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# Generation of monoclonal antibody that distinguishes $PrP^{Sc}$ from $PrP^{C}$ and neutralizes prion infectivity

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ABSTRACT

To establish $PrP^{Sc}$-specific mAbs, we immunized *Prnp*$^{-/-}$ mice with $PrP^{Sc}$ purified from prion-infected mice. Using this approach, we obtained mAb 6H10, which reacted with $PrP^{Sc}$ treated with proteinase K, but not with $PrP^{Sc}$ pretreated with more than 3 M GdnHCl. In contrast, reactivity of pan-PrP mAbs increased with increasing concentrations of GdnHCl used for pretreatment of $PrP^{Sc}$. In histoblot analysis, mAb 6H10 showed a positive reaction on a non-denatured histoblot but reactivity was lower when the histoblot was pretreated by autoclaving. Epitope analysis suggested that the extreme C-terminus of PrP is likely to be part of the epitope for mAb 6H10. MAb 6H10 immunoprecipitated $PrP^{Sc}$ from brains of mice, sheep, and cattle infected with prions. Furthermore, pretreatment of purified $PrP^{Sc}$ with mAb 6H10 reduced the infectious titer more than 1 log. Taken together, these results suggest that mAb 6H10 recognizes a conformational epitope on $PrP^{Sc}$ that is related to prion infectivity.



## Introduction

Prion diseases are fatal neurodegenerative diseases which include scrapie in sheep and goats, bovine spongiform encephalopathy (BSE), and Creutzfeldt-Jakob diseases (CJD) in humans. A hallmark of the diseases is the accumulation of a pathogenic, abnormal isoform of prion protein, designated $PrP^{Sc}$, in the central nervous system of affected animals. $PrP^{Sc}$ is generated from the host-encoded, cellular prion protein, $PrP^{C}$, by certain post-translational modification including conformational transformation (Prusiner et al., 1998). Although the two isoforms are encoded by the host gene, *PrP*, they differ from each other biochemically and biophysically. For example, $PrP^{C}$ is soluble in non-ionic detergents and sensitive to protease treatment, whereas $PrP^{Sc}$ is insoluble due to its propensity to form aggregates that are partially resistant to protease treatment (Oesch et al., 1985; Meyer et al., 1986). $PrP^{C}$ has a high α-helix but low β-sheet content, whereas $PrP^{Sc}$ has a higher β-sheet content (Caughey et al., 1991; Pan et al., 1993; Safar et al., 1993). However, $PrP^{Sc}$ is comprised of PK-sensitive and PK-resistant $PrP^{Sc}$, such that $PrP^{Sc}$ cannot be distinguished from $PrP^{C}$ simply using protease treatment (Bessen and Marsh, 1994; Safar et al., 1998, 2005; Silveira et al., 2005).

To date, there have been a number of reports on the production of monoclonal and polyclonal antibodies against PrP molecules. Most of these are pan-PrP antibodies that recognize either linear or discontinuous epitopes on $PrP^{C}$ and react with $PrP^{Sc}$ pretreated with denaturant (Kascsak et al., 1987; Serban et al., 1990; Williamson et al., 1996; Peretz et al., 1997; Kim et al., 2004a). Because of the co-existence of $PrP^{C}$ and $PrP^{Sc}$, and the propensity of $PrP^{Sc}$ to form aggregates, removal of $PrP^{C}$ by protease treatment and subsequent denaturation are prerequisites for specific detection of $PrP^{Sc}$ by pan-PrP antibodies. However, the ability to analyze the properties of $PrP^{Sc}$ using pan-PrP antibodies is limited, as the biological and biochemical properties of $PrP^{Sc}$ are affected by protease treatment and denaturation.

Molecular probes that specifically react to $PrP^{Sc}$ and distinguish $PrP^{Sc}$ from $PrP^{C}$ can be a powerful tool for analysis of the entity of $PrP^{Sc}$. For instance, $PrP^{C}$ will be act as a molecular probe for $PrP^{Sc}$, as $PrP^{C}$ binds to $PrP^{Sc}$ (Horiuchi et al., 1999). A fusion protein comprised of $PrP^{C}$ and the immunoglobulin Fc region and a genetically modified antibody possessing $PrP^{C}$ segments have been demonstrated to bind to $PrP^{Sc}$ possibly via the $PrP^{C}$ segments (Meier et al., 2003; Moroncini et al., 2004). Moreover, plasminogen binds to $PrP^{Sc}$ not to $PrP^{C}$; however, plasminogen will bind to other serum proteins and thus, selectivity of the binding is obscure (Fischer et al., 2000). In contrast to many reports of pan-PrP mAbs, only a few studies have reported anti-$PrP^{Sc}$ antibodies that specifically discriminate $PrP^{Sc}$ from $PrP^{C}$ (Korth

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et al., 1997; Paramithiotis et al., 2003; Curin Serbec et al., 2004; Jones et al., 2009).

