

A Glycolipid-anchored Prion Protein Is Endocytosed via Clathrin-coated Pits

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Abstract. The cellular prion protein (PrP^C) is a glycolipid-anchored, cell surface protein of unknown function, a posttranslationally modified isoform of which PrP^{Sc} is involved in the pathogenesis of Creutzfeldt-Jakob disease, scrapie, and other spongiform encephalopathies. We have shown previously that chPrP, a chicken homologue of mammalian PrP^C, constitutively cycles between the cell surface and an endocytic compartment, with a transit time of ~60 min in cultured neuroblastoma cells. We now report that endocytosis of chPrP is mediated by clathrin-coated pits. Immunogold labeling of neuroblastoma cells demonstrates that the concentration of chPrP within 0.05 μ m of coated pits is 3–5 times higher than over other areas of the plasma membrane. Moreover, gold particles can be seen within coated vesicles and deeply invaginated coated pits that are in the process of pinching off from the plasma membrane. ChPrP is

also localized to coated pits in primary cultures of neurons and glia, and is found in coated vesicles purified from chicken brain. Finally, internalization of chPrP is reduced by 70% after neuroblastoma cells are incubated in hypertonic medium, a treatment that inhibits endocytosis by disrupting clathrin lattices. Caveolae, plasmalemmal invaginations in which several other glycolipid-anchored proteins are concentrated, are not seen in neuroblastoma cells analyzed by thin-section or deep-etch electron microscopy. Moreover, these cells do not express detectable levels of caveolin, a caveolar coat protein. Since chPrP lacks a cytoplasmic domain that could interact directly with the intracellular components of clathrin-coated pits, we propose that the polypeptide chain of chPrP associates with the extracellular domain of a transmembrane protein that contains a coated pit internalization signal.

THE cellular prion protein (PrP^C)¹ is a plasma membrane glycoprotein that is expressed in the central nervous system and several peripheral tissues beginning early in embryonic development (Bendheim et al., 1992; Manson et al., 1992; Harris et al., 1993a). The physiological function of PrP^C is unknown, although its location on the cell surface suggests a role in adhesion, transmembrane signaling, or uptake of extracellular ligands. Mice in which the PrP^C gene has been deleted show no obvious abnormalities (Büeler et al., 1992). PrP^C is anchored to the plasma membrane by a glycosyl-phosphatidylinositol (GPI) moiety located at the COOH terminus of the polypeptide chain, a feature shared by a heterogeneous group of proteins including lymphocyte and trypanosome surface antigens, adhesion

molecules, exofacial enzymes, and receptors (for review see Low, 1989; Cross, 1990).

The scrapie prion protein (PrP^{Sc}) is a posttranslationally modified isoform of PrP^C that is the major component of the infectious particle (prion) responsible for a group of transmissible neurodegenerative disorders, including kuru, Creutzfeldt-Jakob disease, and Gerstmann-Sträussler syndrome in man, and scrapie in animals (for review see Prusiner, 1991, 1992). Experiments involving mice that do not express the PrP gene, or that express PrP transgenes derived from other species, have suggested that prion replication involves the conversion of endogenous PrP^C into infectious PrP^{Sc} (Prusiner et al., 1990; Scott et al., 1993; Büeler et al., 1993). This conversion has been postulated to result from a species-specific molecular interaction between the two isoforms, although direct biochemical evidence for this is lacking. It has been reported recently that removal of PrP^C from the surface of prion-infected neuroblastoma cells using extracellular phospholipase or proteases blocks prion replication (Caughey and Raymond, 1991; Borchelt et al., 1992). This result suggests that conversion of PrP^C to PrP^{Sc} occurs either on the cell surface, or intracellularly following endocytosis of the cellular isoform. However, the

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1. *Abbreviations used in this paper:* chPrP, chicken prion protein; GPI, glycosyl-phosphatidylinositol; PAI-1, plasminogen activator inhibitor; PIPLC, phosphatidylinositol-specific phospholipase C; PrP^C, cellular isoform of the prion protein; PrP^{Sc}, scrapie isoform of the prion protein; uPA, urokinase-type plasminogen activator.

identity of the cellular compartments in which prion replication occurs is not known.

Endocytic targeting of Pr^{PC} poses an interesting cell biological question. Transmembrane receptors such as those for transferrin, low-density lipoprotein, and mannose 6-phosphate are internalized via clathrin-coated pits, a process that depends on a tyrosine-containing motif in the cytoplasmic tail of the receptor (for review see Trowbridge, 1991). Pr^{PC} and other glycolipid-anchored molecules lack a cytoplasmic domain, and so cannot interact directly with intracellular adaptor molecules that are components of the clathrin coat. It has been reported that several glycolipid-anchored proteins are excluded from coated pits, although the nature of the alternative pathways involved has remained unclear (Bretscher et al., 1980; Rothberg et al., 1990a; Keller et al., 1992).

Recent studies suggest that small, flask-shaped plasma membrane invaginations, called caveolae, might play a role (Anderson et al., 1992; Anderson, 1993). The glycolipid-anchored receptor for the vitamin folate, for example, is concentrated in caveolae (Rothberg et al., 1990a). It has been hypothesized that transient closing of caveolae allows acidification of their interior, followed by dissociation of folate from its receptor, and uptake of the vitamin via a specific membrane transporter (Kamen et al., 1988). Whether caveolae actually pinch off from the plasma membrane, however, and whether they mediate internalization of other glycolipid-anchored proteins, remains unknown (van Deurs et al., 1993).

We have previously reported that chPrP, the chicken homologue of mammalian Pr^{PC}, constitutively cycles between the cell surface and an endocytic compartment, with a transit time of ~60 min in cultured neuroblastoma cells (Shyng et al., 1993). During each passage through the cell, some of the chPrP molecules are proteolytically cleaved within a highly conserved domain in the NH₂-terminal half of the protein. This cleavage process is blocked by lysosomotropic amines and inhibitors of lysosomal proteases (Harris et al., 1993b).

These studies raise the question of what morphological structures mediate the endocytosis of chPrP. Here we use electron microscopic immunolabeling and biochemical analysis to show that, in cultured neuroblastoma cells and neurons, chPrP is concentrated in clathrin-coated pits. Furthermore, we provide evidence that these structures are responsible for the internalization of chPrP. Caveolae are not involved, since by several criteria, these neuroblastoma cells do not contain caveolae. To explain the localization of a glycolipid-anchored protein like chPrP in coated pits, we propose that the polypeptide chain of chPrP binds either directly or indirectly to the extracellular domain of a transmembrane protein that contains a coated pit internalization signal.

Materials and Methods

Reagents and Antibodies

Cell culture reagents were from the Tissue Culture Support Center at Washington University. Phosphatidylinositol-specific phospholipase C (PIPLC) was a generous gift of Dr. Martin Low (Columbia University) and was used at a concentration of 1 U/ml in Opti-MEM (Life Technologies).

Filipin and nystatin were purchased from Sigma Chem. Co. (St. Louis, MO). A rabbit antiserum raised against a bacterial fusion protein encompassing amino acids 35-96 of chPrP has been described previously (Harris et al., 1993). Affinity-purified antibodies raised against a peptide comprising amino acids 14-33 of canine caveolin (VIP21; Dupree et al., 1993) were a gift from Dr. Paul Dupree (EMBL). A rat monoclonal antibody against the mouse transferrin receptor was obtained from Pharmingen. Fluorescein-conjugated secondary antibodies were from Cappel. Gold-conjugated secondary antibodies were from Energy Beam Sciences. Osmium tetroxide, uranyl acetate, and Polybed 812 were from Polysciences Inc. (Niles, IL). ¹²⁵I-transferrin was prepared using chloramine-T (Harlow and Lane, 1988).

