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Prion protein expression in different species: Analysis with a panel of new mAbs

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ABSTRACT **By immunizing prion knockout mice (*Prnp*−/−) with recombinant murine prion protein (PrP^c), we obtained a panel of mAbs specific for murine PrP^c. These mAbs can be applied to immunoblotting, cell surface immunofluorescent staining, and immunohistochemistry at light and electron microscopy. These mAbs recognize both the normal (PrP^c) and protease-resistant (PrP^{res}) isoforms of PrP. Some mAbs are species restricted, while others react with PrP from a broad range of mammals including mice, humans, monkeys, cows, sheep, squirrels, and hamsters. Moreover, some of the mAbs selectively recognize different PrP glycoforms as well as the metabolic fragments of PrP^c. These newly generated PrP^c antibodies will help to explore the biology of PrP^c and to establish the diagnosis of prion diseases in both humans and animals.**

Prion diseases, or transmissible spongiform encephalopathies, are neurodegenerative disorders that affect both humans and animals (1–7). All prion diseases are believed to share the same basic pathogenic mechanism that involves the conversion of the normal cellular prion protein ($PrP^{c)}$ into a form that is infectious, insoluble in nonionic detergents, and partially resistant to proteases (PrP^{res}) (8–10). PrP^c and PrP^{res} share an identical amino acid sequence. The conversion of PrP^c to PrP^{res} may involve a conformational change of PrP^c from a predominantly α-helical form to a β-sheet structure (11–14). The accumulation of PrP^{res} in the brain is a cardinal feature of the prion disease pathology. However, the conditions that trigger and determine the conversion of PrP^c to PrP^{res} remain unclear. One approach to the study of the PrP^c to PrP^{res} conversion is offered by experimental models of inherited prion diseases. Since many of the pathogenic mutations of the *PrP* gene (PrP^M) have high penetrance, it is likely that the change in PrP^M metabolism plays an important role in determining the conversion of PrP^M into PrP^{res}. Detailed studies on cell models of inherited prion diseases have underlined the complexity and the diversity of the metabolic changes affecting the PrP^M (15, 16).

Currently, there is one mAb that reacts with PrP is available commercially (17), however, to understand the biology of PrP and the pathogenesis of prion diseases requires an extensive library of well characterized antibodies to PrP (16). A collection of diverse mAbs would allow identification of the different metabolic products of PrP^c, PrP^M and PrP^{res} providing new insights into the mechanism of prion conversion. Ultimately, these mAbs will facilitate the diagnosis of prion diseases in humans and animals.

We immunized prion knockout mice (*Prnp*−/−) with recombinant murine PrP^c to obtain a panel of mAbs specific for PrP^c and PrP^{res}. These mAbs crossreact with PrP^c from other species of mammals including humans, monkeys, cows, sheep, hamsters, and squirrels. Some selectively recognize PrP according to the degree of glycosylation and size of the metabolic products. Therefore, this panel of mAbs will be useful for characterizing and localizing normal, mutant, and pathogenic PrP.

MATERIALS AND METHODS

Animals and the Generation of mAbs. The creation of 129/Ola *Prnp*−/− mice has been described in detail (18). Recombinant murine PrP was prepared and purified as described (19). *Prnp*−/−mice were immunized with recombinant murine PrP^c in complete Freund's adjuvant. After boosting three times with the corresponding antigen in incomplete Freund's adjuvant, spleen cells from one of the immunized *Prnp*−/− mice were fused with a myeloma, SP2/0, to create hybridomas using a conventional protocol (20). We used an ELISA to screen for potential anti-PrP^c mAbs. Briefly, ELISA plates were coated with 1 μg/ml purified murine PrP^c proteins overnight. Plates were then blocked with 3% BSA. One hundred microliters of culture supernatant was added to each well for 30 min at 37°C. After washing, a goat anti-mouse horseradish peroxidase-labeled Ig antibody (Amersham) was added for 30 min at 37°C. The reaction was visualized by 3,3′,5,5′ tetramethylbenzidine (Sigma) for 30 min at room temperature and blocked with 1 M sulfuric acid. Of the 1000 viable clones tested, 79 positive clones were identified in the first screening. In subsequent screening, 37 clones retained their specificity. Six of these clones (2F8, 5B2, 6G9, 8C6, 8H4, and 9H7) were chosen for further studies.