Availability of a panel of $PrP^{Sc}$-specific antibodies is indispensable for analysis of the biochemical properties of $PrP^{Sc}$. Thus, in order to obtain $PrP^{Sc}$-specific antibodies, we immunized PrP-ablated (*Prnp*$^{-/-}$) mice with $PrP^{Sc}$ purified from prion-infected mouse brains. Prion infectivity is thought to be associated with $PrP^{Sc}$ oligomers; thus, we used non-denatured, purified $PrP^{Sc}$ as the immunogen. One of the mAbs, clone 6H10, showed interesting reactivity to PrP molecules; mAb 6H10 reacted with non-denatured $PrP^{Sc}$ but not with recombinant mouse PrP (rMoPrP) or denatured $PrP^{Sc}$ in an enzyme-linked immunosorbent assay. This pattern of reactivity implied that mAb 6H10 recognizes $PrP^{Sc}$ but not $PrP^{C}$, allowing for detailed characterization of mAb 6H10.

## Results

### *Reactivity of mAb 6H10 to purified $PrP^{Sc}$*

To obtain $PrP^{Sc}$-specific mAbs, we immunized *Prnp*$^{-/-}$ mice with a purified $PrP^{Sc}$ fraction and hybridoma supernatants were screened with the purified $PrP^{Sc}$ and rMoPrP by enzyme-linked immunosorbent assay (ELISA). We established one mAb, 6H10 (isotype: IgG2b), which reacted with purified $PrP^{Sc}$ but not with rMoPrP or $PrP^{Sc}$ denatured with 6 M GdnHCl. This pattern of reactivity suggests that mAb 6H10 might specifically recognize the $PrP^{Sc}$ conformation and thus, we further analyzed mAb 6H10.

Figure 1 shows reactivity of mAb 6H10 and pan-PrP mAbs to purified $PrP^{Sc}$. The mAbs 31C6, 44B1, and 72, which were characterized as pan-PrP mAbs, reacted with purified $PrP^{Sc}$ before proteinase K (PK) treatment (0 μg/ml), but reactivity disappeared when $PrP^{Sc}$ was pretreated with 20 μg/ml or higher concentrations of PK (Fig. 1a). It is known that $PrP^{Sc}$ forms sedimentable aggregates, and $PrP^{C}$ or protease-sensitive PrP molecules, which are expected to expose the epitopes for pan-PrP mAbs, are usually co-purified with $PrP^{Sc}$ during purification. Thus, the drastic decline in reactivity of pan-PrP mAbs with increases in PK concentration is probably due to removal of protease-sensitive PrP species from the purified $PrP^{Sc}$ fraction. In contrast to the results of pan-PrP mAbs, mAb 6H10 reacted with $PrP^{Sc}$ treated with up to 320 μg/ml of PK (Fig. 1a).

Most of epitopes for pan-PrP mAbs are buried in aggregates of purified $PrP^{Sc}$, and become exposed as denaturation reveals cryptic epitopes (Kim et al., 2004a). In contrast, $PrP^{Sc}$-specific conformational epitope(s), if they exist, would be expected to be destroyed upon denaturation. Consistent with this, reactivity of mAb 6H10 gradually decreased and disappeared when PK-treated $PrP^{Sc}$ was treated with more than 3 M GdnHCl (Fig. 1b). This is different from pan-PrP mAbs, which did not react with PK-treated $PrP^{Sc}$ without denaturation (at 0 M GdnHCl) but reactivity became apparent and increased as the GdnHCl concentration is increased (Fig. 1b). Taken together, the results suggested that mAb 6H10 recognizes a conformational epitope on $PrP^{Sc}$. Consistent with this, mAb 6H10 did not react with $PrP^{Sc}$ in immunoblot analysis (Fig. 2a). To confirm reactivity of mAb 6H10 to $PrP^{Sc}$, we performed immunoprecipitation analysis. The mAb 6H10 precipitated $PrP^{Sc}$ into the bead-bound fraction from a suspension of a purified $PrP^{Sc}$ fraction, whereas most of $PrP^{Sc}$ remained in the unbound fraction in the case of mAb 31C6 or a negative control mAb (Fig. 2b). Following immunoprecipitation of $PrP^{Sc}$ by mAb 6H10, the amount of $PrP^{Sc}$ that remained in the corresponding supernatant was decreased.