Cell Lines

Construction of stably transfected clones of mouse neuroblastoma (N2a) cells that express chPrP has been described previously (Harris et al., 1993b). The clone used in the present study is designated A26. MDCK cells were provided by Dr. Robert Mercer, and 3T3-L1 cells differentiated into adipocytes by Drs. David James and Mike Mueckler (Washington University). Cells were maintained in minimal essential medium supplemented with 10% FCS, non-essential amino acids, and penicillin/streptomycin in an atmosphere of 5% CO₂.

Primary Cultures of Glia and Neurons

Brains were dissected from stage E11 chick embryos, the meninges removed, and the brains minced. Minced tissue was incubated in 0.05% trypsin/0.02% EDTA for 30 min at 37°C with occasional agitation. The trypsin-digested tissue was washed once in HBSS, and then resuspended in MEM supplemented with 10% horse serum and 2.5% chick embryo extract. The tissue was further dissociated by trituration through a pasteur pipette. The dissociated cells were centrifuged through 4% BSA in culture medium to remove cell debris. Cells were then plated onto culture dishes coated with collagen. Primary cultures were used for immunogold labeling 4-5 d after plating.

Immunogold Labeling and Electron Microscopy

Living cells in plastic culture dishes were incubated in primary antibody in Opti-MEM at either 4°C or 37°C for 1 h, washed thoroughly with PBS containing 0.1% BSA, and then fixed in 4% paraformaldehyde/PBS at 4°C for 30 min. In some experiments, cells were incubated at 37°C for 10 min in Opti-MEM before fixation, in order to initiate endocytosis. In other experiments, cells were fixed before incubation with primary antibody. After fixation, aldehyde groups were quenched with 0.01% NaBH₄ for 15 min on ice, and cells incubated with gold-conjugated secondary antibodies for 1 h at 4°C. Gold-labeled cells were then fixed with 1% glutaraldehyde in 0.1 M sodium cacodylate buffer for 15 min on ice, rinsed, and postfixed in 1.25% OsO₄ for 45 min at room temperature. After rinsing in 15% ethanol, cells (still attached to the culture dish) were stained with 4% aqueous uranyl acetate for 45 min at room temperature, dehydrated, and embedded in overturned gelatin capsules containing Polybed 812. Thin sections were cut, mounted on grids, and viewed in a Zeiss EM902A electron microscope.

Quantitation of Electron Micrographs

Five grids were analyzed for each experiment, and 5-6 cells chosen randomly from each grid. Photographs at 22,000× magnification were taken along the plasma membrane of each cell, and digitized images of prints were analyzed using SigmaScan/Image (Jandel Scientific). Since gold particles were sometimes adjacent to but not directly within coated pits, we determined the number of gold particles and length of membrane directly over coated areas, as well as the number of gold particles and length of membrane within 0.05 μm of the necks of pits. For each experiment, over 500 μm of membrane was measured.

Quick-Freeze, Freeze-etch Electron Microscopy

Cells were grown as sparse monolayers on 3 × 3 mm #1 coverslips, and then washed in Ringer's, followed by KHMgE (70 mM KCl, 30 mM Hepes buffer [pH 7.4], 5 mM MgCl₂, 3 mM EGTA) in preparation for rupture by brief sonication of the coverslip. Immediately thereafter, the remaining ventral cell fragments, still attached to the coverslip, were fixed in 2% glutaraldehyde in KHMgE for 1-2 h at 25°C. Fixed coverslips were then washed in distilled water, quick-frozen with a homemade liquid helium-cooled, metal-block "cryopress", and freeze-dried for 15 min at -80°C in

a Balzers 400 freeze-etch device. The coverslips were then rotary replicated with 2 nm of Pt-C applied from an electron beam gun mounted 24° above the horizontal, and were "backed" with 6 nm of pure carbon. Replicas were then separated from the coverslips by flotation on hydrofluoric acid, washed by flotation on several changes of water, and picked up on 75-mesh Formvar-coated electron microscopic grids. Grids were viewed at 100KV in a JEOL 100CX electron microscope and photographed at ±10° tilt to create stereo-images. Final electron micrographs were printed in reverse-contrast to highlight platinum-coated elevations such as coated pits and caveolae against a darker plasma membrane background.

Immunofluorescence Microscopy

Cells grown on glass coverslips were incubated with anti-chPrP antiserum for 1 h at 4°C. Primary antibodies were washed off, and the cells fixed in 3% paraformaldehyde for 30 min at 4°C. For studying the effect of cholesterol-binding drugs, cells were treated with the drugs for 15 min at either 4°C or 37°C after fixation. Cells were then incubated with fluorescein-conjugated secondary antibodies for 30 min at room temperature. For localization of caveolin, cells were fixed in 3% paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated with anti-caveolin antibodies for 2 h at room temperature, before staining with fluorescein-conjugated secondary antibodies.

ChPrP Internalization Assay

Internalization of chPrP was quantitated using surface iodination, as described previously (Shyng et al., 1993). Briefly, cells were incubated on ice with PBS containing glucose, lactoperoxidase, glucose oxidase and Na¹²⁵I for 20 min, and the reaction quenched with 1 mM tyrosine and 10 mM sodium metabisulfite. After warming to 37°C for 30 min to allow internalization, cells were treated with PIPLC for 2 h at 4°C before lysis. ChPrP in the PIPLC incubates (surface) and cell lysates (internal) was quantitated by immunoprecipitation. To test the effects of hypertonic treatment, cells were preincubated for 30 min at 37°C in Opti-MEM containing 0.45 M sucrose, and 0.45 M sucrose was also included during the iodination and warm-up.

¹²⁵I-Transferrin Uptake Assay

The assay was performed essentially as described by Ciechanover et al. (1983). Cells were incubated for 1 h at 4°C with PBS containing ¹²⁵I-transferrin (2 μM). After washing, they were transferred to Opti-MEM containing 100 nM unlabeled transferrin at 37°C for 15 min to allow internalization. At the end of the incubation, cells were treated with 0.25% pronase in PBS to remove ¹²⁵I-transferrin on the cell surface, and lysed in buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 0.5% Triton-X100 and 0.5% sodium deoxycholate. The amount of ¹²⁵I-transferrin internalized was determined by measuring the radioactivity in the cell lysates using a gamma counter. Hypertonically treated cells were preincubated for 30 min at 37°C in Opti-MEM plus 0.45 M sucrose, and 0.45 M sucrose was also included during the incubation with ¹²⁵I-transferrin, and the warm-up.

Purification of Clathrin-coated Vesicles

Clathrin-coated vesicles were purified from adult chicken brain using methods described by Maycox et al. (1992). Fifteen frozen adult chicken brains (Pel-Freez) were homogenized in 150 ml 0.1 M MES/NaOH (pH 6.5), 1 mM EGTA, and 0.5 mM MgCl₂ (buffer A) using a Teflon-glass homogenizer. The homogenate was centrifuged at 20,000 g for 20 min, and the resulting supernatant centrifuged at 55,000 g for 1 h. The pellet was resuspended in 10 ml buffer A, rehomogenized, and dispersed by passing through a 27-gauge needle. The suspension was mixed with 10 ml buffer A containing 12.5% Ficoll and 12.5% sucrose and centrifuged for 40 min at 40,000 g. The supernatant was diluted 1:5 in buffer A and centrifuged for 1 h at 100,000 g. The pellet was resuspended in 15 ml buffer A and cleared by centrifugation at 20,000 g for 20 min. The supernatant was then layered on top of buffer A prepared with D₂O containing 8% sucrose, and spun for 2 h at 100,000 g. The final pellet was resuspended in 0.3 ml of buffer A.