Immunofluorescent Staining. Cultured, parental PrP^c-negative human neuroblastoma cell line M17 and a human neuroblastoma M17 cell line that was transfected with a normal human PrP^c gene (15) were harvested and washed with washing medium (PBS supplemented with 5% new born calf serum, 0.1% NaN_3, pH 7.4). A single-cell suspension (1×10^6/ml) was incubated with affinity-purified mAbs or an isotype control antibody on ice for 45 min. Cells were washed three times with washing medium and 25 μl of fluorescein

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Abbreviation: CJD, Creutzfeldt-Jakob disease.
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isothiocyanate-conjugated goat anti-mouse IgG antibody was added for 45 min on ice. Finally, samples were washed and fixed with 1% paraformaldehyde. Cells were analyzed in a FACScan (Becton Dickinson). At least 5,000 cells were analyzed per sample.

Protein Studies and Immunoblotting. Brain tissue from different animals was homogenized in 9 vol of lysis buffer [100 mM sodium chloride, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 10 mM Tris (pH 7.4), and 2 mM phenylmethylsulfonyl fluoride]. For deglycosylation experiments, samples were treated with *N*-glycosidase F (PNGase-F) [1000 units in 1% Nonidet P-40 and 25 mM sodium phosphate (pH 7.5)] as described (21). Proteins were separated in 12% polyacrylamide gels and then transferred to Immobilon P (Millipore) for 2 hr at 60 V. Membranes were incubated overnight at 4°C with the different mAbs. The blots were developed with an enhanced chemiluminescence system (Amersham).

Immunohistochemistry. Paraffin sections of infected brain grafts from a transgenic mouse overexpressing PrP^c (Tg20) mice implanted in the ventricular wall of a Prnp−/− mouse were prepared as described (22, 23). Sections were processed for immunostaining after hydrolytic autoclaving (24). The sections were deparaffinized, rehydrated, and immersed in 98% formic acid for 1 hr at room temperature. Endogenous peroxidase was blocked by immersion in 8% hydrogen peroxide in methanol for 10 min. Sections were completely immersed in 1.5 mM HCl and autoclaved at 121°C for 10 min. After rinsing, they were incubated with the different mAbs. A goat anti-mouse Ig antibody and peroxidase-antiperoxidase was used to detect bound mouse mAb (Sternberg–Meyer, Jarrettsville, MD). Diaminobenzidine tetrahydrochloride was used to visualize the immunoreactivity. Paraffin sections from the brain of a patient with Creutzfeldt-Jakob disease (CJD) were processed and stained the same way.

Electron Microscopy Immunohistochemistry. Neuroblastoma cell cultures (wild type/M) were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 90 min, washed in TBS [0.1 M Tris (pH 7.5) and 0.15 M NaCl], scraped with a rubber policeman, and then centrifuged at 500 × *g* for 10 min at 4°C. The pellet was embedded in 1.5% agarose and cut in small oriented blocks under the light microscope using a razor blade. The floating blocks were immersed in 0.1% Triton X-100 in Tris-buffered saline for 10 min, blocked with 10% nonfat dry milk for 30 min, and incubated with the 8H4 mAb (1:50) overnight at 4°C in TBS, 0.1% Tween 20, 1% normal goat serum, and 1% BSA. The 8H4 mAb was omitted in negative controls. After washes in TBS the blocks were incubated with 5 nm of gold-conjugated goat anti-mouse IgG (Auro Probe; Amersham) for 2 hr at room temperature. The blocks are then fixed in 2.5% glutaraldehyde and postfixed in 1% OsO_4 for 1 h, dehydrated in alcohol, and embedded in Spurr. Ultrathin sections were cut with a ultramicrotome (Ultracut FC4; Reichert), stained with uranyl acetate and lead citrate, and examined with a JEOL 100 electron microscope. For cryosections cells were fixed and scraped as described above. Pellet was incubated in 30% polyvinylpyrrolidone and 2.3 M sucrose, placed on specimen stubs, and frozen in liquid nitrogen. Ultrathin cryosections (approximately 120-nm thick) were cut on glass knives in an ultracryomicrotome (Teichart ultracut S; Leica, Deerfield, IL). The sections were then placed on carbon and Formvar-coated grids and immunostained as described earlier.

