Cell-surface retention of PrP^C by anti-PrP antibody prevents protease-resistant PrP formation

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The C-terminal portion of the prion protein (PrP), corresponding to a protease-resistant core fragment of the abnormal isoform of the prion protein (PrP^{Sc}), is essential for prion propagation. Antibodies to the C-terminal portion of PrP are known to inhibit PrP^{Sc} accumulation in cells persistently infected with prions. Here it was shown that, in addition to monoclonal antibodies (mAbs) to the C-terminal portion of PrP, a mAb recognizing the octapeptide repeat region in the N-terminal part of PrP that is dispensable for PrP^{Sc} formation reduced PrP^{Sc} accumulation in cells persistently infected with prions. The 50 % effective dose was as low as ~1 nM, and, regardless of their epitope specificity, the inhibitory mAbs shared the ability to bind cellular prion protein (PrP^C) expressed on the cell surface. Flow cytometric analysis revealed that mAbs that bound to the cell surface during cell culture were not internalized even after their withdrawal from the growth medium. Retention of the mAb–PrP^C complex on the cell surface was also confirmed by the fact that internalization was enhanced by treatment of cells with dextran sulfate. These results suggested that anti-PrP mAb antagonizes PrP^{Sc} formation by interfering with the regular PrP^C degradation pathway.

INTRODUCTION

Transmissible spongiform encephalopathies (TSEs), also called prion diseases, are fatal neurodegenerative diseases and include scrapie in sheep and goats, bovine spongiform encephalopathy and Creutzfeldt–Jakob disease (CJD) in humans. The causative agent of TSEs, often called a prion, is composed mainly of an abnormal isoform (PrP^{Sc}) of the host cellular prion protein (PrP^C). Mice with genetic knockout of the PrP gene are resistant to prion disease (Bueler *et al.*, 1993) and neurons lacking PrP^C expression are resistant to degeneration, regardless of the presence of PrP^{Sc} (Mallucci *et al.*, 2003). Thus, PrP^C is essential for prion propagation and pathogenesis.

Conversion of PrP^C to PrP^{Sc} is believed to involve direct interaction of the two PrP isoforms. Although the molecular mechanism of conversion is not yet fully understood, it is known that mature PrP^C expressed on the cell surface is a substrate for PrP^{Sc} formation, and a process that involves a conformational transformation takes place in subcellular compartments associated with the degradation pathway of PrP^C, including a sphingolipid-rich membrane microdomain, called a lipid raft (Caughey & Raymond, 1991; Naslavsky *et al.*, 1997; Vey *et al.*, 1996).

Because of the emergence of variant CJD and iatrogenic CJD by dura matter transplantation, especially in Japan, the establishment of therapeutics for prion disease is urgently needed. Therapeutics have been directed at the binding of the two PrP isoforms, as well as the process of conformational transformation, since the conversion of PrP^C to PrP^{Sc} is associated with neuronal pathogenicity. To date, many substances have been reported to inhibit PrP^{Sc} formation in cell culture and/or cell-free systems, including amyloid-binding dyes (Caughey & Race, 1992), sulfated glycosaminoglycans (Caughey & Raymond, 1993), tetrapyrrole compounds (Caughey *et al.*, 1998), cysteine protease inhibitors (Doh-Ura *et al.*, 2000), substituted tricyclic derivatives such as chlorpromazine and quinacrine (Doh-Ura *et al.*, 2000; Korth *et al.*, 2001), branched polyamines (Supattapone *et al.*, 1999, 2001), peptides (Chabry *et al.*, 1998; Soto *et al.*, 2000) and conversion-incompetent PrP (Holscher *et al.*, 1998; Horiuchi *et al.*, 2000; Kaneko *et al.*, 1997). Some of these have already been examined *in vivo*. For instance, sulfated glycosaminoglycans and tetrapyrrole compounds were effective when administered at early stages of infection or simultaneously with the scrapie-affected brain inoculum (Ehlers & Diringer, 1984; Ladogana

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et al., 1992; Priola *et al.*, 2000). Polyene antibiotics prolonged the incubation period, even when administered at the middle-late stage of infection (Demaimay *et al.*, 1997), but the effects appeared to depend on the prion strains and host animals studied (Demaimay *et al.*, 1999; Xi *et al.*, 1992). Recently, Doh-Ura and colleagues (2004) showed that intraventricular administration of pentosan polysulfate and quinine prolonged the incubation periods in a prion-infected transgenic mouse model, even at a late stage of infection (Doh-Ura *et al.*, 2004; Murakami-Kubo *et al.*, 2004). Further *in vivo* studies are expected to lead to the establishment of effective therapeutics for prion diseases. However, to achieve more efficient therapeutics, it is essential to elucidate the mechanisms of action and to investigate proper delivery of drugs based on pharmacokinetics.

Anti-PrP antibodies have also been reported to inhibit the formation of PrP^{Sc} in cultured cells and/or cell-free systems (Enari *et al.*, 2001; Horiuchi & Caughey, 1999; Kaneko *et al.*, 1995; Peretz *et al.*, 2001). Transgenic mice expressing an anti-PrP mAb on B cells (Heppner *et al.*, 2001), immunization with recombinant PrP (Sigurdsson *et al.*, 2002) and passive immunization with an anti-PrP mAb (White *et al.*, 2003) antagonized the peripheral inoculation of scrapie-affected brain inoculum. These *in vivo* experiments suggested the possible use of anti-PrP antibodies as a therapy for prion diseases. However, it remains unclear how anti-PrP antibodies can antagonize PrP^{Sc} formation in cells. To address this point, in the current study, we evaluated a panel of anti-PrP mAbs against diverse epitopes for inhibition of PrP^{Sc} formation. We found that a mAb recognizing the octapeptide repeat sequence, a region that is not essential for PrP^{Sc} formation, reduced PrP^{Sc} accumulation in cells persistently infected with prions. Furthermore, our data suggest a possible link between cell-surface retention of PrP^{C} by anti-PrP antibodies and inhibition of PrP^{Sc} formation in cells.