Western Blot Analysis

Cells were lysed in buffer containing 150 mM NaCl, 50 mM Tris (pH 7.5), 0.5% Triton X-100, 0.5% sodium deoxycholate, and 60 mM octylglucoside. Proteins in the cell lysate were precipitated with 10 vol of methanol at -20°C and separated by SDS-PAGE, followed by immunoblotting,

and visualization with anti-caveolin antibodies and enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL).

Northern Blot Analysis

Total cellular RNA was prepared by the guanidine isothiocyanate method, using a kit from BRL. Northern blots were performed by standard procedures, using a randomly primed cDNA probe encoding canine caveolin (a gift of Dr. Paul Dupree).

Results

Localization of chPrP Using Immunogold Labeling

We used immunogold labeling followed by quantitative electron microscopic analysis to localize chPrP at the ultrastructural level. For these experiments, we employed N2a mouse neuroblastoma cells that were stably transfected with the cDNA for chPrP. This cell line has been characterized in detail before (Harris et al., 1993b; Shyng et al., 1993). Cells were incubated with polyclonal antibodies against chPrP at 4°C, followed by fixation, addition of gold-labeled secondary antibodies, and thin sectioning.

We observed clusters consisting of 2–15 gold particles, as well as some single gold particles, on the cell surface. A number of clusters were associated with clathrin-coated pits. Fig. 1, A and B show representative profiles of clathrin-coated pits, which could be recognized by their characteristic shape, and coating with electron-dense material. Clusters of gold-labeled chPrP molecules were found at the edges of the pits, as well as inside the pits. Quantitative analysis of the micrographs showed that 13.6% of the gold particles were associated with coated pits (Table I, experiment #1); of these particles, about half were directly over the coated area, and the remainder were within 0.05 μm of the neck of the pit. Since 4.4% of the surface membrane was occupied by coated pits (including the 0.05 μm area surrounding the neck of each pit), the concentration of chPrP molecules near clathrin-coated pits was threefold higher than over undifferentiated areas of membrane (Association Index). Neuroblastoma cells that were not transfected with the chPrP cDNA did not show detectable labeling (not shown).

Rothberg et al. (1990a) and Ying et al. (1992) have reported that the temperature at which cells are incubated with primary antibody affects the ultrastructural localization of some glycolipid-anchored proteins. To test for such a temperature-dependent effect, we incubated neuroblastoma cells with anti-chPrP antibody at 37°C instead of 4°C. This modification produced an even higher concentration of chPrP in clathrin-coated pits (4.6-fold; Table I, experiment #2).

To exclude the possibility that the association of chPrP with clathrin-coated pits was induced by antibody binding, we fixed cells with paraformaldehyde before incubating them with primary antibody. We found that there was no significant difference in the distribution of chPrP between living cells and fixed cells (Table I, experiment #3).

We have previously shown using immunofluorescent labeling that chPrP is internalized into cytoplasmic vesicles that contain a fluid-phase marker (Shyng et al., 1993). This observation was substantiated here by monitoring the internalization of chPrP at the ultrastructural level. We warmed cultures to 37°C for 10 min after incubation with primary antibody, thereby initiating endocytosis, and then decorated them with gold-labeled secondary antibody after fixation.

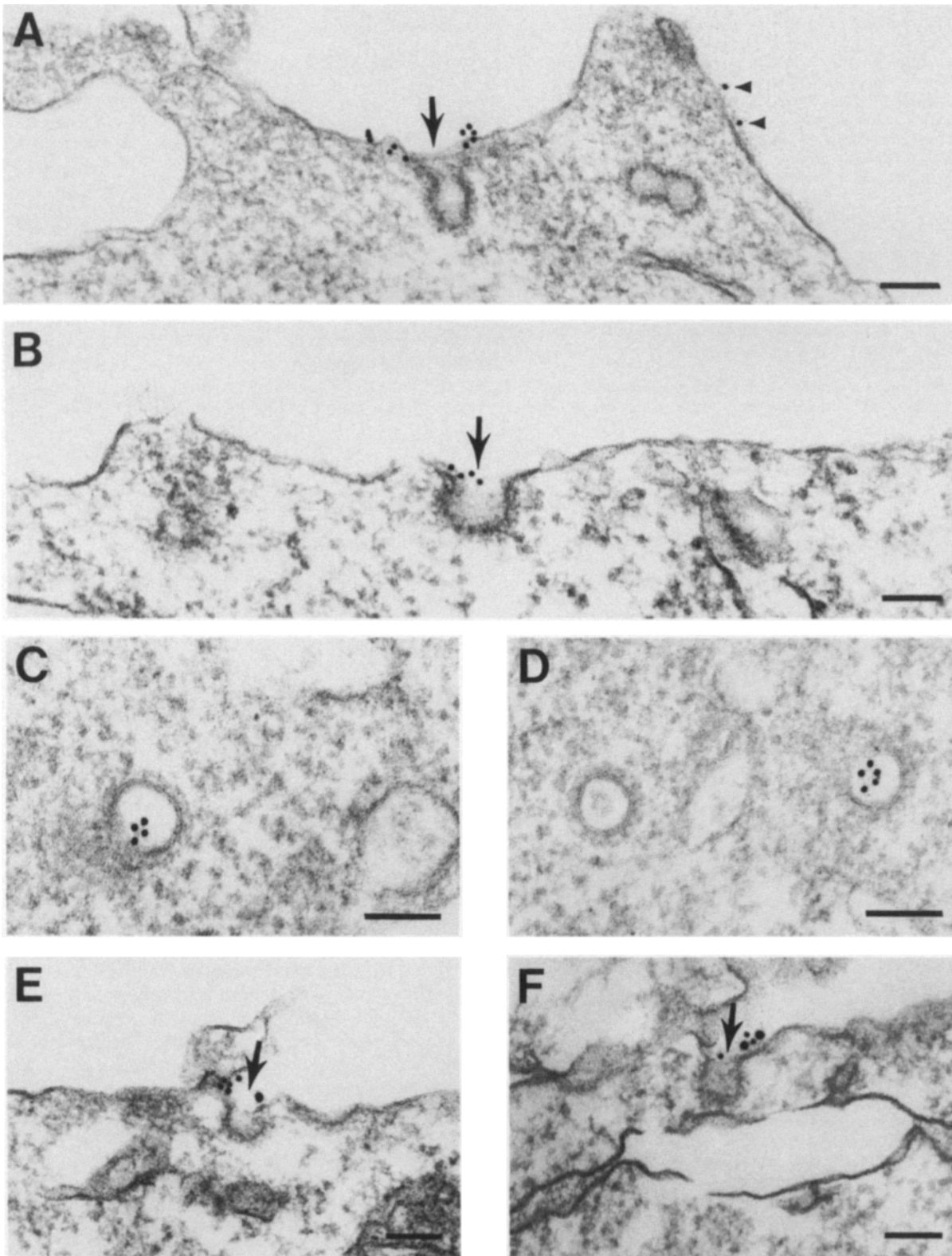


Figure 1. Immunogold localization of chPrP in mouse neuroblastoma cells. In *A* and *B*, cells were incubated with anti-chPrP antibodies at 4°C, and then fixed and processed for immunogold detection using goat anti-rabbit secondary antibodies conjugated to 10-nm gold particles. (*A*) Clusters of gold particles are localized near the edges of a clathrin-coated pit, which is indicated by an arrow. Two isolated gold particles (*arrowheads*) are also seen over an undifferentiated area of membrane. (*B*) A cluster of gold particles within a coated pit (*arrow*). (*C* and *D*) Cells were incubated at 37°C for 10 min after primary antibody incubation to initiate internalization of chPrP; cells were then fixed and decorated with gold-conjugated secondary antibodies. Gold particles are seen within circular, clathrin-coated structures that represent either vesicles that have pinched off from the plasma membrane, or cross-sections through deeply invaginated pits just before budding.

