1) was blunt-ended with Klenow and T4 DNA polymerases and cloned into an expression vector that contains a human cytomegalovirus promoter/enhancer (pBC12/CMV; ref. 12) after the vector had been cleaved with *Hin*dIII and *Eco*RI and made blunt-ended. N2a mouse neuroblastoma cells (ATCC CCL131) were grown in minimal essential medium containing 10% fetal calf serum, nonessential amino acids, and penicillin/streptomycin in an atmosphere of 5% CO_2/95% air. Cells were transfected with the expression construct using Lipofectin (BRL) according to the manufacturer's directions. Cultures were transferred to serum-free medium 1 day after transfection, and after 2 additional days they were labeled for 8 hr with [^{35}S]methionine (200 μCi/ml; 1000 Ci/mmol; 1 Ci = 37 GBq) in serum-free medium lacking unlabeled methionine. Labeling medium was removed, and after rinsing with phosphate-buffered saline (PBS), the cells were incubated for 1.5 hr at 37°C in PBS containing 0.5 unit of phosphatidylinositol-specific phospholipase C per ml from *Bacillus thuringiensis* (ICN). This incubation medium was removed and centrifuged at 16,000 × *g* for 15 min after addition of protease inhibitors (pepstatin and leupeptin, 1 μg/ml; phenylmethylsulfonyl fluoride, 0.5 mM). Prior to immunoprecipitation, samples were made 0.5% in SDS, boiled for 10 min, and diluted with 4 vol of 0.5% Triton X-100/50 mM Tris·HCl, pH 7.5, containing protease inhibitors. Immunoprecipitations were carried out as described (13) with 5 μl of antiserum raised against a synthetic peptide comprising Pro^{47}–Pro^{65} of the ch-PrLP sequence.

Immunocytochemistry. A monoclonal antibody was raised against a synthetic peptide that corresponds to Gln^{213}–Thr^{224} of the ch-PrLP sequence. The peptide was coupled to soybean trypsin inhibitor. Supernatants were screened by ELISA with the peptide conjugated to ovalbumin or with partially purified ARIA as the capture antigen. The monoclonal antibody, 5.1 in our notation, is an IgM. Unfixed spinal cords were rapidly frozen and 10-μm-thick sections were cut on a freezing microtome. The sections were blocked with 10% goat serum in PBS, incubated in monoclonal antibody 5.1 supernatant at a dilution of 1:5 in PBS, rinsed, and, finally, incubated in fluorescein-conjugated goat anti-mouse antiserum.

RESULTS

Protein in one active fraction from a TSK-3000 column, the penultimate step in our purification protocol, was subjected to automated Edman degradation and gas-phase sequencing. Only one amino acid was detected at each cycle, and the following sequence was obtained: KKGKGKPSGGG?GAGSH?QPSYP?QPG. Other than the 35- to 45-kDa band, little protein was evident in a silver-stained gel of this fraction. The same initial sequence was obtained when the purification was "completed" on a microbore C18 column and also when the protein band in an SDS gel of a reverse-phase column active fraction (see ref. 7) was electroblotted onto poly(vinylidene difluoride) membrane (14).

Nested pairs of degenerate oligonucleotide primers that correspond to amino acids 1–7 and 19–23 of the chemically determined sequence were used in the PCR to amplify from chicken brain cDNA a 34-nucleotide sequence that corresponds to the 3′ nucleotide of the 7th amino acid codon through the 3′ nucleotide of the 18th amino acid codon. A synthetic 34-mer with the same sequence was then used to screen 250,000 phage from an E18 chicken brain cDNA library in λgt10. Four positive clones were obtained, three with 2.2-kb inserts and one with a 1.9-kb insert. Restriction maps and sequence analysis revealed that all three 2.2-kb clones were identical and that the 1.9-kb clone shared the same 3′ end but was truncated by 300 nucleotides at its 5′ end.