RESULTS

Immunoblotting of Human and Murine PrP^c. We separated the mAbs according to the pattern of immunoreactivity with native and deglycosylated PrP^c on immunoblots: mAbs 8H4, 8C6, 9H7, and 2F8 recognize full-length and truncated forms of PrP^c; mAb 5B2 reacts only with the full-length PrP^c; and mAb 6G9 is glycosylation specific. mAb 6G9 selectively fails to recognize the highly glycosylated form of PrP^c. None of the mAbs react with the brain homogenate from the *Prnp*−/− mouse, confirming the specificity of all of the mAbs for PrP^c.

In humans and mice, mAb 8H4 representative of the first group reacts equally with the three known PrP^c glycoforms; the unglycosylated form which migrates at 27 kDa, the intermediate form, thought to be monoglycosylated, which migrates at 28–30 kDa; and the highly glycosylated forms which migrate as a band spanning 33–42 kDa (Fig. 1). In addition, mAb 8H4 reacts with two bands at 25 kDa and 18 kDa, respectively. These two bands are known to contain the PrP^c forms truncated at the N terminus which are generated during normal processing of PrP^c (Fig. 1). The 18-kDa protein is likely to be the unglycosylated form of the 25-kDa fragment, since, after deglycosylation, mAb 8H4 reacts only with the 18- and 27-KDa proteins, the latter corresponding to the full-length unglycosylated form. The only difference in mAb 8H4 immunoreactivity between human and mouse PrP^c is the reduced affinity for the mouse 25-kDa fragment (Fig. 1). Among the other mAbs of this group, 8C6 and 9H7 differ from 8H4 in the lack of immunoreactivity with the 25-kDa truncated form. Furthermore, mAb 2F8 has a weaker reaction with the full-length forms and, in addition, fails to react with the truncated forms in the mouse (data not shown).

mAb 5B2 reacts strongly with the highly glycosylated and intermediate isoforms of human and mouse PrP^c but reacts weakly with the unglycosylated isoform (Fig. 1). mAb 5B2 does not react with the truncated PrP^c recognized by the previous group of mAbs. mAb 5B2 reacts strongly with PrP^c after deglycosylation of PrP^c with endoglycosidase F (Fig. 1).

mAb 6G9 consistently failed to react with the highly glycosylated PrP^c but reacts with all of the other forms, including the truncated forms, from both humans and mice (Fig. 1). Upon digestion with endoglycosidase F, mAb 6G9 reacts with both 27-kDa and 18-kDa proteins (Fig. 1).

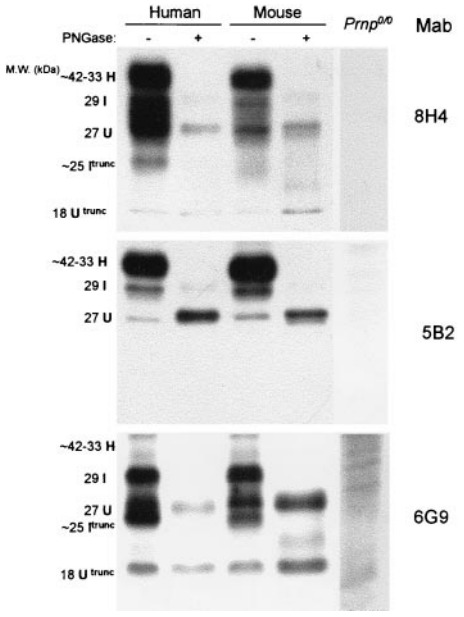

FIG. 1. Human and mouse brain proteins immunoblotted with mAbs to PrP^c raised in this study. *Prnp*−/−, PrP knockout mice; H, I, U, diglycosylated,monoglycosylated, unglycosylated full-length PrP; I trunc and U trunc, monoglycosylated and unglycosylated truncated PrP. mAbs 8H4 and 6G9 react with all PrP forms while mAb 5B2 only reacts with the full-length forms. mAb 6G9 fails to recognize the diglycosylated forms. None of the mAbs react with any protein from the brain of *PrP*−/− mice which does not express PrP.