Table I. Quantitative Analysis of Immunogold-labeling Experiments in Neuroblastoma Cells

Experiment #	Experimental condition	Total # of gold particles	% of gold particles associated with coated pits*	% of membrane in coated pits [‡]	Association index [§]
1	1° Ab 1 h at 4°C on living cells	1137	13.6	4.4	3.0
2	1° Ab 1 h at 37°C on living cells	575	13.1	2.9	4.6
3	1° Ab 1 h at 4°C on fixed cells	515	9.3	2.2	4.2
4	Double labeling: 1° Ab 1 h at 4°C on living cells → 10 min at 37°C	ChPrP:891	14.8	4.4	3.4
		TfR:155	27.9		6.4

Neuroblastoma cells transfected with chPrP were incubated with anti-chPrP antibodies, or a mixture of anti-chPrP and anti-mouse transferrin receptor antibodies under the conditions specified for each experiment. ChPrP was visualized using goat-anti-rabbit antibodies conjugated to 10-nm gold particles, and the transferrin receptor using goat-anti-rat antibodies conjugated to 15-nm gold particles. Over 500 μm of surface membrane area was measured for each experiment.

* Particles that were within 0.05 μm of clathrin-coated pits were counted as associated with the pits. Of these particles, between 40% and 60% were directly over the coated area, and the remainder were within 0.05 μm of the neck of the pit.

‡ Membrane directly over the coated area, as well as 0.05 μm of membrane on either side of the neck of the pit was measured.

§ The association index was calculated by dividing the percentage of gold particles associated with coated pits by the percentage of membrane area that was occupied by clathrin-coated pits. This number represents the concentration of gold particles in clathrin-coated pits compared with undifferentiated areas of membrane.

Gold particles were observed within circular, clathrin-coated structures in the cytoplasm (Fig. 1, C and D). These structures are likely to be either vesicles that had pinched off from the plasma membrane but had not yet been uncoated, or else profiles of deeply invaginated coated pits whose connection to the cell surface was not captured in the plane of section. We note that binding of antibody is unlikely to have substantially perturbed internalization of chPrP in this experiment, since we find that iodinated anti-chPrP antibodies are taken up by cells with a time course which is similar to that for internalization of surface-iodinated chPrP (unpublished data).

Double Labeling of chPrP and Transferrin Receptors

We performed immunogold double labeling to compare the ultrastructural distribution of chPrP with that of the transferrin receptor, which is known to be internalized via clathrin-coated pits. Cells were incubated at 4°C with polyclonal antibodies against chPrP, as well as a rat monoclonal antibody against mouse transferrin receptor. Labeled cells were then cultured at 37°C for 10 min before fixation to allow endocytosis to proceed.

Antigen-antibody complexes were then detected by applying a mixture of goat anti-rabbit antibody conjugated to 10-nm gold particles, plus goat anti-rat antibody conjugated to 15-nm gold particles. As shown in Fig. 1, E and F, many coated pits contain both chPrP and transferrin receptors. Gold particles of both sizes were found near the edges of the pits, within 0.05 μm of the opening, and some were localized inside the pits. We calculated that transferrin receptors were 6.4 times more concentrated in coated pits than over other areas of the plasma membrane, (Table I, experiment #4).

This number was 1.9-fold higher than that calculated for chPrP in the same experiment.

ChPrP Is Concentrated in Clathrin-coated Pits in Primary Cultures of Neurons and Glia

To demonstrate that the localization of chPrP in coated pits was not a phenomenon restricted to transfected mouse neuroblastoma cells, we performed immunogold-labeling experiments on primary cultures containing a mixture of neurons and glia from the brains of E11 chick embryos. We have previously shown by *in situ* hybridization and Northern analysis that the mRNA for chPrP is easily detectable in chick brain at this stage (Harris et al., 1993a).

Primary cultures were fixed with paraformaldehyde, incubated with antibodies against chPrP and processed for immunogold detection. Although the amount of labeled chPrP in these primary cultures was less than in the transfected neuroblastoma cells, the distribution of the protein was very similar. Gold particles were clustered on the surface of both neurons and glial cells, which could be distinguished from each other by their morphology. Clusters were present on the cell bodies, as well as the processes of neurons. On both cell types, gold particles were clustered in or near clathrin-coated pits (Fig. 2). We quantitated the distribution of gold particles in neurons and glial cells separately, and found that in both the concentration of chPrP in coated pits was even greater than in transfected neuroblastoma cells (Table II).

Internalization of chPrP Is Inhibited by Treating Cells with Hypertonic Medium

To gather further evidence that clathrin-coated pits are responsible for the internalization of chPrP, we treated neu-

(E and F) Cells were incubated at 4°C with rabbit anti-chPrP antibody and a rat monoclonal antibody against the mouse transferrin receptor. After incubation at 37°C for 10 min, the cells were fixed, and chPrP was visualized using a goat anti-rabbit antibody conjugated to 10 nm gold, and transferrin receptors with a goat anti-rat antibody conjugated to 15 nm gold. The two types of gold particles are concentrated in the same coated pits, which are indicated by arrows. Scale bars in all panels, 0.1 μm.

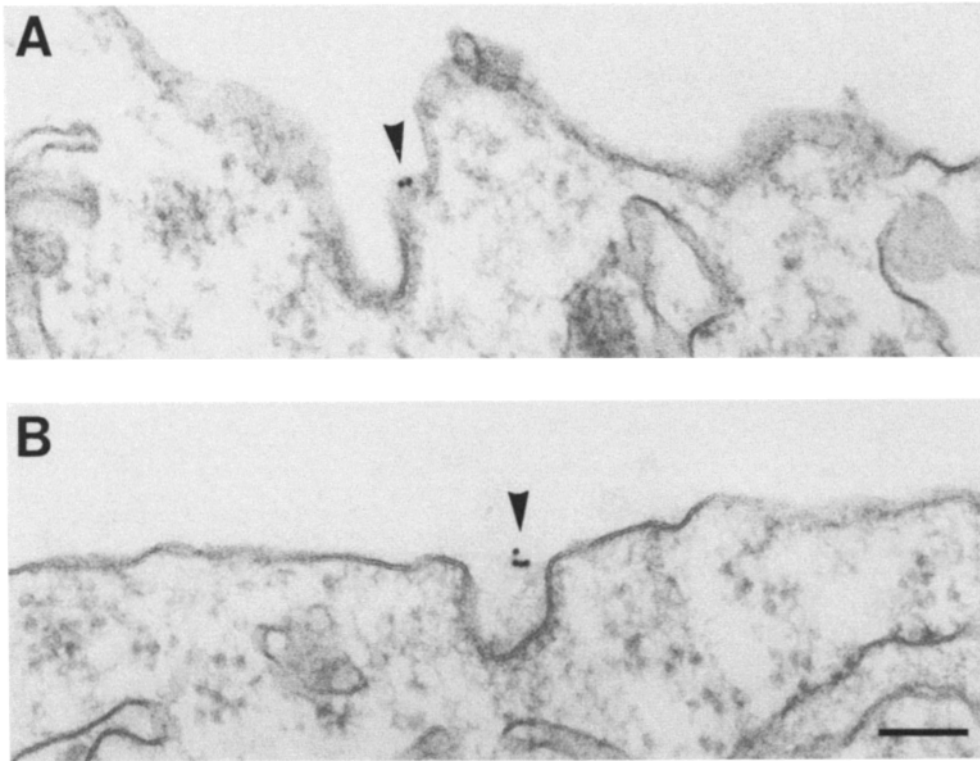


Figure 2. Immunogold localization of chPrP in a primary culture of neurons and glia. Cells cultured from E11 chick brain were fixed, incubated with anti-chPrP antibodies, and processed for immunogold detection. Clusters of gold particles (arrowheads) were localized at clathrin-coated pits on both neurons (A) and glial cells (B), which could be distinguished by their morphology. (Neurons had a more rounded nucleus, and a larger nuclear/cytoplasmic ratio). Scale bar, 0.1 μm .