One of the 2.2-kb inserts (in a plasmid designated p65-21) was sequenced by the dideoxynucleotide chain-termination method in its entirety (Fig. 1). The cDNA predicts a protein of 267 amino acids. Although the presumed initiator methionine is not preceded by an in-frame stop codon, it occurs in a favorable context for translation initiation (15). Moreover, this methionine begins a typical signal peptide sequence, whose predicted cleavage site (16, 17) between residues Ser^{24} and Lys^{25} is followed by the N-terminal amino acid sequence determined by Edman degradation. Thus, there can be no doubt that the p65-21 insert encodes the partially sequenced

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                                                                  GAATTCCCTCGGC  -159
AGCCAGCTCCTCCCTCTCGCTATTTATTCCTTTCTCCCCCCCCTACGCTGGATCTGGATCATCTCAAGCCGAGCGGTGA  - 80
CGGCTTCTTGGATCGCTCATACATAAATATCTGTGAGTCAGAGGAAGCAACCACCGACCCCAAGACCTCACCCCGAGCC  - 1

ATG GCT AGG CTC CTC ACC ACC TGC TGC CTG CTG GCC CTG CTG CTC GCC GCC TGC ACC GAC    60
Met Ala Arg Leu Leu Thr Thr Cys Cys Leu Leu Ala Leu Leu Leu Ala Ala Cys Thr Asp    20

GTC GCC CTC TCC AAG AAG GGC AAA GGC AAA CCC AGT GGT GGG GGT TGG GGC GCC GGG AGC   120
Val Ala Leu Ser [Lys] Lys Gly Lys Gly Lys Pro Ser Gly Gly Gly Trp Gly Ala Gly Ser    40

CAT CGC CAG CCC AGC TAC CCC CGC CAG CCG GGC TAC CCT CAT AAC CCA GGG TAC CCC CAT   180
His Arg Gln Pro Ser Tyr Pro Arg Gln Pro Gly Tyr Pro His Asn Pro Gly Tyr Pro His    60

AAC CCA GGG TAC CCC CAC AAC CCT GGC TAT CCC CAT AAC CCC GGC TAC CCC CAG AAC CCT   240
Asn Pro Gly Tyr Pro His Asn Pro Gly Tyr Pro His Asn Pro Gly Tyr Pro Gln Asn Pro    80

GGC TAC CCC CAT AAC CCA GGT TAC CCA GGC TGG GGT CAA GGC TAC AAC CCA TCC AGC GGA   300
Gly Tyr Pro His Asn Pro Gly Tyr Pro Gly Trp Gly Gln Gly Tyr Asn Pro Ser Ser Gly   100

GGA AGT TAC CAC AAC CAG AAG CCA TGG AAA CCC CCC AAA ACC AAC TTC AAG CAC GTG GCG   360
Gly Ser Tyr His Asn Gln Lys Pro Trp Lys Pro Pro Lys Thr Asn Phe Lys His Val Ala   120

GGG GCA GCA GCG GCG GGT GCT GTG GTG GGG GGC TTG GGG GGC TAC GCC ATG GGG CGC GTT   420
Gly Ala Ala Ala Ala Gly Ala Val Val Gly Gly Leu Gly Gly Tyr Ala Met Gly Arg Val   140

ATG TCA GGG ATG AAC TAC CAC TTC GAT AGA CCC GAT GAG TAC CGA TGG TGG AGT GAG AAC   480
Met Ser Gly Met Asn Tyr His Phe Asp Arg Pro Asp Glu Tyr Arg Trp Trp Ser Glu Asn   160

TCG GCG CGT TAT CCC AAC CGG GTT TAC TAC CGG GAT TAC AGC AGC CCC GTG CCA CAG GAC   540
Ser Ala Arg Tyr Pro Asn Arg Val Tyr Tyr Arg Asp Tyr Ser Ser Pro Val Pro Gln Asp   180

GTC TTC GTG GCC GAT TGC TTT AAC ATC ACA GTG ACT GAG TAC AGC ATT GGC CCT GCT GCC   600
Val Phe Val Ala Asp [Cys] Phe [Asn] Ile Thr Val Thr Glu Tyr Ser Ile Gly Pro Ala Ala   200