Reactivity with PrP^c from other Mammals. In the macaque, spider, and Capuchin monkeys, mAb 8H4 from the first group reacts with the three full-length glycoforms and the 25-kDa and 18-kDa truncated fragments similar to that observed in humans and mice (Fig. 2). However, two additional, slightly different patterns can be seen in other animals. In the chimpanzee, cow, sheep, and squirrel, mAb 8H4 appears to react less with the intermediate form and the 25-kDa truncated form. In the squirrel monkey, mAb 8H4 recognizes weakly the highly glycosylated PrP^c. mAb 2F8 recognizes PrP^c from all of the animal species studied reproducing a pattern which resembles that of mAb 8H4. Among the other mAbs of this group, 8C6 and 9H7 show a generally weaker and more species-dependent reaction than 8H4. mAb 2F8 recognizes PrPc from all of the animal species examined with a pattern similar to that of mAb 8H4 (data not shown).

mAb 5B2 shows a strong reactivity with all of the full-length forms as in humans and mice. However, mAb 5B2 appears to recognize the intermediate and the unglycosylated forms less well than the highly glycosylated form in the other species. mAb 5B2 does not recognize the truncated forms in any of the tested species (Fig. 2).

The distinctive characteristic of mAb 6G9 is the lack of a reaction with the highly glycosylated form in all species. mAb 6G9 also fails to react with PrP^c from the squirrel monkey and the squirrel. Moreover, in all species of animals tested, mAb 6G9 recognizes the intermediate form as one band rather than two bands as demonstrated by the other mAbs (Fig. 2).

When the patterns of immunoreactivity of the mAb panel in the various species are compared, the overall immunoreactivity is higher with the human and mouse PrP^c and lower with the squirrel monkey PrP^c than with that of other species. As to the individual PrP^c forms, the highly glycosylated form is generally well recognized by most of the mAbs across species, but there are differences in immunoreactivity for the intermediate, unglycosylated, and truncated forms. These forms are well recognized in humans and less well recognized in cow, sheep, squirrel, squirrel monkey, and chimpanzee, perhaps because these forms are underrepresented in these species.

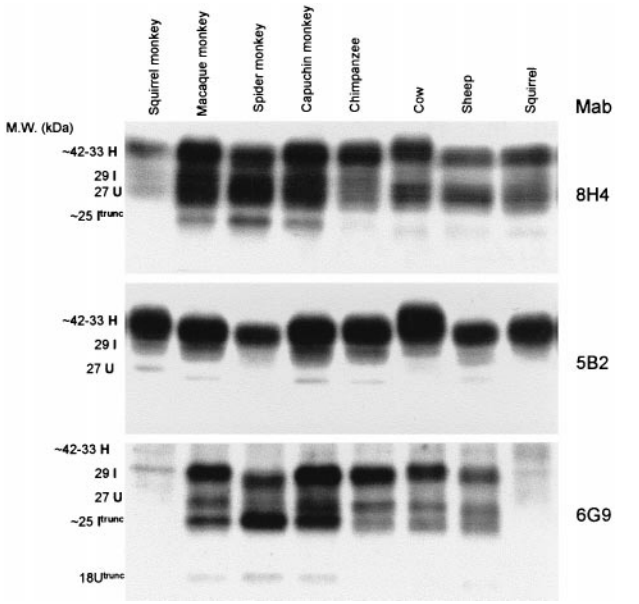

FIG. 2. Brain proteins from different mammals immunoblotted with mAbs to PrP^c. Abbreviations as in the legend to Fig. 1. The three mAbs react well with all species except the squirrel monkey, which is recognized poorly by both mAb 8H4 and 6G9 and the squirrel which is not recognized by 6G9. Both 8H4 and 6G9 but not 5B2 recognize the truncated forms in most species. mAb 6G9 fails to react with the H form in all species examined.

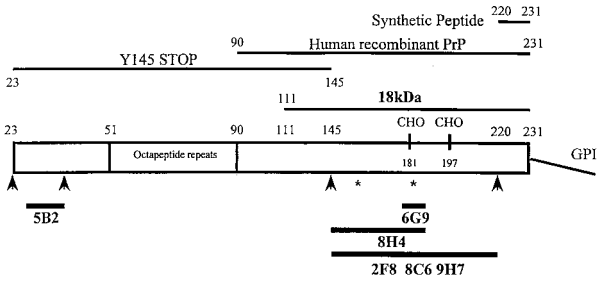

FIG. 3. Epitope mapping of anti-PrP^c mAbs. Diagrammatic representation of mAb epitope topography in the 23–220 PrPc sequence. *, indicates the human PrP^c residues 159 and 182, respectively, which correspond to 166 and 189, respectively, in the squirrel monkey.