neuroblastoma cells with hypertonic medium, which is known to disrupt clathrin lattices and inhibit the internalization of ligands via coated pits (Heuser and Anderson, 1989; Hansen et al., 1993). Cells were preincubated in MEM containing 0.45 M sucrose for 30 min at 37°C, surface-iodinated on ice, and then reincubated at 37°C in the hypertonic medium for 30 min to allow endocytosis to proceed. The amounts of chPrP present on the cell surface and intracellularly were then quantitated by immunoprecipitation. We found that hypertonic treatment reduced the internalization of chPrP to 29% of the control value (Fig. 3). This inhibition was readily reversed by replacing the hypertonic medium with isotonic medium during the second 37°C incubation, indicating that the effect was unlikely to be due to irreversible damage to the cells (data not shown). To confirm that incubation in hypertonic medium was interrupting clathrin-mediated endocytosis, we measured the uptake of ^{125}I -transferrin, and

found that it was reduced to 15% of control after this treatment.

ChPrP Is Present in Clathrin-coated Vesicles Purified from Adult Chicken Brain

Using immunoblotting, we have also detected chPrP in a purified coated vesicle fraction prepared from adult chicken brain. The purity of the vesicle fraction was determined by both electron microscopy and by SDS-PAGE. After embedding in plastic and thin-sectioning, we found that ~90% of the membranous vesicles were surrounded by a complete clathrin cage (Fig. 4 A). SDS-PAGE analysis of purified vesicles showed a major protein of 180 kD, most likely representing the clathrin heavy chain (Fig. 4 B). To solubilize contaminating proteins from uncoated membranes, the vesicle fraction was treated with Triton X-100, and the clathrin cages collected by centrifugation (Pearse, 1982; Makiya et al., 1992). The detergent-extracted cages contained chPrP (Fig. 4 C), confirming that this protein is present in coated vesicles, and is closely associated with components of the clathrin cage.

Table II. Quantitative Analysis of Immunogold Labeling of chPrP in Primary Cultures of Neurons and Glia

Cell type	Total # of gold particles	% of gold particles associated with coated pits	% of membrane in coated pits	Association index
neurons	186	8.6	1.7	5.2
glia	169	19.5	3.0	6.5

Primary cultures containing a mixture of neurons and glia from the brains of E11 chick embryos were fixed with paraformaldehyde 4–5 d after plating. They were labeled with anti-chPrP antibodies and detected using goat-anti-rabbit secondary antibodies conjugated to 10-nm gold particles. Neurons and glia were distinguished by their morphology. Over 500 μm^2 of plasma membrane of each cell type was surveyed. Quantitation was performed as described in the legend to Table I.

Electron Microscopy Fails to Detect Caveolae in Neuroblastoma Cells

Several GPI-anchored proteins, most notably the receptor for the vitamin folate, are concentrated in non-clathrin-coated membrane invaginations called caveolae. These structures are most abundant in endothelial cells, adipocytes, fibroblasts, and smooth muscle cells, but are also present in several other cell types (Rothberg et al., 1992; Anderson, 1993). Although we observed numerous clathrin-coated pits in thin-section electron micrographs of our neuroblastoma

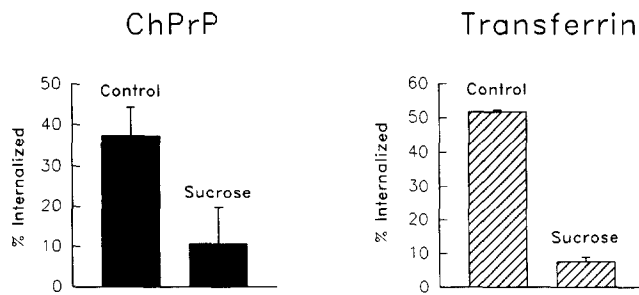


Figure 3. Internalization of chPrP and transferrin is inhibited by treating cells with hypertonic solution. (**LEFT**) Cells were iodinated at 4°C, and after warming to 37°C for 30 min, were treated with PIPLC at 4°C before lysis. ChPrP in the PIPLC incubates (*surface*) and cell lysates (*internal*) was quantitated by immunoprecipitation. Internalization is expressed as $(\text{internal}/\text{internal} + \text{surface}) \times 100$. For hypertonic treatment, incubations were done in medium containing 0.45 M sucrose. The amount of chPrP internalized in cells treated with sucrose is 29% of that in control cells. (**RIGHT**) Cells that had bound ^{125}I -transferrin at 4°C were incubated at 37°C for 15 min, and after stripping the surface label with pronase, the amount of internalized ^{125}I -transferrin was measured by gamma counting. Internalization is expressed as a percentage of the amount of ^{125}I -transferrin bound at 4°C without pronase stripping. For hypertonic treatment, incubations were done in medium that contained 0.45 M sucrose. The amount of ^{125}I -transferrin internalized in cells treated with sucrose is 15% of that in control cells. Standard errors are indicated.

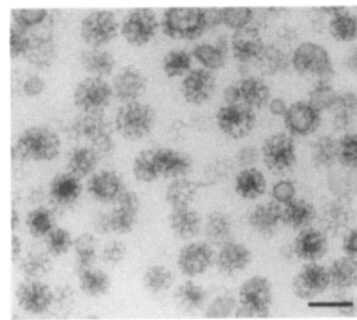
cells (Fig. 1), we were unable to find any plasmalemmal invaginations with the structural features of caveolae, which lack a thick cytoplasmic coat, occur in clusters, and have a flask-shaped profile with a smaller diameter than coated pits.

In addition, we could not find any caveolae after searching extensively through untransfected and chPrP-expressing N2a cells that were sheared open, and then quick-frozen, freeze-dried, and platinum-replicated (Fig. 5). In such replicas, caveolae display a striated coat that is easily distinguished from a clathrin lattice (see Fig. 5 inset; Steer and Heuser, 1991; Rothberg et al., 1992). Although no caveolae were found, numerous clathrin lattices were observed.

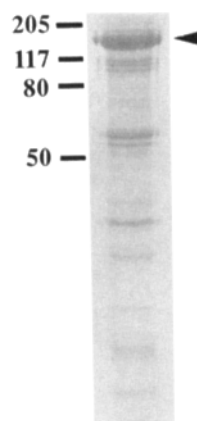
Neuroblastoma Cells Do Not Express Caveolin

Caveolin is a 21-kD protein that appears to be a component of the caveolar cytoplasmic coat (Rothberg et al., 1992; Dupree et al., 1993). To determine whether murine N2a cells express caveolin, even though they do not display caveolae, we used rabbit polyclonal antibodies raised against an NH₂-terminal peptide of canine caveolin for immunofluorescence staining and for immunoblotting. This antiserum has been shown to cross-react with mouse caveolin (Dupree et al., 1993). As positive controls, we used two cell lines which are known to contain caveolae: canine MDCK cells, and murine 3T3-L1 cells that were differentiated into adipocytes (Fan et al., 1983; our unpublished observations). Fig. 6 shows that N2a cells exhibited only background staining for caveolin, whereas MDCK and 3T3-L1 cells stained intensely. Furthermore, a 21-kD caveolin band was not detected on immunoblots of N2a cell lysates, and a caveolin mRNA of 3 kb was not observed on Northern blots of total RNA from N2a cells (Fig. 7). In contrast, caveolin protein

