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Supplemental materials and methods

Chemicals and reagents

PIPLC was purchased from Boehringer. Reagents for cell culture were purchased from GIBCO BRL. Protein quantification was performed using the bicinchoninic acid (BCA) assay from Pierce Chemical Co. FITC-conjugated protein A and all other chemicals and inhibitors were purchased from Sigma-Aldrich. PVDF membranes were obtained from Millipore. The ECL detection kit and X-ray films were purchased from Amersham Biosciences. Molecular weight standards were obtained from Bio-Rad Laboratories. Protein A–gold, 5-nm gold particles coupled to protein A molecules in a 1:1 anticipated ratio, was produced by J. Griffiths from the Utrecht University Medical School Department of Cell Biology . H68-4 antitransferrin receptor antibodies were purchased from Zymed Laboratories. Filipin was purchased from Sigma-Aldrich and used at 50 μg/ml. Aurolysin probe (Fivaz et al., 2002) was a gift from G. van der Goot (Geneva, Switzerland).

Cultured cells

CHO/K1 cells were obtained from the American Type Culture Collection. Production of CHO cells expressing SHaPrP^C (CHO/30C3) was reported previously (Blochberger et al., 1997). CHO cells expressing MH2MPrP^C (Scott et al., 1992), designated CHO/MH2M cells, and CHO cells expressing the glutamine synthetase vector pEE12 (Celltech), designated CHO/EV cells, were constructed in the same manner as the CHO/30C3 cells, as described elsewhere (Blochberger et al., 1997). Immunoblot analysis confirmed that CHO/30C3 and CHO/MH2M cells express SHaPrP^C and MH2MPrP^C, respectively. CD59-transfected CHO cells were provided by J. Fennelly (JR2 Hospital, Oxford, UK) (Wheeler et al., 2002).

Antibodies and purified PrP^C

The mAb 3F4 was raised against SHaPrP (Kascsak et al., 1987). Its epitope is present on SHaPrP^C and MH2MPrP^C but not on Chinese hamster PrP^C. The RO73 antiserum, raised in rabbit against SDS-purified SHaPrP, reacts with SHaPrP^C, Chinese hamster PrP^C, mouse (Mo) PrP^C, and MH2MPrP^C (Serban et al., 1990). The recombinant antibody fragments (Fabs) R1 and R2 were produced using a phage display system; the epitope is located at the COOH-terminal end (residues 225–231) of PrP adjacent to the GPI anchor (Peretz et al., 1997). For all studies described here, data using Fab R1 are shown. Identical results were obtained with R2 and D13 and with Fab D18, which recognizes residues 133–157 (Williamson et al., 1998).

The polyclonal antiserum against caveolin-1 was purchased from Transduction Laboratories. The mAb against caveolin-1 was a gift from M. Lisanti (Albert Einstein College, New York, NY). HRP-conjugated secondary antibodies used for immunoblotting were purchased from Amersham Biosciences. The gold-conjugated goat IgG was obtained from Sigma-Aldrich. Purified SHaPrP^C used for binding studies was isolated from hamster brain and from 30C3 cells according to previously established procedures (Pan et al., 1992). Anti-CD59 was purchased from Serotec.

Western blotting

Proteins from the different cell lysates and gradient fractions were separated on SDS gels and transferred to nylon membranes. After blocking, membranes were probed with 3F4, RO73, and α-caveolin antibodies (1:5,000) for 2 h at RT and detected with HRP-conjugated secondary antibodies (1:5,000) for 1 h at RT before visualization on X-ray films using ECL. To measure PrP^C degradation after protein A–gold uptake, CHO cells expressing SHaPrP^C and control cells were incubated at 37°C for 1 h with and without protein A–gold. After incubation, cells were lysed, proteins were separated on SDS gels, and blots were probed for D18 anti-PrP.

Binding studies

Binding of labeled conjugates was performed on the different cells in culture and on purified proteins immobilized on nitrocellulose. Confluent cells were washed with media twice before incubation for 1 h with media containing either protein A–gold (OD 0.3), protein A–FITC (10 μg/ml) or gold-conjugated goat IgG (10 μg/ml). Cells tested for protein A–gold and goat IgG–gold binding were then washed twice with media, fixed with 2% paraformaldehyde/0.2% glutaraldehyde, scraped from the plates, and inspected after sedimentation. Cell pellets positive for bound gold label turned red. For protein A–FITC binding studies, cells were grown on coverslips, and then washed twice with PBS before fixation with 2% paraformaldehyde. Binding was determined by inspection on a Leica fluorescence microscope.

Binding of protein A–gold to purified SHaPrP^C and BSA (Sigma-Aldrich) was tested by transferring 8 μg of SHaPrP^C purified from hamster brain and from 30C3 cells to nitrocellulose in a dot blot apparatus (Schleicher & Schuell). The nitrocellulose was blocked with media containing 10% FBS for 30 min at RT. Blots were incubated with media containing protein A–gold for 1 h at RT. Blots were washed with PBS twice and were developed with a silver enhancement kit (Nanoprobes) to enhance the gold signal.

Immunogold labeling of living cells

To study uptake of protein A–gold, the different cell lines were incubated for 10 min with media containing protein A–gold and then fixed or chased for an additional 50 min at 37°C before fixation. To analyze the temperature dependence of protein A–gold uptake, cells were incubated with protein A–gold for 10 min at 4°C before fixation. To examine the PrP^C-dependent uptake, each cell type was incubated with and without PIPLC (0.2 U/ml) for 2 h at 37°C before uptake experiments. To demonstrate that protein A–gold binds specifically to PrP^C, cells were preincubated at 4°C with and without a cocktail of anti-PrP Fabs (R1, D13, and D18) in order to mask the PrP^C exclusively, followed by sheep and mouse Fab incubation of 30 min at 4°C before a 10-min incubation at 4°C with media containing protein A–gold. After this incubation, pellets were made and observed for color. Pellets that appeared red contained a high concentration of gold probe.

Cryoimmunogold electron microscopy

Subcellular localization of antigens using high-resolution cryoimmunogold electron microscopy was performed as previously described (Peters and Hunziker, 2001). In brief, the different confluent 30C3 and CHO/MH2M, CHO/K1, and CHO/EV cells were washed and then fixed either with 2% paraformaldehyde for 24 h or with 2% paraformaldehyde/0.2% glutaraldehyde for 2 h. Fixed cells were embedded in gelatin; ultrathin sections were generated and mounted on grids. Sections were immunolabeled with primary antibody and then with protein A–gold. For labeling experiments using recombinant anti-PrP Fabs, sections were immunolabeled with Fabs and then with a rabbit anti-Fab followed by protein A–gold. Ultrathin cryosections were examined at 80 kV by Jeol 1010, Philips CM10, and Technia 12 electron microscopes.

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