AAG AAG AAC ACC TCC GAG GCT GTG GCG GCA GCA AAC CAA ACG GAG GTG GAG ATG GAG AAC   660
Lys Lys [Asn] Thr Ser Glu Ala Val Ala Ala Ala [Asn] Gln Thr Glu Val Glu Met Glu Asn   220

AAA GTG GTG ACG AAG GTG ATC CGC GAG ATG TGC GTG CAG CAG TAC CGC GAG TAC CGC CTG   720
Lys Val Val Thr Lys Val Ile Arg Glu Met [Cys] Val Gln Gln Tyr Arg Glu Tyr Arg Leu   240

GCC TCG GGC ATC CAG CTG CAC CCT GCT GAC ACC TGG CTC GCC GTC CTC CTC CTC CTC CTC   780
Ala Ser Gly Ile Gln Leu His Pro Ala Asp Thr Trp Leu Ala Val Leu Leu Leu Leu Leu   260

ACC ACC CTT TTT GCC ATG CAC TGATGGGATGCCGTGCCCCGGCCCTGTGGCAGTGAGATGACATCGTGTCCC   852
Thr Thr Leu Phe Ala Met His                                                       267

CGTGCCCACCCATGGGGTGTTCCTTGTCCTCGCTTTTGTCCATCTTTGGTGAAGATGTCCCCCCGCTGCCTCCCCGCAG   931
GCTCTGATTTGGGCAAATGGGAGGGGATTTTTGTCCTGTCCTGGTCGTGGCAGGACGGCTGCTGGTGGTGGAGTGGGATG  1010
CCCAAAAAATGGCCTTCACCACTTCCTCCTCCTCTTCCTTTCTGGGGCGGAGATATGGGCTCGTCCAGCCCTTATTGTC  1089
CCTGCAAGAGCGTATCTGAAAATCCTCTTTGCTAACAAGCAGGGTTTTACCTAATCTGCTTAGCCCCAGTGACAGCAGA  1168
GCGCCTTTCCCCAGGGCACACCAACCCCAAGCTGAGGTGCTTGGCAGCCACACGTCCCATGGAGGCTGATGGGTTTTGG  1247
GGCGTCCCAAGCAACACCCTGGGCTACTGAGGTGCAATTGTAGCTCTTTAATCTGCCAATCCCAACCCTACCGTGTAGA  1326
TAGGAACTGCCTGCTCTGCATTTTGCATGCTGCAAACACCTCCTGCCGCAGCGCCCCCAAAATAGAGTGATTTGGGAAT  1405
AGTGAGGCTGAAGCCACAGCAGCTTGGGATTGGGCTCATCATATCAATCCATGATGCTTTGCTTCCAGCTGAGCCTCAC  1484
TGCCCTTTTATAGCCTGCCCAGAGGAAGGGAGCGCTGCTAAATGCCCAAAAAGGTAACACTGAGCAAAAGCTTATTTCA  1563
ATGTATGATAGAGAACGAGTGCATCTCGCACAGATCAGCCATGGGAGCATCGTTTGCCATCAGCCCCAAAACCCAAAGG  1642
ATGCTAAAATGCAGCCAAAGGGGAATCAAGCACGCAGGGAAGGACTTGAATCAGCTCAACTGGATTGAAATGGCAAAAG  1721
GCATGAGTAGAACGAACGGCAAGGGGATGCTGGAGATCCACCTCCTGTGAGCAAATTGTTCGATGCAGCCAATGGAACT  1800
ATTGCTTCTTGTGCTTCAGTTGCTGCTGATGTGTACATAGGCTGTAGCATATGTAAAGTTACACGTGTCAAGCTGCTCG  1879
CACCGCGTAGAGCTAATATGTATCATGTATGTGGGCACTGAATGCCACCGTTGGCCATACCCAACCGTCCTAAACGATT  1958
TTCACGTCGCTGTAACTTAAGTGGAGATACACTTTCAGTATATTCAGCAAAAGGAATTC                       2017
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FIG. 1. Nucleotide and deduced amino acid sequence of the 2.2-kb p65-21 insert. Three potential asparagine-linked glycosylation sites and two cysteine residues are shaded. The eight imperfect hexapeptide repeats are doubly underlined and the two hydrophobic regions are singly underlined. Amino acid residues 25 (shaded) through 51 correspond to the N-terminal sequence determined by Edman degradation.

protein. There is a 5′ untranslated region of 171 nucleotides and a 3′ untranslated region of 1216 nucleotides that does not include a poly(A) tail or a polyadenylylation signal.