### *Immunoprecipitation of $PrP^{Sc}$ from brain homogenates by mAb 6H10*

Next we performed immunoprecipitation from brain homogenates. The mAb 31C6 immunoprecipitated bands corresponding to $PrP^{C}$ from brain homogenates of uninfected mouse, whereas mAb 6H10 did not. PrP precipitated from brain homogenates of mice infected with the Obihiro or Chandler strain by mAb 31C6 disappeared after PK treatment. In contrast, 6–7 kDa smaller PrP bands were detected when fractions immunoprecipitated from brain

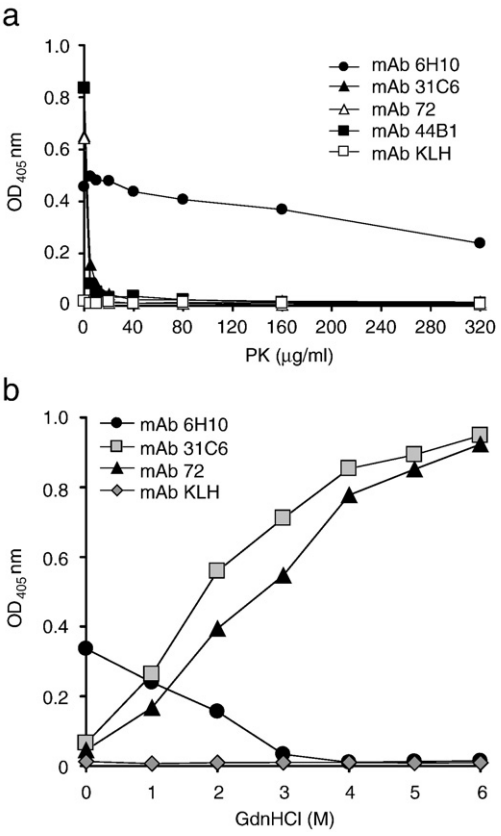


**Fig. 1.** Reactivity of mAb 6H10 to $PrP^{Sc}$ in ELISA. (a) Reactivity to PK-treated $PrP^{Sc}$. Wells were coated with 200 ng/well of purified, PK-untreated $PrP^{Sc}$ of the Obihiro strain. After adsorption, wells were treated with the indicated concentrations of PK. After terminating PK activity with Pefabloc, the wells were subjected to the antibody reaction. The anti-PrP mAbs used were 6H10, 31C6, 72, and 44B1. Anti-KLH mAb was used as a negative control mAb. (b) Effect of denaturation of $PrP^{Sc}$. Purified $PrP^{Sc}$ adsorbed to the wells was digested with 20 μg/ml PK and treated with GdnHCl (0–6 M) at r.t. for 1 h. Then wells were subjected to the antibody reaction.

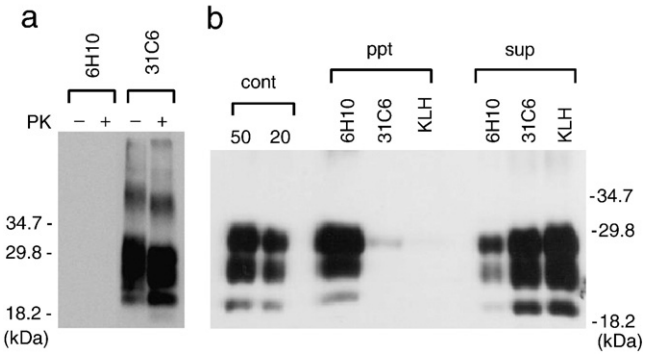


**Fig. 2.** Reactivity of mAb 6H10 to $PrP^{Sc}$ in Immunoblotting. (a) Reactivity of mAbs 6H10 and 31C6 on an immunoblot. PK-treated (+) and PK-untreated (-) brain homogenates of mice infected with the Obihiro strain were subjected to immunoblotting. (b) Immunoprecipitation. PK-digested purified $PrP^{Sc}$ of the Obihiro strain in PBS containing 1% Triton X-100 was incubated with antibodies as indicated at the top. The antigen–antibody complexes were collected with magnetic beads coated with protein G. $PrP^{Sc}$ in precipitates (ppt) and supernatants (sup) were detected by immunoblotting using HRP-conjugated mAb 31C6. Anti-KLH mAb (KLH) was used as a negative control mAb. PK-digested $PrP^{Sc}$ fractions at 50 and 20 μg brain equivalents were loaded as controls.