METHODS

Antibodies and chemicals. The properties of anti-PrP mAbs used in this study have been described elsewhere (Kim *et al.*, 2004). The mAb against sarcomeric actin (clone alpha-Sr-1) was purchased from DAKO. Stock solutions of chlorpromazine, dextran sulfate 500 (DS500) and polyethyleneimine were prepared in deionized water, while E-64d was dissolved in DMSO and quinacrine in methanol. Culture medium containing each chemical compound or mAb was prepared freshly for each experiment.

Cell culture. The mouse neuroblastoma cell line Neuro2a (CCL-131; ATCC) was cultured in Dulbecco's modified Eagle's medium (ICN Biomedicals) with 10 % fetal bovine serum (FBS) and non-essential amino acids. Mouse neuroblastoma cells persistently infected with prions, originally established by Race *et al.* (1987), were cloned by limiting dilution. Subclone I3/I5-9, which possessed a high level of PrP^{Sc}, was used in this study. I3/I5-9 cells were maintained in Opti-MEM (Invitrogen) containing 10 % FBS and cells passaged fewer than 20 times were used for experiments.

Treatment of cells persistently infected with prions and sample preparation. Almost-confluent I3/I5-9 cells in 25 cm^2 flasks were split 1 : 20 into 35 mm tissue culture dishes. On day 2, the medium was replaced with 3 ml Opti-MEM containing 4 % FBS and each test compound or mAb, and the cells were cultured for a further 3 days. For PrP^{C} detection, the cells were washed with PBS and lysed with 300 µl lysis buffer A (1 % Zwittergent 3-14, 150 mM NaCl, 50 mM Tris/HCl, pH 7·5) supplemented with protease inhibitors (2 mM EDTA, 1 µg pepstatin ml^{-1}, 2 µg leupeptin ml^{-1}, 2 µM bestatin and 1 µg aprotinin ml^{-1}). After the removal of cell debris by low-speed centrifugation, samples were centrifuged at 45 000 r.p.m. for 30 min at 4 °C using the TLA 100.3 rotor of a Beckman Optima TLX and the resulting supernatants were used as a source of PrP^{C}. For the detection of PrP^{Sc}, cells were lysed with 300 µl lysis buffer B (5 mM EDTA, 0·5 % Triton X-100, 0·5 % sodium deoxycholate, 150 mM NaCl, 10 mM Tris/HCl, pH 7·5) and kept on ice for 30 min. Cell debris was removed by centrifugation for 5 min at 1000 r.p.m. A portion of the sample (10 %) was removed for determination of protein concentration using the DC protein assay (Bio-Rad) and the remaining portions were treated with 20 µg proteinase K ml^{-1} for 20 min at 37 °C. Proteolysis was terminated by the addition of 1 mM Pefabloc (Roche). The samples were then treated with DNase I (100 µg ml^{-1}) and RNase A (5 µg ml^{-1}) for 15 min at room temperature and centrifuged at 70 000 r.p.m. for 2 h at 4 °C using the TLA 100.3 rotor of a Beckman Optima TLX. The resulting pellets were dissolved in SDS-PAGE sample buffer.

SDS-PAGE and immunoblotting. SDS-PAGE was carried out using NuPAGE 12 % Bis-tris gels and MOPS-SDS running buffer according to the manufacturer's instructions (Invitrogen). After SDS-PAGE, proteins were transferred on to Immobilon-P PVDF membranes (Millipore) using a Transblot Mini Cell wet-type blotting apparatus (Bio-Rad) and NuPAGE transfer buffer (Invitrogen) at 60 V for 2 h. Immunoreactive proteins were detected using X-ray film as described elsewhere (Kim *et al.*, 2004). For quantitative analysis, immunoreactive proteins were visualized using the Western-Star Protein detection kit (TROPIX) according to the supplier's instructions and processed with an LAS-1000 lumino image analyser (Fujifilm). The intensity of the bands was quantified using Science Lab 98 Image Gauge software (Fujifilm).

Flow cytometric analysis. Adherent cells were treated with ice-cold PBS containing 0·1 % collagenase (Wako) and dispersed by pipetting. Cells were washed with 0·5 % FBS in PBS (FBS/PBS) and incubated with anti-PrP mAbs diluted with 0·5 % FBS/PBS for 30 min on ice. Cells were washed three times with 0·5 % FBS/PBS and incubated with 1 : 2000-diluted Alexa 488-labelled Fab fragment of goat anti-mouse IgG (Molecular Probes) for 30 min. After washing, cells were stained with 5 µg propidium iodide ml^{-1} in 0·5 % FBS/PBS for 5 min and analysed using an EPICS XL-ADC flow cytometer (Beckman Coulter). All procedures were carefully carried out under chilled conditions.

Indirect immunofluorescence assay. Cells grown in eight-well slides (Nunc) were fixed with 100 % methanol for 20 min at −20 °C. Fixed cells were blocked with 5 % FBS/PBS for 30 min at room temperature, after which they were incubated with hybridoma supernatants or mAbs diluted in 1 % FBS/PBS for 30 min at room temperature. After washing with PBS, cells were incubated with 1 : 1000-diluted Alexa 488-labelled Fab fragment of goat anti-mouse IgG for 30 min. Finally, the slides were mounted with PBS containing 50 % glycerol and 1 % n-propyl gallate (Wako) and examined using a fluorescence microscope equipped with a cooled CCD unit (*Cool*SNAP HQ; Roper).