The mobility of the highly glycosylated and the intermediate forms are also heterogeneous. PrP^c from the cow and squirrel monkey migrates slower and PrP^c from the spider monkey migrates faster than all other species because of the insertion or deletion of one of the octapeptide repeats, respectively.

PrP^c Epitopes Recognized by the mAb Panel. mAbs 8H4, 8C6, 9H7, and 2F8, the four mAbs of the first group, must be directed to an epitope located in the C-terminal region of PrPc between residues 145 and 220 (Fig. 3) for two reasons: (*i*) they react with the truncated PrP^c forms which have been shown to be generated by the cleavage of the full-length PrP^c at amino acid 111/112 (25), and (*ii*) they do not react with either the synthetic human PrP peptide 220–231 or with the mutant PrP expressed in neuroblastoma cells transfected with the 145 stop codon PrP gene construct which is truncated at residue 145 (G.Z., R.B.P., P.G., and N. Singh, unpublished data). The epitope recognized by 8H4 might be further deduced by the lack of recognition of PrP^c from the squirrel monkey. The squirrel monkey carries two unique amino acid substitutions in the PrP sequence at residues 166 and 189.

mAb 5B2 does not immunoreact with the truncated PrP^c forms and also fails to recognize the recombinant protein with amino acids 90–231 (ref. 26 and unpublished results). Therefore, the epitope recognized by mAb 5B2 resides at the N-terminal half, amino acids 23–90 of the molecule. This interpretation is supported by our recent finding that mAb 5B2 binds specifically to a synthetic peptide corresponding to amino acids 23–40 of the PrP (results not shown).

The epitope bound by mAb 6G9 is most likely to reside at or very close to the *N*-glycosylation site that in humans is

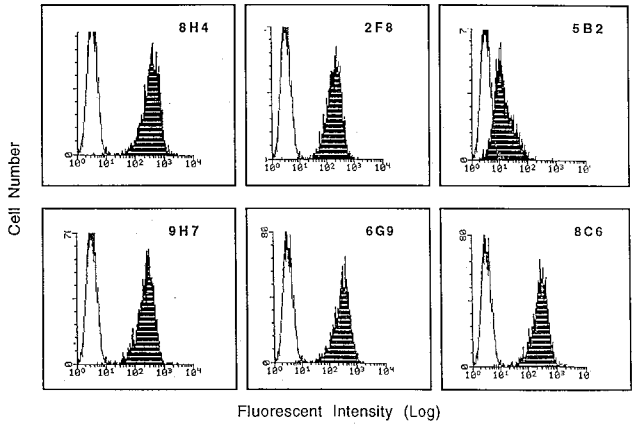

FIG. 4. Expression of PrP^c on the surface of transfected human neuroblastoma cell line analyzed by FACScan. Clear areas represent staining of PrP^c-negative parental M17 cells. Shaded areas represent staining of PrP-positive transfectants. mAb 5B2 stains less intensely than other mAbs probably because it does not react with truncated PrP^c forms.