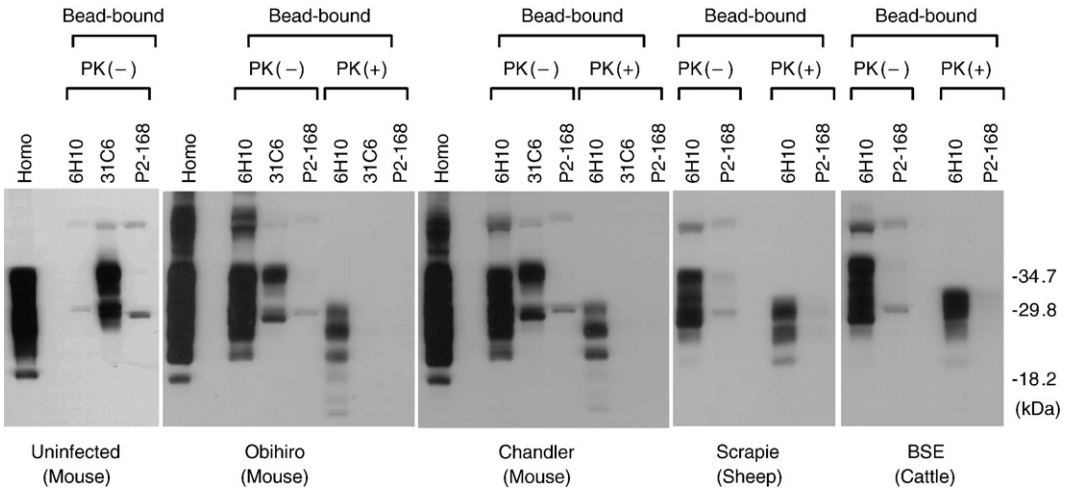


**Fig. 3.** Immunoprecipitation of $PrP^{Sc}$ by mAb 6H10. Brain homogenates (2.5%) of prion-infected or uninfected animals were incubated with 10 μg of mAbs (6H10, 31C6, P2-168) and then immunoprecipitated with protein G coupled magnetic beads. Bead-bound fractions were treated with PK [PK(+)] or not [PK(-)] and then subjected to immunoblotting. The blots were probed with HRP-conjugated mAb 31C6. MAb P2-168 was used as a negative control mAb. Homo, brain homogenates of uninfected mice or mice infected with prions.

homogenates of the Obihiro or Chandler strain-infected mice with mAb 6H10 were treated with PK (Fig. 3). Thus, at least, some fraction of the PrP immunoprecipitated with mAb 6H10 was resistant to PK treatment. These results suggest that mAb 6H10 selectively recognizes an epitope specific to $PrP^{Sc}$ and thus has a potential to distinguish $PrP^{Sc}$ from $PrP^{C}$. Moreover, mAb 6H10 exhibited broad host specificity; the mAb reacted with $PrP^{Sc}$ in the brains of scrapie-affected sheep and BSE-affected cattle (Fig. 3). These results suggest that, if not all, at least a certain population of $PrP^{Sc}$ in the brains of prion-infected animals possesses the epitope for mAb 6H10 on its surface.

Recently, Biasini et al. (2008) reported that mAb 15B3, which is purported to be specific to $PrP^{Sc}$ (Korth et al., 1997), also reacted with non-infectious PrP aggregates. Thus we analyzed reactivity of mAb 6H10 to PrP aggregates prepared from rMoPrP89-231 purified from *Escherichia coli* (Fig. 4). The mAb 31C6 immunoprecipitated both non-aggregated and aggregated rMoPrP89-231. In contrast, mAb 6H10 did not immunoprecipitate non-aggregated rMoPrP89-231, but showed weak reaction to aggregated rMoPrP89-231 at the same level as an isotype-matched negative control (mAb P2-168). Thus, the reaction of mAb 6H10 to aggregated rMoPrP89-231 appears to be a non-specific reaction that is possibly caused by a hydrophobic property of aggregated rMoPrP89-231. The same results were obtained when rMoPrP23-231 was used (data not shown). Considering that mAb P2-168 did not immunoprecipitate $PrP^{Sc}$ from brains of prion-infected animals, again, the reactivity of mAb 6H10 to $PrP^{Sc}$ in immunoprecipitation is thought to be a specific reaction between mAb 6H10 and the epitope on $PrP^{Sc}$.