Cell growth and cytotoxicity. The effect of mAbs on cell growth was analysed using the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) assay (Ishiyama *et al.*, 1996) and cytotoxicity was analysed by lactate dehydrogenase (LDH) release assay using the LDH-Cytotoxic Test (Wako).

RESULTS

Anti-PrP mAbs inhibit PrP^{Sc} accumulation in cultured cells

Several antibodies recognizing regions in the C-terminal portion of PrP have been reported to inhibit PrP^{Sc} accumulation in neuroblastoma cells persistently infected with prions (Enari *et al.*, 2001; Peretz *et al.*, 2001). We recently established a panel of diverse anti-PrP mAbs including those recognizing the octapeptide repeat in the N-terminal region of PrP (Kim *et al.*, 2004). In the current studies, we investigated whether they would effect PrP^{Sc} accumulation in prion-infected neuroblastoma cells. Fig. 1(a) shows the effect of mAbs recognizing linear epitopes on PrP^{Sc} accumulation in I3/I5-9 cells persistently infected with prions. Following a 3-day treatment, only two mAbs reduced PrP^{Sc} accumulation: 31C6, which recognizes aa 143–149 of mouse PrP, and 110, which recognizes PHGGGWG at aa 59–65 and aa 83–89 in the octapeptide repeat. Quantitative analysis revealed that other mAbs did not affect the total amount of PrP^{Sc}, or the ratio of di-, mono- and non-glycosylated PrP^{Sc}.

Flow cytometric analysis showed that mAbs 110 and 31C6 bound PrP^{C} on the cell surface, although the fluorescence intensity of mAb 110 was weaker than that of mAb 31C6 (Fig. 1b, left panel). In contrast, mAbs that had no effect on PrP^{Sc} accumulation did not appear to bind to PrP^{C} on the cell surface (Fig. 1b, right panel). Two other mAbs, 44B1 and 72, which are thought to recognize discontinuous epitopes (Kim *et al.*, 2004), reacted with PrP^{C} on the cell surface (Fig. 1b) and inhibited PrP^{Sc} accumulation (Fig. 2). These results suggested that mAbs that can bind to PrP^{C} on the cell surface have the potential to antagonize PrP^{Sc} accumulation in cells persistently infected with prions.

Fig. 2 shows the dose-dependence of the effect of the anti-PrP mAbs. The four effective mAbs (110, 31C6, 44B1 and 72) reduced the amount of PrP^{Sc} in a dose-dependent manner, although PrP^{Sc} was not completely eliminated following the 3-day treatment. The 50 % effective dose (EC_{50}) of mAbs 110, 31C6, 44B1 and 72 was estimated to be 0·2 μg ml^{-1}(1·2 nM), 0·1 μg ml^{-1} (0·7 nM), 0·3 μg ml^{-1} (1·7 nM) and 0·6 μg ml^{-1} (4·1 nM), respectively (Fig. 2b).

Fig. 3 shows the long-term effect of mAbs on PrP^{Sc} formation. Treatment for 6 days with mAb 110, 44B1, 31C6 (Fig. 3) or 72 (data not shown) reduced PrP^{Sc} to an almost undetectable level, and no re-emergence of PrP^{Sc} was observed in the following 6 and 12 days of incubation in the absence of mAbs. On the contrary, mAbs that did not bind to cell-surface PrP^{C} showed little effect on PrP^{Sc} accumulation even after long-term treatment.

The influence of mAbs on cell growth and acute toxicity was examined by WST-1 assay and LDH release assay, respectively. No significant effect on cell growth was observed, even with long-term treatment (5 μg ml^{-1} for 6 days) and mAbs did not demonstrate any acute toxicity (10 μg ml^{-1}) following 2 h of treatment.

Effect of anti-PrP mAbs on total amount of PrP^{C}

Fig. 1(a, lower panel) shows total PrP^{C} in the I3/I5-9 cells treated with mAbs for 3 days. The intensities of PrP^{C} bands were normalized with α-sarcomeric actin on the same blot and PrP^{C} levels relative to cells treated with negative control mAb (P1-284) are indicated at the bottom. Although there was a certain degree of variation, no marked difference was observed in the total amount of PrP^{C}. In contrast, after long-term treatment (6 days), the total amount of PrP^{C} in I3/I5-9 cells treated with mAb 110 or 44B1 appeared to be higher than that with the negative-control mAb or other anti-PrP mAbs (Fig. 3, top right panel). To confirm this further, we repeated the same experiment at least three times for the four inhibitory mAbs, 110, 31C6, 44B1 and 72. Relative PrP^{C} levels in cells treated with these four mAbs were 168 ± 38, 88 ± 23, 183 ± 54 and 103 ± 33 %, respectively. These results suggested that the effect of mAbs on PrP^{C} level varied depending on the mAb: mAbs 110 and 44B1 increased total PrP^{C} levels following long-term treatment, while mAbs 31C6 and 72 did not affect the total PrP^{C} level.

Cell-surface localization of the mAb–PrP^{C} complex

The N-terminal portion of PrP, including the octapeptide repeat, is not essential for PrP^{Sc} formation and/or prion propagation (Flechsig *et al.*, 2000; Rogers *et al.*, 1993). The finding that not only the mAbs recognizing the C-terminal part of PrP, such as 31C6 and 44B1, but also mAb 110 inhibited PrP^{Sc} accumulation in the neuroblastoma cells, together with the fact that only the mAbs that bound to cell-surface PrP^{C} showed an inhibitory effect, implied that the mAb–PrP^{C} interaction on the cell surface is essential for inhibition of PrP^{Sc} accumulation. To investigate this further, we analysed the dynamics of anti-PrP mAbs after their binding to the cell surface (Fig. 4). Neuro2a cells were treated with 10 μg mAb 31C6 ml^{-1} for 1 h, after which the cells were cultured for an additional 4 h without mAb. Cells were then harvested and stained with an Alexa 488-conjugated secondary antibody. As a control, cells cultured with mAb 31C6 for 1 h were immediately stained with the secondary antibody. Flow cytometric analysis showed no difference in fluorescence intensity between the two preparations, suggesting that the mAb–PrP^{C} complex remained on the cell surface, even after the additional 4 h culture in the absence of mAb. As I3/I5-9 cells are established by repeated limiting dilution, Neuro2a cells may not be a suitable uninfected control for I3/I5-9 cells. Hence, we carried out the same experiment using I3/I5-9 cells. It is known that elimination of PrP^{Sc} parallels the reduction of prion infectivity. Considering biosafety issues, we used I3/I5-9 cells cured of PrP^{Sc} by long-term treatment with mAb 44B1 for flow cytometric analysis. mAb 31C6 (Fig. 4) and