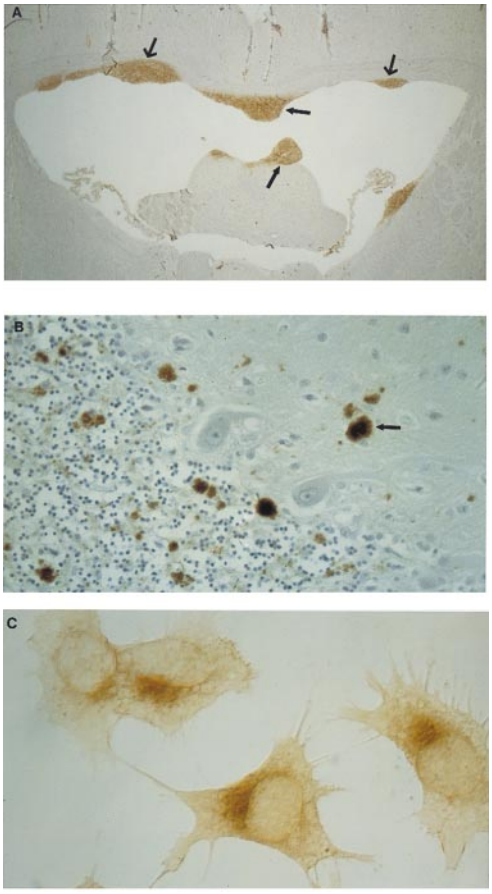

FIG. 5. Immunohistochemistry of PrP^{res} in mouse brain, PrP^{res} in CJD brain, and PrP^{c} in a human neuroblastoma cell line. (*A*) Infected brain grafts from a transgenic mouse overexpressing PrP^{c}, Tg20 mice, implanted in the ventricular wall of a *Prnp*−/− mouse. Paraffin sections were processed and stained with mAb 8H4. mAb 8H4 only stained the neurografts (arrows). (×20.) (*B*) Cerebellar tissues from the brain of a patient with CJD were processed and stained with mAb 8H4. Plaque-like PrP deposits (arrows) are diffusely distributed in the molecular and granular layers of the cerebellum. (× 150.) (*C*) A neuroblastoma cell line transfected with a construct expressing normal human PrP^{c} was stained with 8H4. PrP^{c} is distributed in the intracellular compartment with a Golgi-like distribution. (×430.)

located at residue 181. This inference is based on the finding that mAb 6G9 does not recognize the highly glycosylated PrP^{c} form in which both glycosylation sites are occupied. The epitope localization at or close to human PrP residue 181 is further supported by the lack of reactivity of mAb 6G9 with all forms of PrP^{c} from the squirrel monkey. This PrP^{c} carries an amino acid substitution at residue 189, corresponding to residue 182 in humans (27).

Immunofluorescent Staining and Immunocytochemistry of PrP^{c} or PrP^{res} in Unfixed and Fixed Cells and Tissues. All six of our mAbs reacted with PrP^{c} expressed at the surface of living human neuroblastoma cell transfectants overexpressing PrP^{c}. The fluorescence-activated cell sorting profiles of six of these mAbs are shown in Fig. 4. The staining intensity of mAb 5B2 is less than that of all of the other mAbs, suggesting that the epitope recognized by 5B2 may be less abundant. This observation is in good accordance with our earlier observation that mAb 5B2 does not recognize the truncated PrP^{c} forms which can account for approximately ≈30% of the total PrP^{c} at the cell surface (N. Singh, G.Z., R.B.P., and P.G., unpublished results). The same panel of mAbs did not stain the parental M17 cell line in which PrP^{c} is not detectable.

Brain tissues from Tg20 transgenic mice that had been implanted in *Prnp*−/− mice and then infected with PrP^{sc} (22, 23) were processed and stained with mAb 8H4. mAb 8H4 intensely immunostained the infected murine implants, especially their neuropils and the surface of neurons (Fig. 5*A*). A similar result was obtained with mAbs 2F8, 5B2, 8C6, 9H7, and 6G9 although the intensity of the staining of some of these antibodies was less than that of mAb 8H4 (results not shown). We also stained tissue sections from fixed cerebellar cortex from a patient with CJD. All mAbs except for mAb 6G9 also immunostained PrP^{sc} in the three cortical layers of the cerebellum in the affected CJD subject with a plaque-like pattern (Fig. 5B). These anti-PrP^{c} mAbs did not stain unaffected regions of the brain. The procedures of hydrolytic autoclaving used to enhance staining of PrP^{sc} may have destroyed the epitopes present in normal PrP^{c}. Furthermore, mAbs 8H4, 5B2, and 2F8 also react with the PrP^{c} present in fixed and permeabilized neuroblastoma transfectants (Fig. 5*C*). As reported earlier with another antibody, staining is seen in the cytoplasm and is most pronounced in the Golgi apparatus (28).

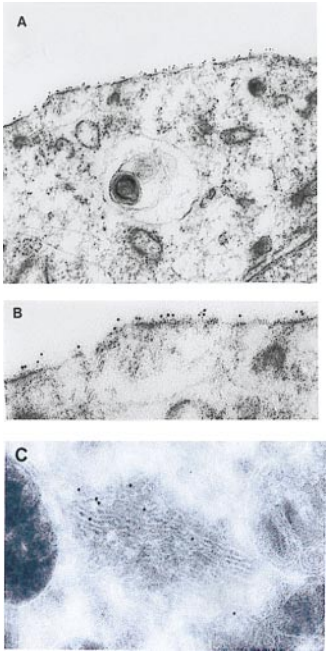

FIG. 6. Immunoelectron microscopy of neuroblastoma cells. (*A*) Gold particles line up the cell surface. (×39,000.) (*B*) Same as *A*. (×78,000.) (*C*) Gold particles are distributed over the Golgi compartment. (× 59,000.)