A



B



C

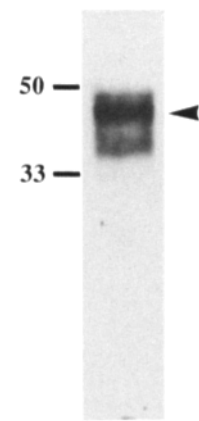


Figure 4. ChPrP is present in clathrin-coated vesicles purified from adult chicken brain. (**A**) Electron micrograph of a thin plastic section of the purified, clathrin-coated vesicle fraction. More than 90% of the vesicles are surrounded by a clathrin cage. Scale bar, 0.1 μm . (**B**) Coomassie-stained SDS polyacrylamide gel of proteins in the purified coated vesicle fraction. The most prominent protein is the clathrin-heavy chain, with an M_r of 180 kD (arrowhead). Bands near 100 and 55 kD may represent subunits of adaptor proteins. (**C**) The coated vesicle fraction was treated with 1% Triton X-100 for 30 min at 25°C to solubilize contaminants from uncoated membranes. The clathrin cages were then collected by centrifugation at 100,000 g and analyzed by immunoblotting using anti-chPrP antibodies. A band of 35–45 kD corresponding to chPrP is detected (arrowhead).

and mRNA were easily detectable in both MDCK and 3T3-L1 cells.

The Distribution of chPrP Is Not Affected by Cholesterol-binding Drugs

Several drugs that chelate cholesterol disrupt caveolae (Steer and Heuser, 1991), and cause clusters of glycolipid-anchored folate receptors to disperse on the surface of cultured kidney epithelial cells (Rothberg et al., 1990b). We tested the effect of two of these agents, filipin and nystatin, on the distribution of chPrP in neuroblastoma cells by immunofluorescence microscopy. The drugs were applied at either 4°C or 37°C to paraformaldehyde-fixed cells that had been stained with anti-

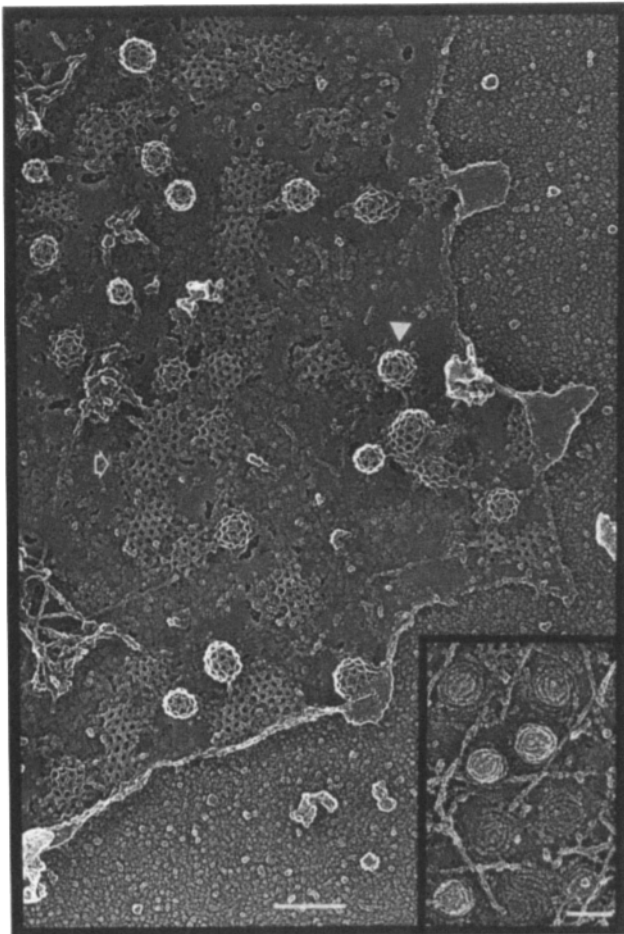


Figure 5. Electron micrographs of cells that have been sheared open and then quick-frozen, freeze-dried, and platinum-replicated. The main field shows the inner surface of the plasma membrane of a chPrP-expressing N2a neuroblastoma cell, upon which numerous polygonal lattices are seen. These display all of the structural features of clathrin-coated pits (Heuser, 1980, 1989). Some lattices are relatively flat (*open arrowhead*), while others curve around a budding vesicle (*filled triangle*). These polygonal lattices are easily distinguished from the striped coats that characterize caveolae. Caveolae are displayed in the inset, which shows a region of plasma membrane from a 3T3-L1 cell. Despite extensive examination, no structures that display such a striped coat could be found in either transfected or untransfected neuroblastoma cells. Scale bars, 0.2 μm .

chPrP antibody (Fig. 8). In neither case did the punctate staining pattern of chPrP change from that observed in control cells, in terms of either the size or the density of fluorescent dots. Likewise, no changes in chPrP distribution were observed when these two drugs were applied to living cells at 37°C (data not shown).

Discussion

We have previously reported that chPrP, the avian homologue of mammalian PrP^C, continuously cycles between the cell surface and an acidified endocytic compartment in cultured neuroblastoma cells (Shyng et al., 1993). During each cycle, which requires ~60 min, a small percentage of the protein molecules are proteolytically cleaved within a highly conserved region of the amino acid sequence (Harris et al., 1993b).

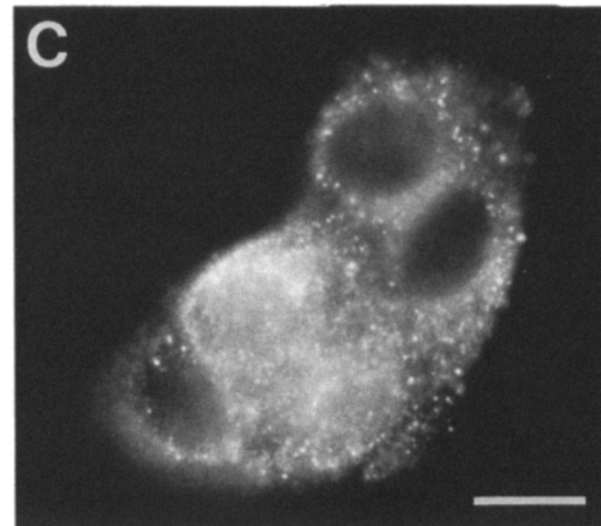
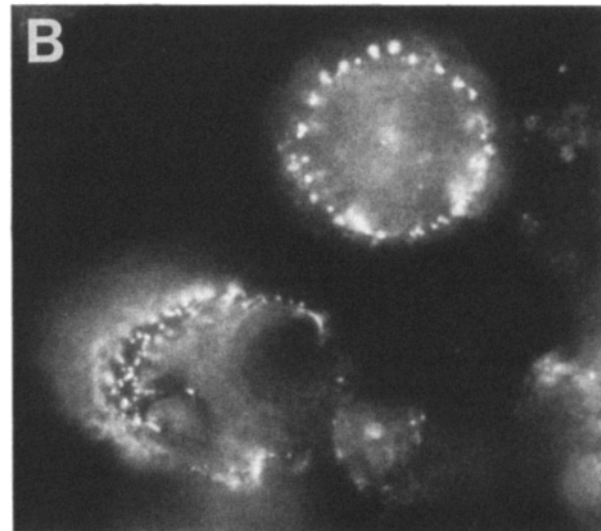


Figure 6. Immunofluorescent staining of caveolin in chPrP-expressing neuroblastoma cells, 3T3-L1 adipocytes, and MDCK cells. Cells were fixed in 3% paraformaldehyde and permeabilized with 0.1% Triton X-100 before labeling with an antibody to canine caveolin, followed by a fluorescein-conjugated secondary antibody. Only background staining was observed in the neuroblastoma cells (A), while the 3T3-L1 adipocytes (B) and MDCK cells (C), both of which are known to have caveolae, stained intensely. Scale bar, 10 μm .