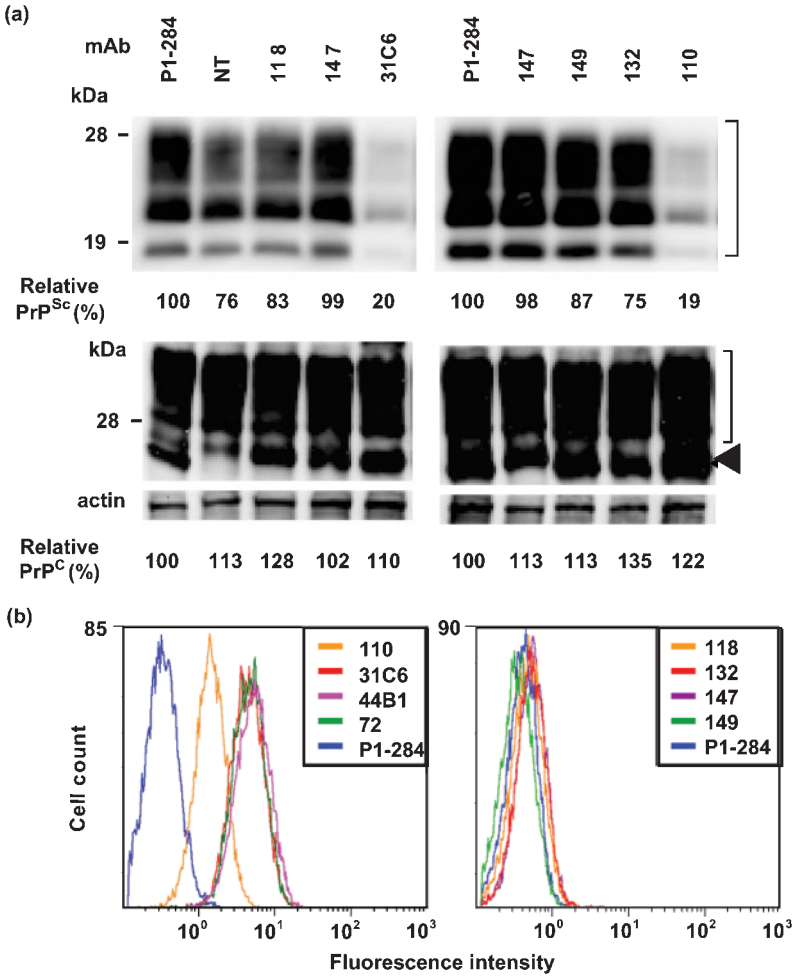

Fig. 1. Inhibition of PrP^{Sc} accumulation in prion-infected I3/I5-9 cells by anti-PrP mAbs. (a) Detection of PrP^{Sc} (upper panels) and PrP^{C} (lower panels). I3/I5-9 cells were cultured for 3 days with 4 % FBS in Opti-MEM containing 5 μg mAbs ml^{-1}. The level of PrP^{Sc} in the cells was determined by immunoblot analysis using mAb 44B1. Antibodies added to the culture are indicated above the panels. mAb P1-284 against feline panleukopenia virus was used as a control for non-specific effects. For detection of PrP^{Sc}, the load volume of each sample was adjusted based on the protein concentration of the corresponding cell lysate that had not been treated with proteinase K. For quantitative analysis of PrP^{Sc}, the three PrP^{Sc} bands indicated by a square bracket (right-hand side, upper panels) were grouped together. To check the ratios of the three PrP^{Sc} bands, each was selected separately. For PrP^{C}, the PrP^{C} bands indicated by a square bracket (right-hand side, lower panels) were quantified. The bands indicated by the arrowhead were excluded from the quantitative analysis, as they overlapped with immunoglobulin light chains that were detected by secondary antibodies. The blot used for PrP^{C} detection was also probed with anti-sarcomeric actin mAb for normalization. The levels of PrP^{Sc} and PrP^{C} relative to cells treated with negative-control mAb (P1-284) are indicated below the panels. NT, cells cultured without mAbs. Molecular mass markers are shown in kDa on the left. Epitopes for mAbs were as follows: 110, aa 56–89; 132, aa 119–127; 118, aa 137–143; 31C6, aa 143–149; 149, aa 147–151; 147, aa 219–229 (Kim *et al.*, 2004). (b) Binding of mAbs to the surface of Neuro2a cells examined by flow cytometry. The left panel shows mAbs that bound to the cell surface, while the right panel shows mAbs that did not bind. mAb P1-284 was used as a control for non-specific binding.

the three other inhibitory mAbs, 110, 44B1 and 72 (data not shown), showed the same retention of mAb–PrP^{C} complexes as observed with Neuro2a cells.