Immunogold Electron Microscopy. To identify the cellular organelle in which the conversion of PrP^{c} to PrP^{res} takes place, we need antibodies suitable for electron microscopic immunolocalization in well preserved cells and tissues. Neuroblastoma transfectants were immunostained with mAb 8H4 and 5B2. mAb 8H4 immunolocalizes PrP^{c} on the cell surface (Fig. 6 *A* and *B*). mAb 5B2 labeled the Golgi compartment confirming our earlier observation using immunohistochemistry (Fig. 6*C*).

DISCUSSION

Immunochemical tests are the most efficient and reliable diagnostic procedures for identifying humans and animals affected by prion diseases. Only one mAb, 3F4, has been extensively used to date. It reacts with an epitope residing between amino acids 109 and 112 (17). mAb 3F4 does not recognize the C-terminal PrP^{c} fragments which are generated during the normal metabolism of PrP or the small pathological fragments containing the C-terminal region (26). Furthermore, mAb 3F4 detects PrP^{c} in humans and hamster but not in mouse, cow, sheep, Capuchin monkey, and squirrel. This is

a significant limitation since transgenic mice are currently the most widely used animal models for studying prion diseases. The ability to detect cow and squirrel PrP will also help document the potential transmission of prion diseases from these animals to humans and will allow routine diagnosis of prion diseases in a number of mammals (6, 29). Therefore, a panel of anti-PrP mAbs which recognize epitopes distributed over the PrP^c will permit detection of PrP^c and PrP^{res} as well as monitoring of PrP^c processing under normal or pathological conditions.

The mAbs raised in our study are directed to distinct epitopes throughout the 23–231 PrP^c fragment. One of the mAbs, mAb 5B2, reacts with the N terminus of PrP^c. The remaining mAbs recognize the PrP^c C-terminal fragments as well as other C-terminal fragments generated under pathological conditions. Moreover, our mAbs also recognize PrP^c from many different species of mammals including humans, mouse, cattle, sheep, squirrel, and hamster. Because our mAbs recognize PrP^c from evolutionary diverse species, it is likely that these mAbs will also react with PrP^c from other mammals. We have shown that a single amino acid substitution might compromise the epitope recognition by several mAbs; more frequently when the substitutions are not conservative. For example, mAb 6G9 selectively fails to recognize the squirrel monkey PrP^c which is the only known mammal with a Val at PrP position 189 corresponding to the human PrP 182 residue. Thus, since 6G9 also fails to recognize PrP^c in the squirrel we can also predict the presence of an amino acid substitution at, or close to, the residue corresponding to the human residue 182 in this species whose PrP^c has yet to be sequenced.

Some of the mAbs react differently with the various PrP glycoforms. One mAb, 6G9, preferentially recognizes the monoglycosylated and the unglycosylated forms but not the highly glycosylated forms. In contrast, another mAb 8H4 recognizes all three isoforms equally well in humans and macaque, spider, and Capuchin monkeys, but only two glycoforms from cow, sheep, and squirrel. These antibodies will be useful in comparing glycoforms in different species and in identifying the individual glycoforms under conditions in which they are altered (28).

The study of PrP^c metabolism and the PrP^c to PrP^{res} conversion has been impaired by the difficulty to identify cellular locales of PrP^c processing and conversion (30). All of the mAbs we developed are suitable for immunohistochemistry of PrP^c and PrP^{res} on fixed and unfixed tissues. More important, we have been able to use immunoelectron microscopy with mAbs 8H4 and 5B2 to demonstrate the presence of the prion protein on the cell surface and in the Golgi apparatus of neuroblastoma cells fixed in glutaraldehyde. The ability to conduct ultrastructural immunolocalization following glutaraldehyde fixation is especially critical. Glutaraldehyde is the fixative of choice for electron microscopy but commonly cannot be used in immunocytochemistry because such treatment denatures the epitopes. Thus, our mAbs should provide an opportunity to map the ultrastructural distribution of the PrP^c and PrP^{res} isoforms in pathological tissues.

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