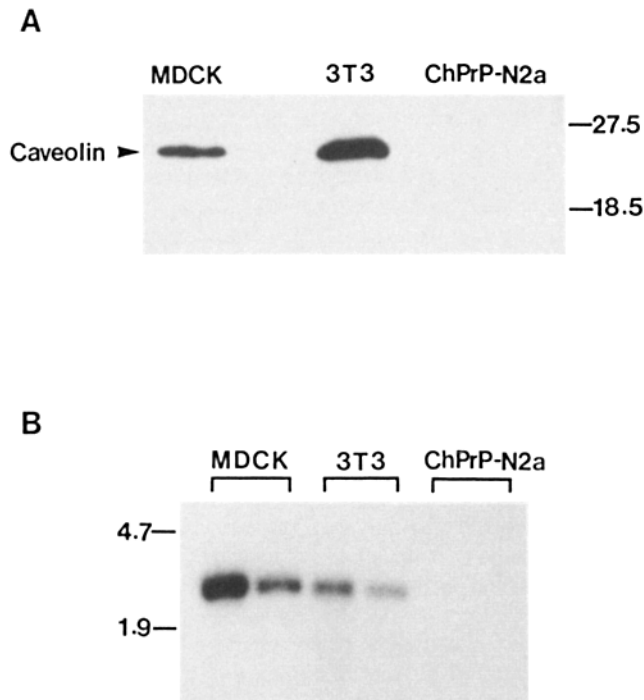


Figure 7. Expression of caveolin protein and mRNA in chPrP-expressing neuroblastoma cells, 3T3-L1 adipocytes, and MDCK cells. **(A)** Membrane proteins from the three cell lines were solubilized, methanol precipitated, and analyzed by immunoblotting using an antibody to canine caveolin. A 21-kD band corresponding to caveolin is detectable in MDCK cells, 3T3-L1 adipocytes, but not in N2a neuroblastoma cells. **(B)** Total cellular RNA (5 and 10 μ g) from each of the three cell lines was analyzed by Northern blotting using a cDNA probe encoding canine caveolin. A 3-kb caveolin mRNA is detectable in MDCK and 3T3-L1 cells, but not in N2a neuroblastoma cells.

In the present study, we have investigated the mechanism by which chPrP, a glycolipid-anchored protein, is endocytosed. Our combined electron microscopic and biochemical evidence demonstrates that chPrP is internalized via clathrin-

coated pits in cultured neuroblastoma cells. First, quantitative immunogold labeling shows that the concentration of chPrP molecules within 0.05 μ m of clathrin-coated pits is 3–5 times higher than over other areas of the plasma membrane. Second, in cells that have been warmed to 37°C after antibody labeling, gold particles can be seen in coated vesicles and deeply invaginated coated pits that are in the process of pinching off from the plasma membrane. Third, internalization of chPrP is blocked by incubation of cells in hypertonic medium, a treatment which causes disruption of clathrin lattices and inhibition of endocytosis (Heuser and Anderson, 1989). Finally, we show that chPrP is concentrated in coated pits in primary cultures of neurons and glia, and is found in coated vesicles purified from chicken brain.

A number of well-known transmembrane receptors are endocytosed via clathrin-coated pits, including those for transferrin, low-density lipoprotein, and mannose 6-phosphate. It is now clear that specific amino acid motifs in the cytoplasmic tails of these receptors, usually containing tyrosine residues within a so-called tight-turn configuration, serve as endocytic targeting signals (for example see Davis et al., 1987; Collawn et al., 1990; Jadot et al., 1992; for review see Trowbridge, 1991). These signals are thought to interact with cytoplasmic proteins such as adaptor molecules that are components of the clathrin coat.

A heterogeneous group of proteins, including chPrP and mammalian PrP^Cs, are anchored to the plasma membrane by a glycosyl-phosphatidylinositol moiety which is attached to the COOH terminus of the polypeptide chain (for review see Low, 1989; Cross, 1990). The anchor is added posttranslationally in the endoplasmic reticulum, following cleavage of a COOH-terminal hydrophobic sequence which serves as a signal for anchor attachment. Because they lack a cytoplasmic polypeptide domain, GPI-anchored proteins cannot interact directly with components of the clathrin coat. The question thus arises whether these proteins are endocytosed at all, and if so, what morphological structures are involved. Although some GPI-anchored proteins, such as thy-1, appear to undergo little internalization (Lemansky et al., 1990), a number of others, including chPrP, 5'-nucleotidase, and a

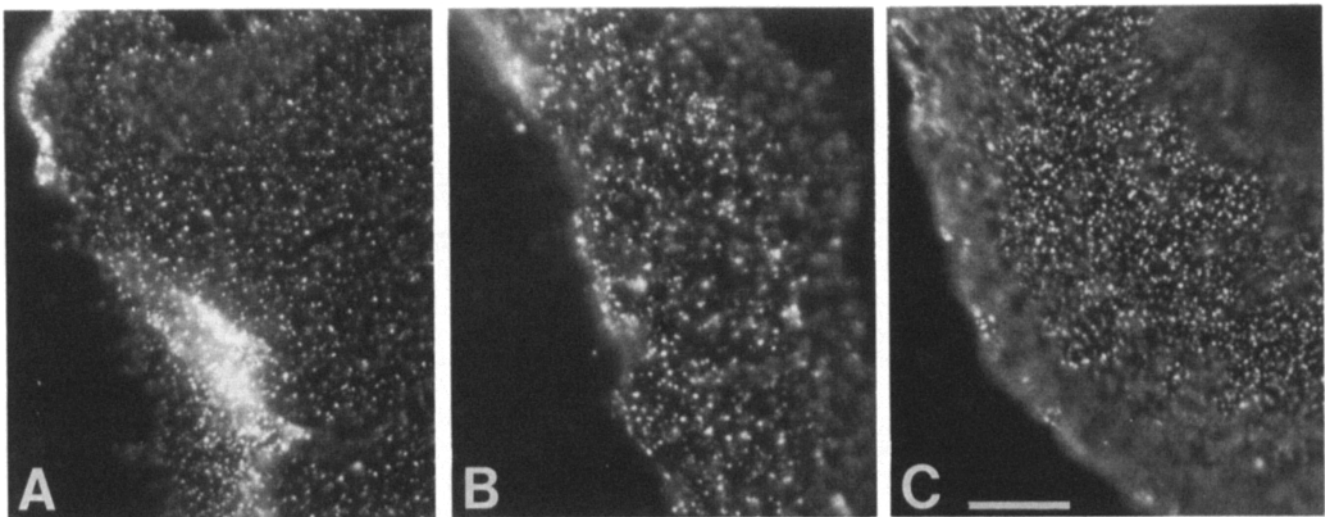


Figure 8. Filipin and nystatin do not alter the surface distribution of chPrP. Neuroblastoma cells were stained with anti-chPrP antibody at 4°C and fixed in paraformaldehyde. They were then either left untreated **(A)**, or were incubated at 4°C with 5 μ g/ml filipin **(B)** or 100 μ g/ml nystatin **(C)** before application of secondary antibody. ChPrP is stained in a punctate pattern, and neither drug alters the size, density, or distribution of the fluorescent dots. Scale bar, 10 μ m.

CD4/DAF chimera, are constitutively endocytosed and recycled to the cell surface (van den Bosch et al., 1988; Keller et al., 1992; Shyng et al., 1993).