To confirm further the retention of mAb–PrP^{C} complexes on the cell surface, Neuro2a and I3/I5-9 cells were cultured for 1 h with mAbs 110, 31C6, 44B1 and 72, and, in some cases, the cells were cultured for an additional 4 h with mAb-free medium. The cells were then fixed with ice-cold methanol and mAb–PrP^{C} complexes were detected using secondary antibody (Fig. 5). All mAbs bound to the cell surface (Fig. 5a–e) and membrane staining could be detected, even after 4 h incubation in the absence of mAbs (Fig. 5f–j). To characterize further the retention of

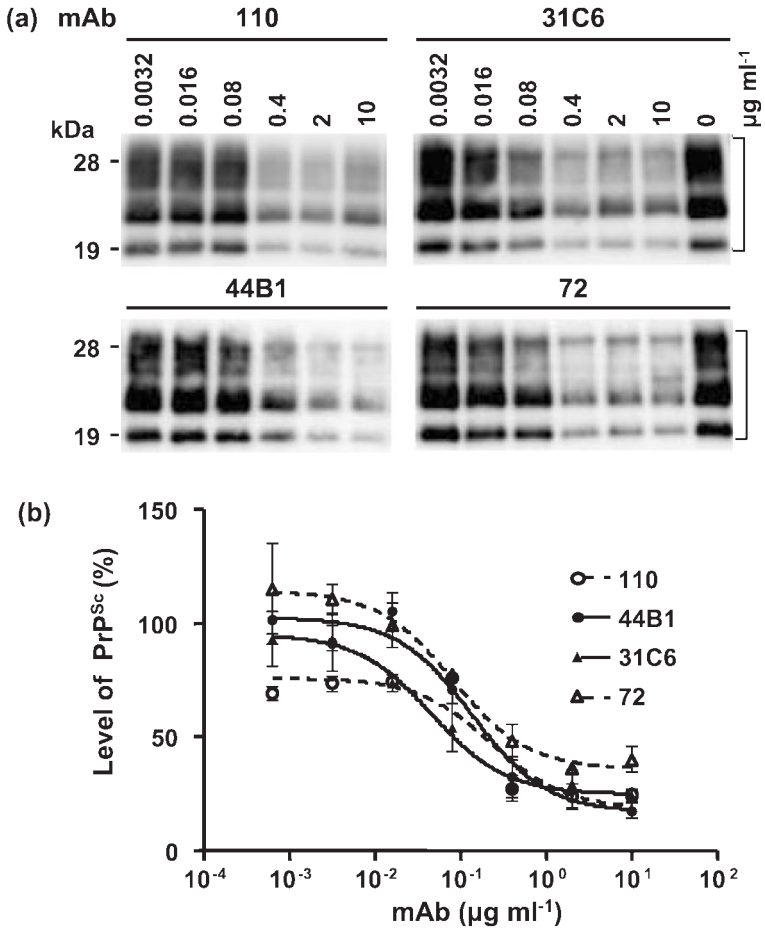

Fig. 2. Dose-dependent inhibition of PrP^{Sc} accumulation by anti-PrP mAbs. (a) Representative results from immunoblotting. I3/I5-9 cells were cultured for 3 days with various concentrations of mAb as indicated above the panels. The level of PrP^{Sc} was determined by immunoblot analysis using mAb 44B1. (b) Dose–response curve. The intensity of the PrP^{Sc} bands in the blots was quantified using an LAS-1000 lumino image analyser. The PrP^{Sc} level in the absence of mAbs was assigned a value of 100 % in each experiment. The graph shows means ± SD from at least three independent experiments. EC_{50} values were estimated using GraphPad PRISM (GraphPad Software).

mAb–PrP^C complexes on the cell surface, we examined the effect of DS500, which is reported to accelerate PrP^C endocytosis (Shyng *et al.*, 1995). Following treatment with DS500, the mAb–PrP^C complexes on the cell surface were internalized and detected as intracellular granules (Fig. 5k–o). These results demonstrated that the mAbs bound to the cell-surface PrP^C remained there, regardless of their epitope specificity.

Effect of other compounds on PrP^C expression

Our results indicated a possible link between cell-surface retention of PrP^C by anti-PrP antibodies and the inhibition of PrP^{Sc} formation in cells, and suggested that the mAb treatment altered the total amount of PrP^C at least for mAbs 110 and 44B1. In order to examine whether compounds that inhibit PrP^{Sc} accumulation in prion-infected cells affect PrP^C level in the cells, we tested DS500, E-64d, quinacrine, chlorpromazine and polyethyleneimine. We confirmed that these compounds inhibited PrP^{Sc} accumulation in I3/I5-9 cells (data not shown). Using the concentrations at which these compounds caused >90 % inhibition, we examined their effects on cellular levels of PrP^C following a 3-day treatment (Fig. 6a). Immunoblot analysis revealed that only DS500 reduced the PrP^C level (to ~30 % that of untreated cells) among the compounds tested. Flow cytometric analysis with mAb 110 (Fig. 6b) confirmed that DS500 reduced the level of cell-surface PrP^C.

Since sulfated glycosaminoglycans like DS500 may bind to the N-terminal region of PrP^C (Pan *et al.*, 2002), the reduction in fluorescence intensity may be due to blocking of mAb 110 binding. For this reason, we used mAbs 31C6 and 44B1 to detect PrP^C instead of mAb 110. Table 1 shows the mean relative amount of PrP^C on the cell surface calculated from at least three independent experiments. Regardless of the mAb used for detection, DS500 reduced the PrP^C level to ~50 % of the untreated control. No significant change in cell-surface expression of PrP^C was observed with the other compounds tested.