The predicted amino acid sequence contains a series of eight imperfect hexapeptide repeats (Arg^{42}–Pro^{89}) in the N-terminal half of the molecule, in which every third residue is proline and every sixth residue is glycine (Fig. 2). An uninterrupted stretch of 20 nonpolar amino acids (Val^{119}–Gly^{138}) flanked by charged residues is located near the middle of the molecule, and there is a shorter hydrophobic region at the C terminus (Trp^{252}–Met^{266}). There are three potential asparagine-linked glycosylation sites in the molecule (asparagine residues 188, 203, and 212). A fourth asparagine residue, Asn^{96}, is separated from a serine residue by proline, and therefore is unlikely to be utilized as a glycosylation site (18). N-glycanase (Genzyme) digestion experiments indicate that at least some of the sites are, in fact, glycosylated (7). The sequence also contains two cysteine residues (residues 186 and 231) that provide the potential for intramolecular disulfide bond formation.

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42  Arg Gln Pro Ser Tyr Pro  47
    Arg Gln Pro Gly Tyr Pro  53
    His Asn Pro Gly Tyr Pro  59
    His Asn Pro Gly Tyr Pro  65
    His Asn Pro Gly Tyr Pro  71
    His Asn Pro Gly Tyr Pro  77
    Gln Asn Pro Gly Tyr Pro  83
    His Asn Pro Gly Tyr Pro  89
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FIG. 2. ch-PrLP proline- and glycine-rich repeat region.

Computer searches of protein data bases revealed that the predicted protein is homologous to the mammalian PrP (PrP^C) (4–6). We therefore refer to the p65-21 insert-encoded protein as the ch-PrLP. The sequences of ch-PrLP and a mouse PrP (19) are shown aligned in Fig. 3*A*. With one gap in the ch-PrLP sequence and five gaps in the mouse PrP sequence, the two are identical at 33% of the amino acid positions. The degree of similarity increases to 43% if conservative substitutions are taken into account. An uninterrupted stretch of 24 amino acids (ch-PrLP residues Pro^{112}–Tyr^{135}), which includes 17 of the 20 nonpolar residues in the central region, is identical in the chicken and mouse proteins. It is noteworthy that amino acid substitutions at several positions within the highly conserved region (Asn^{107}, Phe^{108}, Val^{111} of mouse PrP) have been associated with variations in the incubation time for experimental scrapie in rodents (19, 22), and a substitution at Ala^{117} of human PrP (homologous to Ala^{124} of ch-PrLP) has been identified in two patients with Gerstmann–Sträussler syndrome (23). In addition, this region is part of a segment that appears to regulate the membrane topology of mammalian PrP during *in vitro* translation (24).

One of the two cysteine residues in the mouse PrP^C (residue 178) occurs in the same position (after alignment) as one of the cysteines in the ch-PrLP and the other (PrP Cys^{213}) is displaced by only 4 residues. In hamster PrP, the cysteines form an intramolecular disulfide bond (25). The two predicted