### *Reactivity of mAb 6H10 to prion-infected and uninfected brain in histoblot*

To further analyze the reactivity of mAb 6H10 to $PrP^{Sc}$, we performed histoblot analysis (Fig. 5a). Two pan-PrP anitbodies, one of which is antiserum of $Prnp^{-/-}$ mice immunized with rMoPrP (NIAH), and the other is mAb 110, showed positive reaction to histoblot of prion-infected and uninfected mouse brains. However, the positive signals nearly disappeared when the blots were pretreated with PK, indicating that the signals represented protease-sensitive PrP. When the PK-treated histoblots were further processed by autoclaving, which partially denatures $PrP^{Sc}$ and thus exposes cryptic epitopes, these antibodies reacted only with prion-infected mouse brains. In contrast, mAb 6H10 reacted intensely to a histoblot of prion-infected mouse brain pretreated with PK and the intensity was drastically reduced by autoclaving of the histoblot. These results indicated that $PrP^{Sc}$ accumulated in the brain possessed the epitope for mAb 6H10. Unexpectedly, mAb 6H10 showed weak reaction to PK-untreated, uninfected mouse brain sample, although the reaction was not observed on a PK-treated histoblot. To examine whether mAb 6H10 reacts not only with $PrP^{Sc}$ but also $PrP^{C}$ in a histoblot, we used brains of wild-type ($Prnp^{+/+}$) and $Prnp^{-/-}$ mice. The mAb 44B1, classified as pan-PrP mAb, reacted only with the brain of $Prnp^{+/+}$ mice as expected. However, mAb 6H10 reacted weakly with both $Prnp^{+/+}$ and $Prnp^{-/-}$ mice brains (Fig. 5b). Thus, the weak reaction of mAb 6H10 to the brains of uninfected mice may be the result of the presence of a host antigen(s) other than $PrP^{C}$ that shares an epitope similar to that for mAb 6H10.

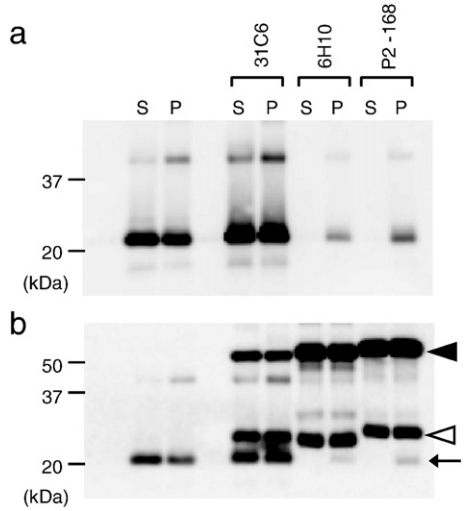


**Fig. 4.** Reactivity of mAb 6H10 to non-infectious PrP aggregates. (a) Immunoprecipitation of non-aggregated rMoPrP89-231 (S) and aggregated rMoPrP89-231 (P) with mAbs 31C6, 6H10, and P2-168. The mAb P2-168 is an isotype-matched negative control for mAb 6H10. Samples were prepared as described in Materials and Methods, and 10 μl of bead-bound fraction was loaded in each lane. The blot was probed with HRP-conjugated mAb 44B1. (b) After chemiluminescence reaction, the same blot in (a) was washed with PBST and re-probed with HRP-conjugated goat anti-mouse Igs (GE Healthcare) to ensure comparable amount of mAbs were used for each immunoprecipitation. Closed and open arrowhead indicate immunoglobulin heavy and light chain, respectively. Arrow indicates rMoPrP89-231 detected in the re-probing process.

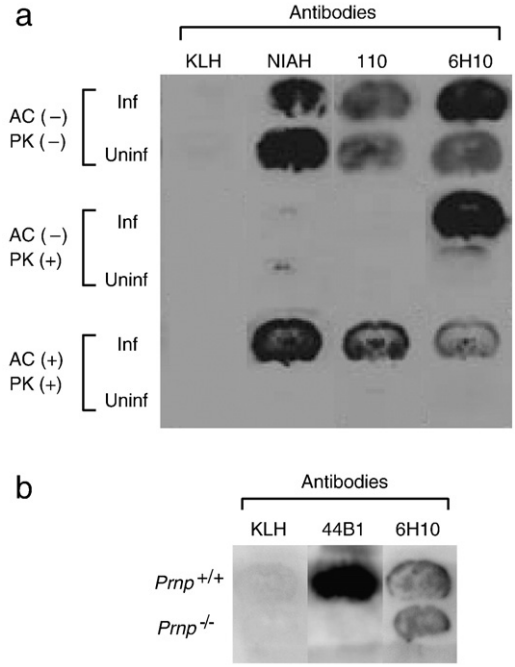


**Fig. 5.** Histoblot analysis. (a) Cryosections of Obihiro strain-infected (Inf) and uninfected mice brains (Uninf) were blotted onto PVDF membranes. The histoblots on the 1st row were neither treated with autoclave (AC-) nor PK (PK-). The blots on the 2nd row were treated with 50 μg/ml PK (PK+) but not with autoclave (AC-), and those on the 3rd row were treated with PK (PK+) and then autoclaved (AC+). Antibodies: KLH, negative control mAb; NIAH, serum of *Prnp*-/- mouse immunized with rMoPrP; 110, mAb 110; 6H10, mAb 6H10. (b) Histoblots of wild-type ($Prnp^{+/+}$) and $Prnp^{-/-}$ mouse brains were stained with antibodies as indicated.