DISCUSSION

Anti-PrP antibodies that react with the C-terminal portion of PrP inhibit PrP^{Sc} formation in cultured cells (Enari *et al.*, 2001; Peretz *et al.*, 2001). One explanation for the inhibitory effect of these antibodies is that the binding of mAb to the corresponding epitope on PrP^C directly inhibits PrP^C–PrP^{Sc} interaction by occupying their binding domains. Fab D18, the most effective mAb reported by Peretz *et al.* (2001), reacts with the region spanning aa 132–156 in mouse PrP. In this study, we examined three mAbs

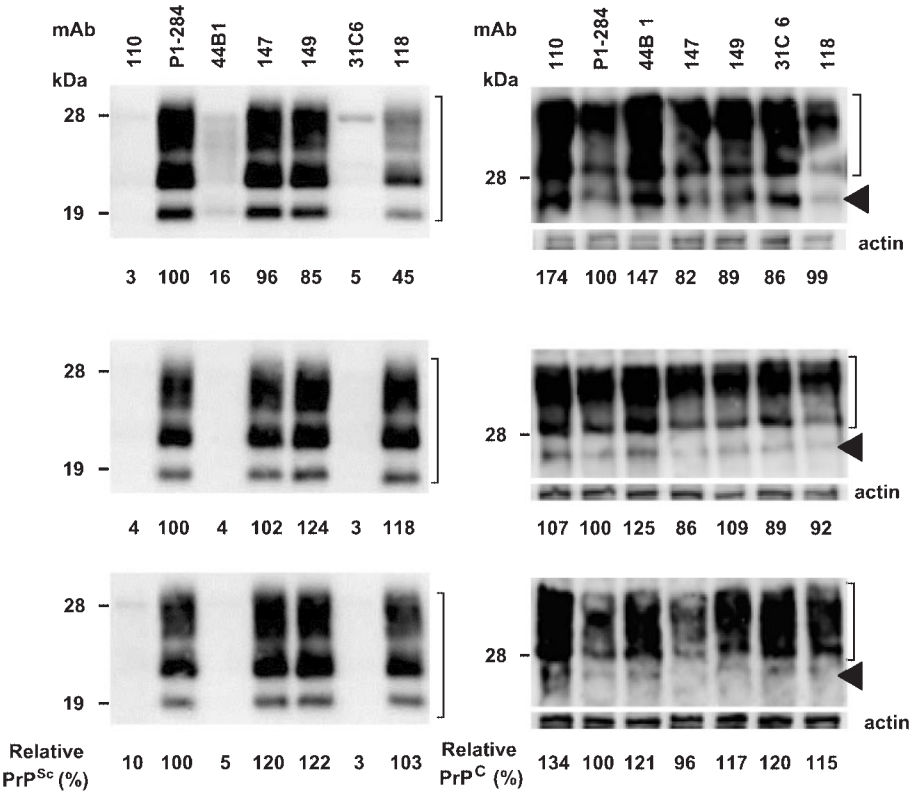

Fig. 3. Clearance of PrP^{Sc} by long-term antibody treatment. I3/I5-9 cells were cultured for 6 days with 5 μg mAb ml^{-1} (top panels). After withdrawal of the mAb, cells were cultured for an additional 6 (middle panels) or 12 (bottom panels) days in the absence of mAb. Quantitative analysis was carried out as described in the legend to Fig. 1 and relative PrP^{Sc} (left panels) and PrP^{C} (right panels) levels are indicated below the corresponding images.

recognizing epitopes within this region, but only mAb 31C6, which recognizes aa 143–149, displayed inhibitory activity. The remaining mAbs, 118 and 149, which bind adjacent epitopes aa 137–143 and aa 147–151, respectively, did not inhibit PrP^{Sc} formation in the cells. The main difference among these three mAbs was their ability to bind mature PrP^{C}; only mAb 31C6 bound PrP^{C} on the cell surface. Although it is well known that the N-terminal portion of PrP, including the octapeptide repeat, is not essential for prion propagation and/or PrP^{Sc} formation (Flechsig *et al.*, 2000; Rogers *et al.*, 1993), mAb 110, which recognizes the sequence in the octapeptide repeat, also antagonized PrP^{Sc} formation. This implied that there are mechanisms of inhibition other than blocking of the specific epitopes. Indeed, four of eight anti-PrP mAbs recognizing different epitopes inhibited PrP^{Sc} formation, suggesting that a common feature of the inhibitory mAbs is their ability to bind PrP^{C} on the cell surface. Taken together, our results suggest that inhibition of PrP^{Sc} formation by mAbs depends on their binding to mature PrP^{C} on the

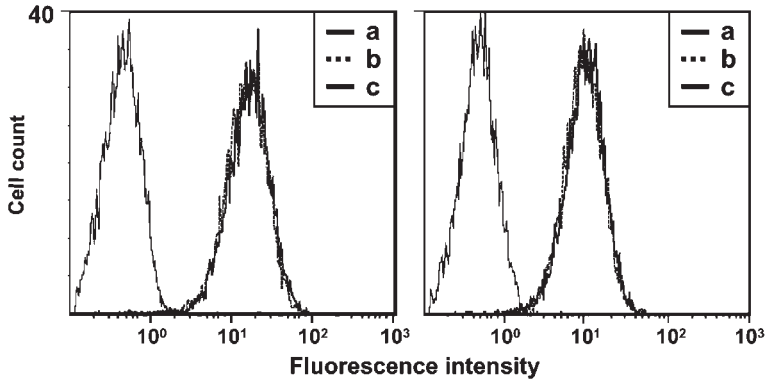

Fig. 4. Retention of mAb–PrP^{C} complexes on the cell surface. Neuro2a (left panel) or I3/I5-9 cells cured of PrP^{Sc} by mAb treatment (right panel) were cultured for 1 h in the presence of 10 μg negative control mAb P1-284 (a) or mAb 31C6 (b, c) ml^{-1}. Cells were harvested immediately and stained with Alexa-488-conjugated secondary antibody (a, b). Alternatively, after the removal of mAb, the cells were cultured for an additional 4 h in the absence of mAb and then harvested and stained with the secondary antibody (c).