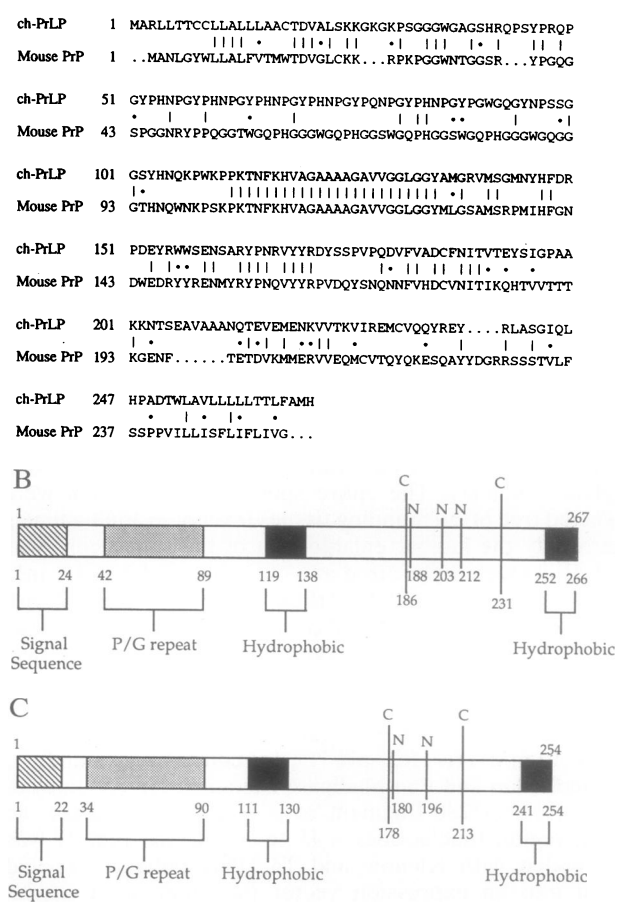

FIG. 3. (*A*) Alignment of the amino acid sequences of ch-PrLP and I/Ln strain mouse PrP^C (19). Identities are indicated by solid vertical lines, and conservative substitutions (20) are indicated by solid circles. The University of Wisconsin GAP computer program (21) was used to generate the alignment (gap weight, 5; length weight, 0.3). (*B*) Schematic diagram of the structure of ch-PrLP. (*C*) Schematic diagram of the structure of mouse PrP^C for comparison (based on data from refs. 4, 5, and 19).

N-linked glycosylation sites in the PrP (residues 180 and 196) occur at positions close to two of the three sites in ch-PrLP (residues 188 and 203). The ch-PrLP cDNA predicts that the residues at positions 42 and 48 that were not definitely identified by gas-phase sequencing are arginines. Arginine residues at positions 25 and 37 of hamster PrP were also not identified by sequencing (25). Perhaps they bear the same modifications that prevent detection.

The mammalian PrP also displays the same structural domains as the ch-PrLP (Fig. 3 *B* and *C*), including proline- and glycine-rich repeats in the N-terminal half of the molecule as well as central and C-terminal hydrophobic regions (4, 5). Furthermore, like PrP^C (26, 27), a fraction of the ch-PrLP molecules appear to be anchored to the surface membrane via a glycosyl phosphatidylinositol anchor. Transfected neuroblastoma N2a cells express recombinant ch-PrLP, and immunoreactive material can be released into the medium after the cells are exposed to bacterial phosphatidylinositol-specific phospholipase C (Fig. 4). Thus, the C-terminal hydrophobic region of ch-PrLP is likely to be the signal for attachment of a glycosyl-phosphatidylinositol anchor (28). We have no evidence that the central hydrophobic region (Val^{119}–Gly^{138}) spans the membrane.

The major mRNA detected on Northern blots of embryonic and adult chicken spinal cord and brain is 2.9 kb (Fig. 5*A*). A band at 2.9 kb was evident in E6 spinal cords, the earliest time examined, and the amount of this message increased during embryonic development, reaching highest levels in the adult chicken. The same time course of gene expression was observed in the brain (Fig. 5*A*), the source of the purified protein. Small amounts of a 3.5-kb mRNA are also apparent in both spinal cord and brain, especially in poly(A)$^+$ mRNA (data not shown). The significance and origin of the larger mRNA remain to be determined. A band of ≈1 kb varied from preparation to preparation and probably represents a degradation product.

In adult chickens, the 2.9-kb mRNA is highly concentrated in the central nervous system (Fig. 5*B*). Among the nonneural tissues examined, the kidney exhibited the highest levels. The message was more widely distributed in E17 embryos.