### *Neutralization of prion infectivity by mAb 6H10*

Next, we examined whether mAb 6H10 can neutralize prion infectivity. Purified $PrP^{Sc}$ from mice infected with the Obihiro strain was pre-incubated with mAbs and then inoculated intracerebrally to mice for a bioassay (Table 1). Pre-incubation of $PrP^{Sc}$ with mAb 6H10 prolonged the mean incubation time by 19 days compared to $PrP^{Sc}$ pretreated with negative control mAbs anti-KLH ($p<0.01$), whereas pre-treatment with mAb 31C6 or 110 appeared to prolong the incubation time a little but the differences were not significant. A prolongation of 19 days was inferred as corresponding to a more than 95% reduction in prion infectivity based on the infectivity–incubation time standard curve obtained from bioassays of serially diluted brain homogenates from the Obihiro strain-infected mice (Table 1). Several pan-PrP mAbs have been reported to inhibit the accumulation of $PrP^{Sc}$ in prion persistently infected neuroblastoma cells when the cells were incubated with those mAbs (Enari et al., 2001; Peretz et al., 2001; Perrier et al., 2004; Kim et al., 2004b). Cells persistently infected with the Obihiro strain were unavailable so far, we used mouse neuroblastoma cells persistently infected with the Chandler strain (I3/I5-9 cells, Kim et al., 2004b) to examine whether mAb 6H10 inhibits $PrP^{Sc}$ formation in cells. Cells were cultured with mAb 6H10 (up to 20 μg/ml) for 4 days; however, mAb 6H10 did not affect the $PrP^{Sc}$ formation in I3/I5-9 (data not shown). Our previous study showed that anti-PrP mAbs strongly reacted with $PrP^{C}$ on the cell surface could inhibit $PrP^{Sc}$ formation in I3/I5-9 cells (Kim et al., 2004b). However, mAb 6H10 did not react with cell surface of I3/I5-9 cells by flowcytometirc analysis (data not shown).

**Table 1**
Neutralization of prion infectivity by mAb 6H10.

| mAb | Numbers of mice | | Incubation time to terminal stage [mean ± SD (days)] |
|---|---|---|---|
| | Inoculated | $PrP^{Sc}$ positive | |
| Anti-KLH | 5 | 5 | 161 ± 4 |
| mAb 31C6 | 5 | 5 | 166 ± 6 |
| mAb 110 | 5 | 5 | 169 ± 7 |
| mAb 6H10 | 5 | 5 | 180 ± 8[a] |
| *Brain homogenate*[b] | | | |
| 10% | 10 | 10 | 161 ± 6 |
| 1% | 12 | 12 | 164 ± 6 |
| 0.1% | 12 | 12 | 175 ± 6 |
| 0.01% | 11 | 11 | 189 ± 18 |
| 0.001% | 12 | 12 | 212 ± 12 |
| 0.0001% | 11 | 11 | 245 ± 31 |
| 0.00001% | 6 | 3 | 278 ± 52 |
| 0.000001% | 6 | 0 | >393 |

[a] $p<0.01$ by ANOVA followed by Dunnett's *post hoc* test.
[b] To obtain an infectivity–incubation time standard curve, 10-fold serial dilutions of brain homogenates of mice infected with the Obihiro strain were bioassayed. The 50% lethal dose ($LD_{50}$) of the original homogenates (10% brain homogenates) was estimated to be $10^6$ $LD_{50}$/20 μl by the Reed-Muench method. The standard curve for incubation periods ($x$) shorter than 190 days was fitted by the approximation of $LD_{50}=e^{41.48\,-\,0.183x}$. The standard curve for incubation periods ($x$) longer than 190 days was fitted by the approximation of $LD_{50}=e^{21.07\,-\,0.076x}$.