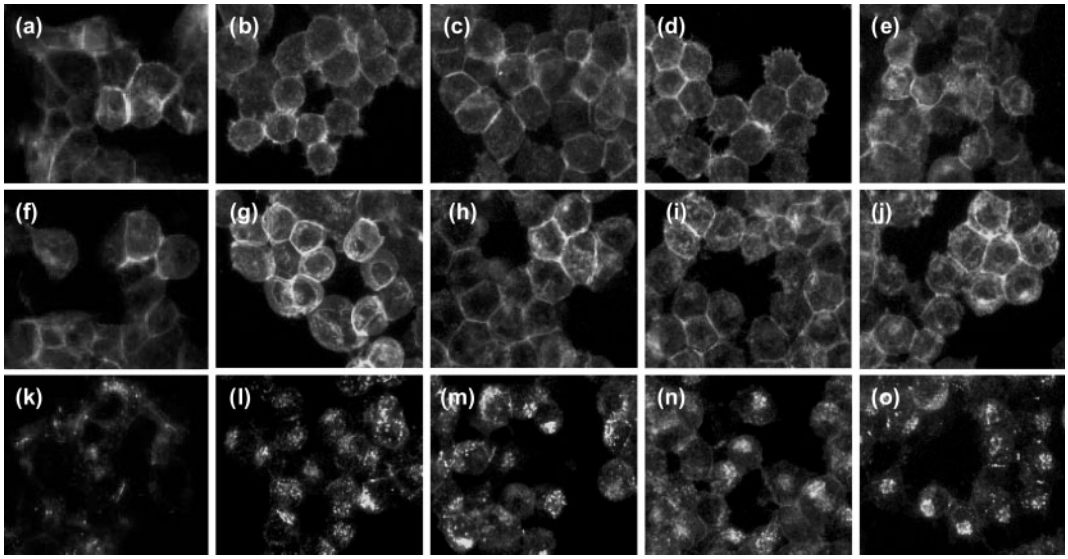

Fig. 5. Internalization of mAb–PrP^C complexes by treatment with DS500. Neuro2a cells (a, f and k) and I3/I5-9 cells (b–e, g–j, l–o) were cultured for 1 h with mAb 110 (b, g and l), 31C6 (a, f and k for Neuro2a cells; c, h and m for I3/I5-9 cells), 44B1 (d, i and n) or 72 (e, j and o). After removal of the mAb, cells were washed with ice-cold PBS and fixed with ice-cold methanol (a–e). Alternatively, after removal of mAb, cells were cultured with mAb-free medium for 4 h and fixed with ice-cold methanol (f–j). For DS500 treatment (k–o), after removal of mAb, cells were cultured for 3 h in mAb-free medium and then treated for 1 h with 25 μg DS500 ml^{-1}, after which they were fixed with ice-cold methanol. The fixed cells were directly stained with Alexa-488-conjugated secondary antibody to detect bound anti-PrP mAb.

cell surface rather than their binding to specific epitopes. On the other hand, transient interaction between the flexible N-terminal region and the second α-helix in the C-terminal globular domain has been postulated (Zahn *et al.*, 2000), and antibody binding to the N terminus of PrP prevents binding of C terminus-specific mAb (Li *et al.*, 2000). Hence, it cannot be excluded that binding of mAb 110 to the octapeptide repeat might sterically influence a particular domain involved in binding to PrP^{Sc}.

Although the cell-surface binding of mAb 110 was lower than that of the other mAbs (Fig. 1b, left panel), it inhibited PrP^{Sc} formation as efficiently. This may be explained by the presence of an 18 kDa N-terminally truncated PrP^C. This truncated PrP^C fragment is produced by cleavage of PrP^C around residue 112 during the recycling process (Chen *et al.*, 1995) so that it is not recognized by mAb 110. Recently, Mishra *et al.* (2002) reported that the N-terminally truncated form comprised as much as 40–50 % of PrP^C on the cell surface. This could account for the lower signals obtained using mAb 110. Because N-terminally truncated PrP^C is unlikely to act as a substrate for prion propagation and/or PrP^{Sc} formation (Lawson *et al.*, 2001; Weissmann, 1999), the binding of mAb 110 to PrP^C possessing the N-terminal portion is apparently sufficient for the inhibition of PrP^{Sc} formation.

In this work, we have demonstrated both quantitatively and qualitatively that mAbs that bind to cell-surface PrP^C remain attached to the membrane, even after withdrawal of the mAbs from the culture medium. This suggests that the mAb–PrP^C complex on the cell surface is not preferentially internalized into the cell. Mature PrP^C expressed on the cell surface is thought to be internalized via either clathrin-coated or -uncoated vesicles from which it enters the degradation pathway (Peters *et al.*, 2003; Shyng *et al.*, 1994; Sunyach *et al.*, 2003). Because PrP^{Sc} formation is believed to take place in the subcellular compartments that include cell membrane during the degradation pathway (Borchelt *et al.*, 1992; Caughey & Raymond, 1991), it is possible that mAb treatment could interfere with the regular PrP^C metabolism simply by retaining it on the cell surface. We suspected that the cell-surface retention of PrP^C would result in an increase in total PrP^C. Actually, two mAbs, 110 and 44B1, obviously increased the total amount of PrP^C, while two other mAbs 31C6 and 72 did not influence the total amount of PrP^C. It is conceivable that binding of mAbs to specific epitopes of cell-surface PrP^C might result in downregulation of PrP^C synthesis; however, further experiments are required to resolve this.