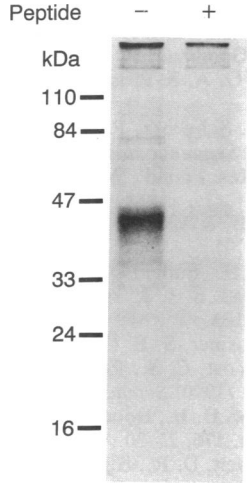

FIG. 4. Release of ch-PrLP from transfected N2a mouse neuroblastoma cells by phosphatidylinositol-specific phospholipase C. After metabolic labeling with [^{35}S]methionine, protein released from intact cells by incubation with the phospholipase was immunoprecipitated with an antiserum raised against a synthetic peptide corresponding to Pro^{47}–Pro^{65} of the ch-PrLP sequence. In parallel cultures incubated without phospholipase, a much smaller signal is seen (data not shown). Immunoprecipitation is specifically blocked when the antiserum is preincubated with the peptide immunogen (1 mg/ml) (lane +).

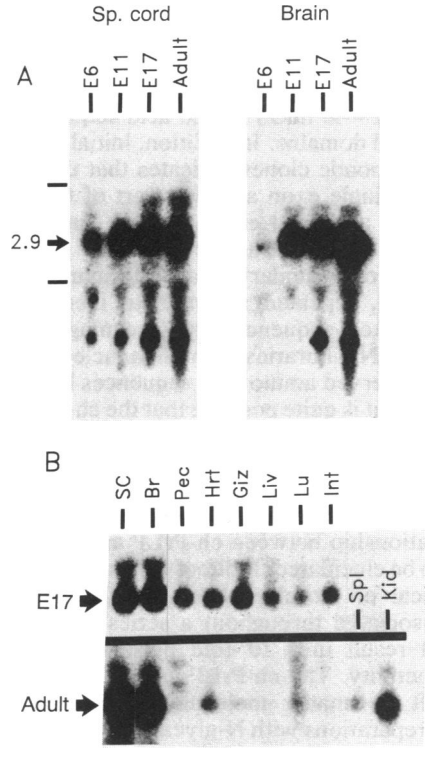

FIG. 5. (*A*) Northern blots of ch-PrLP mRNA in spinal cord and brain at the indicated stages. (*B*) Tissue distribution of ch-PrLP mRNA in E17 and adult animals. Only the region of the blot surrounding the 2.9-kb mRNA is shown. SC, spinal cord; Br, brain; Pec, pectoral muscle; Hrt, heart; Giz, gizzard; Liv, liver; Lu, lung; Int, intestine; Spl, spleen; Kid, kidney.

Immunohistochemical experiments indicate that ch-PrLP is concentrated in the ventral horn of the spinal cord. The section shown in Fig. 6 was labeled with antibodies directed against a synthetic peptide corresponding to residues Gln^{213}–Thr^{224} of the ch-PrLP. The staining was markedly reduced when the antibodies were preincubated with excess peptide. Label, above background, was observed in the dorsal horn, but it was less intense than that present in motor neurons. *In situ* hybridization experiments indicate that the mRNA for ch-PrLP is also concentrated in ventral grey matter, although it is detected at lower levels elsewhere in the cord (ref. 7; unpublished data).

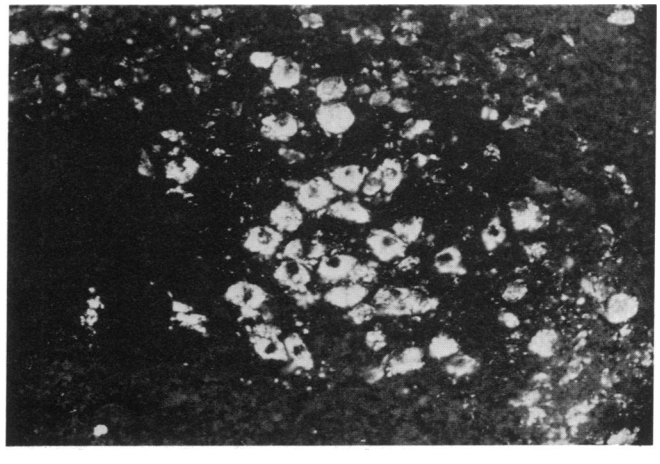

FIG. 6. Fluorescence micrograph of a section of lumbar spinal cord from an E18 chicken stained with a monoclonal antibody directed against a synthetic peptide corresponding to residues Gln^{213}–Thr^{224} of ch-PrLP. Dorsal to the top; lateral to the right.