A number of studies have suggested that membrane glycolipids and some GPI-anchored proteins are excluded from coated pits, or at least are not concentrated there (Bretscher et al., 1980; Montesano et al., 1982; Tran et al., 1987; Keller et al., 1992; Bamezai et al., 1992). Although originally derived from an analysis of thy-1 and ganglioside-binding toxins, this notion has received renewed attention as a result of studies on the receptor for the vitamin folic acid. The GPI-anchored folate receptor is absent from coated pits in cultured kidney epithelial cells, and is instead localized to flask-shaped plasma membrane invaginations called caveolae (Rothberg et al., 1990a). These structures display a striated cytoplasmic coat that is composed in part of a 21-kD protein called caveolin (Rothberg et al., 1992). Whether or not caveolae actually pinch off from the plasma membrane, and serve as endocytic organelles, has remained an unsettled issue (Severs, 1988; van Deurs et al., 1993). One proposal has been that caveolae close transiently without budding, thereby sequestering receptor-bound folate at a high concentration, and allowing it to enter the cytoplasm via a transmembrane carrier (Anderson et al., 1992; Anderson, 1993). In addition to this putative role in the uptake of nutrients (termed "pocytosis"), caveolae have been proposed to function in calcium regulation and transmembrane signaling (Stefanova et al., 1991; Fujimoto et al., 1992; Fujimoto, 1993; Sargiacomo et al., 1993).

Our results make it clear that not all glycolipid-anchored proteins are found in caveolae. We did not observe caveolae in cultured neuroblastoma cells by electron microscopy after thin-sectioning or freeze-etching, we failed to detect caveolin mRNA or protein in these cells, and we found that cholesterol-binding drugs which disrupt caveolae in other cell types did not alter the distribution of chPrP. These results are consistent with previous reports that morphologically identifiable caveolae, as well as caveolin mRNA and protein, are most abundant in endothelial cells, fibroblasts, and muscle (Glennay, 1989, 1992; Rothberg et al., 1992). We have never seen caveolae in neurons or neuronal cell lines (Heuser, J. E., unpublished observations). Of course, these results do not rule out the possibility that chPrP might be localized to caveolae, or to both caveolae and coated pits, in those cell types where caveolae are found. We are currently investigating the ultrastructural localization of chPrP expressed in other cell types.

A recent study by Ying et al. (1992), also using immunogold labeling, concluded that endogenously expressed murine PrP^C was localized to caveolae in 3T3 fibroblasts. However, that study also claimed that PrP^C was localized to caveolae in N2a neuroblastoma cells. On this point we have to disagree, since we do not find caveolae in our own N2a cells from the ATCC nor in N2a cells sent to us by Ying et al. (data not shown). We suggest that this discrepancy arises from our more stringent definition of caveolae, which is based on identification of a striated caveolar coat in deep-etch EM replicas (see Steer and Heuser, 1991). The study of Ying et al. (1992) employed thin-sections of N2a cells, wherein identification of caveolae is more problematic.

Although chPrP appears to begin its journey along the endocytic pathway in clathrin-coated pits and vesicles, its sub-

sequent fate remains to be determined. Whether it is sorted away from other recycling proteins like the transferrin receptor, and is delivered to distinct endocytic compartments, is unknown. We have previously shown that the bulk of internalized chPrP molecules do not colocalize with a lysosomal marker, suggesting that the protein is restricted to structures earlier along the endocytic pathway (Shyng et al., 1993). More precise identification of the compartments involved will require the use of additional markers, and the application of subcellular fractionation. It will be particularly interesting to determine whether chPrP in neurons becomes packaged into synaptic vesicles, whose membrane components are retrieved from the nerve terminal via coated pits (Heuser and Reese, 1973; Maycox et al., 1992).

Our results raise the possibility that the biosynthetic targeting of chPrP, as well as its endocytic targeting, might differ from that of other glycolipid-anchored proteins. Most newly synthesized GPI-anchored proteins are delivered preferentially to the apical surface of polarized epithelial cells, and to the axonal surface of neurons (Lisanti and Rodriguez-Boulan, 1990; Dotti et al., 1991). It has been suggested that this phenomenon results from incorporation of the lipid anchor into detergent-resistant complexes of sphingolipids and cholesterol that form in the Golgi, and that interact with an apical sorting machinery (Simons and Wadinger-Ness, 1990; Brown and Rose, 1992). It will therefore be interesting to analyze the targeting of chPrP in polarized cell types, and to determine whether the protein becomes resistant to detergent extraction during its biosynthesis. Our observation that the distribution of chPrP was not affected by treatment of cells with cholesterol-binding drugs, which are thought to disrupt glycolipid-containing complexes, suggests that the protein is not associated with these complexes on the cell surface.

The mechanism by which chPrP associates with clathrin-coated pits remains to be explored. Preliminary results suggest that the polypeptide chain of chPrP plays an important role. We have found that deletion of the NH₂-terminal 67 amino acids of chPrP markedly reduces the efficiency with which the molecule is endocytosed, and also diminishes its association with clathrin-coated pits (Harris, D. A., A. Lesko, and S.-L. Shyng. 1993. *Mol. Biol. Cell.* 4:436a.). This truncated chPrP molecule retains its GPI anchor, consistent with the hypothesis that the polypeptide chain rather than the anchor is critical in endocytic targeting. Moreover, we have found that an unrelated GPI-anchored protein, decay-accelerating factor, is poorly endocytosed in N2a cells (unpublished data). These results argue that clathrin-mediated endocytosis is a distinctive feature of chPrP, and is not shared by all glycolipid-anchored proteins expressed in N2a cells.

Presumably, specific components associated with coated pits must be responsible for localizing chPrP molecules in these structures. One attractive model is that the polypeptide chain of chPrP binds to the extracellular domain of a transmembrane protein that contains a coated pit localization signal in its cytoplasmic domain. This binding might be direct, or via intermediate proteins. Such a mechanism appears to be responsible for the localization of the glycolipid-anchored receptor for urokinase-type plasminogen activator (uPA). Upon binding a complex of uPA and plasminogen activator inhibitor (PAI-1), the uPA receptor associates with the LDL

receptor-related protein, and both receptors are subsequently internalized via coated pits (Nykjaer et al., 1993). Whether binding of an extracellular ligand also plays a role in the localization of chPrP to coated pits remains to be determined. In any case, the identification of molecules in coated pits that associate with chPrP is likely to provide important clues to the physiological function of PrP^C. In addition, these binding molecules might play a role in the entry of infectious PrP^{Sc} into cells.

We observed that chPrP was about twofold less concentrated in coated pits than the transferrin receptor (Table I, Experiment #4). In addition, we have reported previously that the rate at which chPrP is endocytosed ($t_{1/2} = 20$ min; Shyng et al., 1993) is considerably slower than that for the transferrin receptor ($t_{1/2} = 3.5$ min; Ciechanover et al., 1983). Both of these results might be explained by postulating that chPrP associates more weakly with coated pit components than do transmembrane receptors that contain a cytoplasmic localization signal.

Our results are relevant to proposed cellular mechanisms for the replication of mammalian prions. Preliminary evidence suggests that murine PrP^C, like chPrP, is endocytosed via clathrin-coated pits in neuroblastoma cells (unpublished data). Based on studies of scrapie-infected cells, it has been suggested that endogenous PrP^C is converted to infectious PrP^{Sc} either on the cell surface, or following endocytosis of the cellular isoform (Caughey and Raymond, 1991; Borchelt et al., 1992). We have previously argued that this conversion occurs as PrP^C cycles between the cell surface and early endocytic compartments (Shyng et al., 1993). The present work now extends these conclusions, and suggests the possibility that clathrin-coated pits and vesicles are involved in the generation of PrP^{Sc}. If this were so, then inhibition of clathrin-mediated endocytosis of PrP^C might represent a therapeutic strategy for blocking prion replication.

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