It was recently reported that polyclonal antibodies against dimeric recombinant PrP inhibited PrP^{Sc} formation in the cell, while the corresponding Fab fragments had little effect on PrP^{Sc} formation (Gilch *et al.*, 2003). This suggests that antibody-mediated cross-linking of PrP^C on the cell surface is important for inhibition of PrP^{Sc} formation. Whether cross-linking of PrP^C by IgG is required for the retention of the mAb–PrP^C complex under our experimental conditions remains to be determined. Treatment of cells persistently

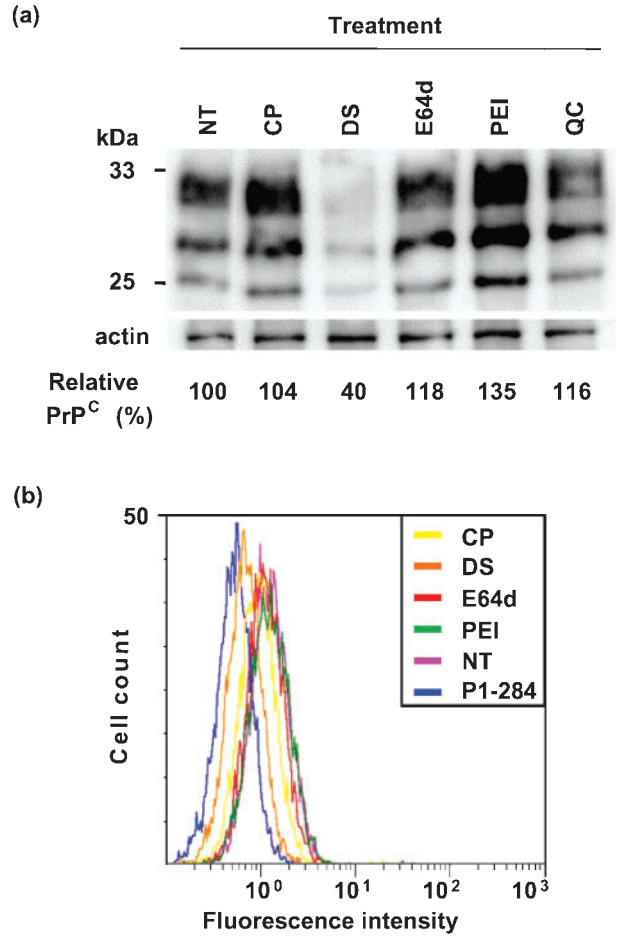

Fig. 6. Influence of chemical treatments on the expression of PrP^C. (a) Total amount of PrP^C. Neuro2a cells were treated for 12 h with various chemical compounds as indicated above the panel. Final concentrations were 3 μg chlorpromazine (CP) ml^{-1}, 25 μg DS500 (DS) ml^{-1}, 50 μM E-64d (E64d), 3 μg polyethyleneimine (PEI) ml^{-1} and 2 μM quinacrine (QC). Total PrP^C was detected in cell lysates by immunoblot analysis using mAb 31C6 (upper panel). The same blot was probed with anti-sarcomeric actin mAb to normalize for loading (lower panel). The intensity of the bands was quantified using an LAS-1000 lumino image analyser, and the relative amount of PrP^C compared with untreated control (NT) was calculated for each experiment. The data below the panel are means from three independent experiments. (b) Representative flow cytometric analysis of the cell-surface expression of PrP^C. Neuro2a cells were treated with compounds as described in (a), harvested, stained with mAb 110 followed by Alexa-488-conjugated secondary antibody and analysed by flow cytometry. The mean fluorescence intensity of the untreated control (NT) was assigned a value of 1 and the relative fluorescence intensities were calculated from the mean fluorescence intensity from each histogram. Quinarine was excluded from this experiment because of its autofluorescence. mAb P1-284 was used as a negative control for flow cytometric analysis.

Table 1. Effects of chemical treatment on cell-surface expression of PrP^C

Data represent means ± SD (minimum of $n=3$) of relative fluorescence intensity compared with control (NT).

Treatment	mAb for detection		
	110	31C6	44B1
NT	1·00	1·00	1·00
Chlorpromazine	0·77 ± 0·05	0·95 ± 0·03	0·92 ± 0·03
DS500	0·52 ± 0·03*	0·55 ± 0·10*	0·48 ± 0·04*
E-64d	0·99 ± 0·17	0·99 ± 0·06	0·97 ± 0·11
Polyethyleneimine	1·01 ± 0·16	1·04 ± 0·03	1·07 ± 0·11

*Statistically significant differences ($P<0·05$). The conditions of the treatments are described in the legend to Fig. 6.

infected with prions using antibodies against the laminin receptor precursor/laminin receptor (LRP/LR) reduced PrP^{Sc} accumulation (Leucht *et al.*, 2003). Because binding of LRP/LR to PrP^C could be involved in PrP metabolism (Gauczynski *et al.*, 2001), it is conceivable that antibodies interfere with the interaction between PrP^C and a molecule(s) that participates in PrP^C internalization.

Many reagents, including small molecules, recombinant PrP and anti-PrP antibodies, have been identified as potential inhibitors of prion propagation. It is important to elucidate their mechanisms of action, not only for the establishment of therapeutics but also for an understanding of prion replication. In the present study, we have demonstrated that blocking of the internalization of PrP^C with anti-PrP mAbs prevents PrP^{Sc} accumulation. Although anti-PrP mAbs recognizing specific epitopes have recently been reported to induce neuronal death in the hippocampus and cerebellum (Solforosi *et al.*, 2004), we have not found an apparent adverse effect on the cell growth and clinical manifestation by intraventricular inoculation of the anti-PrP mAbs used in this study (data not shown). Further analyses using prion-infected animals are necessary for evaluation of anti-PrP antibodies as therapeutics for treating prion diseases.

After the submission of this paper, a paper was published by Perrier *et al.* (2004) in which it was described that recognition by mAb SAF34 of the octapeptide repeat region on the N-terminal part of human PrP inhibited PrP^{Sc} formation in prion-infected neuroblastoma cells.

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