DISCUSSION

ch-PrLP, the major protein in purified preparations of a 42-kDa AChR-inducing activity, is remarkably similar to the mammalian PrP—in linear amino acid sequence and in several specialized domains. In addition, initial characterization of ch-PrLP genomic clones indicates that the coding region lies within a single exon and that part of the 5′ noncoding region lies on a second exon (D.A.H., unpublished data; J.-M. Gabriel and S. Prusiner, personal communication). This organization is similar to that of the mammalian PrP gene (5). Moreover, repeated attempts to identify related but different chicken sequences by screening embryonic and adult brain cDNA libraries with oligonucleotides that correspond to conserved amino acid sequences have been unsuccessful. Thus, it is quite possible that the ch-PrLP is the avian equivalent of the mammalian protein: the 270 million years that separate birds and mammals can account for the sequence divergence. However, additional experiments designed to identify related molecules are needed.

The relationship between ch-PrLP and the 42-kDa ARIA remains to be elucidated. Indirect evidence suggests that they are identical or closely related. ARIA and the ch-PrLP remain associated throughout a series of chromatographic steps that result in a 10^6-fold purification of the AChR-inducing activity. The ch-PrLP and the AChR-inducing activity shift to smaller molecular mass after digestion of purified preparations with N-glycanase (7). The ch-PrLP and its mRNA are concentrated in motor neurons, consistent with the hypothesis that this protein is involved in the formation and maintenance of nerve–muscle synapses. It is significant that the ch-PrLP mRNA is evident in E6 spinal cords because it is at this time that motor axons begin to form functional contacts in the periphery (29) and the first muscle cell AChR clusters appear surrounding the nerve trunk (30). Finally, it is perhaps not a coincidence that reducing agents destroy the activity of ARIA (7, 8) and that the two cysteines in the mammalian PrP have been shown to be disulfide linked (25).

However, attempts to precipitate ARIA with a serum, raised against a ch-PrLP fusion protein, that removes most of the ch-PrLP from solution have been unsuccessful. ARIA may reside in a small fraction of the ch-PrLP molecules that are not recognized by the antiserum, perhaps because they bear a crucial posttranslational modification or because they comprise an alternative isoform. Transfection experiments are complicated by the fact that N2a cells secrete material that decreases levels of AChRs on cultured myotubes, and we have not yet demonstrated that transfected N2a cells secrete more AChR-inducing activity than do nontransfected controls. Thus, despite the strong, indirect evidence that the ch-PrLP is a form of ARIA, we cannot rule out the possibility that they are unrelated molecules. In any case, the early appearance of the ch-PrLP in the spinal cord, and its concentration in motor neurons, suggest that it may play a role in neuromuscular junction formation.

Beyond the neuromuscular junction, the role of the ch-PrLP (and its mammalian counterpart) in the normal brain remains to be determined. Preliminary results (unpublished data) have shown that the ch-PrLP is concentrated in cholinergic neurons in the brainstem, telencephalon, and optic lobes as well as in spinal cord motor neurons. It is noteworthy that PrP mRNA increases in parallel with choline acetyltransferase activity in the basal forebrain during postnatal development, and that both PrP mRNA and choline acetyltransferase activity increase after intraventricular administration of nerve growth factor (31). The results reported here raise the possibility that the ch-PrLP and, by analogy, the PrP play a trophic role in the brain (the source of the purified proteins), perhaps in regulating the number and distribution of chemoreceptors. It is attractive to think that the scrapie isoform of PrP lacks this trophic activity and may, in fact, act as an antagonist.

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