### *Epitope for mAb 6H10*

To investigate the epitope for mAb 6H10, we adopted phage display analysis. Fig. 5a shows consensus amino acid (aa) residues deduced from selected phage clones with mAbs 31C6 or 110, or rabbit polyclonal antibodies (pAb) B103. Three or four aa residues of the phage clones selected with mAbs 31C6 and 110 were identical to the aa residues in regions determined by pepspots analysis (Kim et al., 2004a). Similarly, three or four aa residues of selected phage clones with pAb B103 were identical to the synthetic peptide used as the immunogen. These results suggest that at least three residues are required to form an epitope for antibody. In total, 56 phage clones selected after five rounds of panning with mAb 6H10, and these were subjected to DNA sequencing so that the aa sequences could be deduced. The consensus aa residues of two abundant phage clones are listed in Fig. 6a. The most abundant phage clone ph#121 contained four residues identical to the C-terminus of MoPrP (SPSQAWLYMRHE, underlined residues). The second most abundant phage clone ph#125 also had three residues identical to the C-terminus of MoPrP (TQNWSMSMLLKQ, underlined residues). In contrast, no aa sequence identity to MoPrP was observed in the third most abundant phage clone ph#98 (IPLTGKYLDEQS, 6/56, 11%). Binding of antibodies to selected phage clones was confirmed by captured ELISA (Fig. 6b). The mAb 6H10 reacted with phage clones ph#121 and ph#125, originally selected by mAb 6H10, but not with ph#6, originally selected by mAb 31C6. On the other hand, mAb 31C6 reacted only with phage clone ph#6. Taken together, these data demonstrate the specificity of phage clone selection. Moreover, the results suggest that the C-terminus of MoPrP is involved in the formation of the epitope for mAb 6H10. We also analyzed the epitope for mAb 6H10 using a pepspots membrane; however, mAb 6H10 did not react with any spots (data not shown).

## Discussion

Immunization of $Prnp^{-/-}$ mice and screening of hybridomas with non-denatured $PrP^{Sc}$ is one of the ways to establish $PrP^{Sc}$-specific mAbs. Using this approach, we obtained mAb 6H10, which reacted with non-denatured $PrP^{Sc}$ but not with denatured $PrP^{Sc}$. It is well known that epitopes for pan-PrP antibodies on $PrP^{Sc}$ become accessible by antibodies after dissociation of $PrP^{Sc}$ aggregates and/or denaturation of $PrP^{Sc}$ (Kascsak et al., 1987; Serban et al., 1990; Williamson et al., 1996). However, the reactivity of mAb 6H10 to $PrP^{Sc}$ decreased with the increases in GdnHCl concentration for the pretreatment of $PrP^{Sc}$ to 3 M, indicating that the properties of mAb

a

***Antibody: mAb 31C6***

| | |
|---|---|
| aa sequence of epitope | 143-DWEDRYYRE-231 |
| Deduced aa sequences | **D**xxx**R**xxx**E** (6/11)<br>**DW**xx**R** (2/11) |
| Possible epitope | **DW**ED**R**YYR**E** |

***Antibody: mAb 110***

| | |
|---|---|
| aa sequence of epitope | 83-PHGGGWG-89 |
| Deduced aa sequences | **P**x**G**x**GW** (6/10)<br>**P**x**G**xx**W** (1/10) |
| Possible epitope | **P**HG**G**G**W**G |

***Antibody: pAb B103***

| | |
|---|---|
| aa sequence of epitope | 90-QGGTHGQWNKPSKPKTNMK-109 |
| Deduced aa sequences | **NK**xxx**P** (7/11)<br>H**NK**xxx**P** (2/11) |
| Possible epitope | QGGT**H**GQW**NK**PSK**P**KTNMK |

***Antibody: mAb 6H10***

| | |
|---|---|
| aa sequence of epitope | NA |
| Deduced aa sequences | **SQA**xxxx**R** (24/56)<br>**TQ**xxxx**S** (13/56) |
| Possible epitope | 213-CV**TQ**YQKE**SQA**YYDG**R**RSS-231 |

b

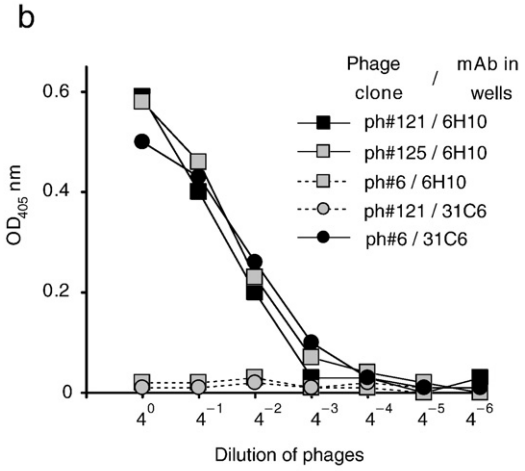


**Fig. 6.** Epitope analysis by peptide phage display. (a) Identity of aa sequences between selected phage clones and the region to which antibodies are expected to bind. The aa sequence of the epitopes of mAbs 110 and 31C6 were determined by pepspots analysis (Kim et al., 2004a), whereas that of pAb B103 corresponds the synthetic peptide used for immunization (Horiuchi et al., 1995). The deduced aa sequences indicate that consensus aa residues of selected phage clones. Shown are two abundant consensus sequences deduced from selected phage clones, which were obtained after three rounds (for pAb B103, and mAbs 110 and 31C6) or five rounds (for mAb 6H10) of panning. Amino acid residues identical to the expected region are indicated in bold. Numbers in parentheses indicate the total number of phage clones analyzed for each antibody (denominators) and the numbers of phage clones that possess the corresponding consensus aa sequence (numerators). The residues that are expected to constitute the epitope for each antibody are underlined (possible epitope). (b) Binding of antibodies to selected phage clones. Wells coated with mAb 6H10 or 31C6 were incubated with four-fold serial dilutions of the selected phage clones (ph#121, ph#125, or ph#6). Phage–antibody complexes were detected as described in Materials and Methods. Phage clones ph#121 and ph#125, selected by mAb 6H10, express the peptides SPSQAWLYMRHE and TQNWSMSMLLKQ, respectively. Phage ph#6, selected by mAb 31C6, expresses peptide S*DW*HT*R*FHYSMN (underlining indicates the consensus aa residues for the corresponding epitope).

6H10 differ from those of pan-PrP mAbs. The infectivity of the Obihiro strain, which was used as a source of $PrP^{Sc}$ in this study, was dramatically reduced by the treatment with 3 M GdnHCl (Shindoh et al., 2009), consistent with a decrease in the reactivity of mAb 6H10 to $PrP^{Sc}$ of the Obihiro strain pretreated with 3 M GdnHCl. Furthermore, mAb 6H10 neutralized infectivity of $PrP^{Sc}$ purified from mice infected with the Obihiro strain (Table 1). Thus, mAb 6H10 may recognize a conformational epitope on non-denatured $PrP^{Sc}$ that is related to the oligomerization interface on the $PrP^{Sc}$ molecule. Reduction of the hydrophobic surface on $PrP^{Sc}$ correlated with the dissociation and/or denaturation of $PrP^{Sc}$ by GdnHCl at concentrations between 0 and 3 M (Safar et al., 1994). Circular dichroism analysis also revealed that GdnHCl dissociated $PrP^{Sc}$ aggregates with a midpoint of transition around 2 M (Safar et al., 1993). The half-maximal GdnHCl concentrations, which are required to denature 50% of $PrP^{Sc}$, have been reported to range from 1.5 to 3 M for most of mouse-adapted scrapie and BSE prions (Legname et al., 2006; Shindoh et al., 2009). In addition, treatment of samples with 2–4 M GdnHCl resulted in decrease of prion infectivity (Caughey et al., 1997; McKenzie et al., 1998). Although these reports used different prion strains, the decrease in reactivity of mAb 6H10 to $PrP^{Sc}$ with an increase in GdnHCl concentration for pretreatment of $PrP^{Sc}$ concurs with structural alteration of $PrP^{Sc}$ or the decrease of infectivity induced by GdnHCl at similar concentrations.

The results of phage display analysis suggested the possibility that the extreme C-terminus of PrP molecule is part of the epitope for mAb 6H10. Among the amino acid residues expected to compose the epitope for mAb 6H10 (aa215-TQxxxxSQAxxxxR-aa228 of MoPrP), the underlined residues are identical to those of sheep and bovine PrP. This may be consistent with the fact that mAb 6H10 could immunoprecipitate $PrP^{Sc}$ from sheep and cattle samples. The mAb 6H10 showed no reaction with denatured $PrP^{Sc}$ by immunoblotting or ELISA (Figs. 1 and 2), or with the corresponding C-terminal peptide by pepspots analysis (data not shown). In contrast, reactivity of mAbs that recognize linear epitopes on the extreme C-terminus increased after pretreatment of $PrP^{Sc}$ with denaturants (Peretz et al., 1997; Kim et al., 2004a). Thus it is unlikely that mAb 6H10 binds linear epitope composed of the C-terminal residues. One mAb, V5B2, which was raised against a synthetic peptide corresponding to the C-terminal residues of human PrP (aa214-CITQYERESQAYY-aa226), is reported to discriminate $PrP^{Sc}$ in brains of CJD patients from $PrP^{C}$ in non-CJD brains possibly by recognizing the oligomerized C-terminal region in $PrP^{Sc}$ oligomers (Curin Serbec et al., 2004; Ulrih et al., 2006). Therefore, the C-terminal regions of two PrP molecules may cooperate to form a conformational epitope; however, the exact properties of the epitope for 6H10 are not yet fully elucidated. Alternatively, in the proposed model of $PrP^{Sc}$ protofibrils, the C-terminal region is thought to be located in close proximity to other regions on the same or different PrP molecule (DeMarco and Daggett, 2004; Govaerts et al., 2004). Cross-linking of recombinant PrP oligomers revealed that an interaction between the N- and C-terminal regions (Kaimann et al., 2008). Thus, the C-terminal region and other regions on the same or different PrP molecule may participate in constituting the epitope for mAb 6H10. The mAb 15B3, which was produced by immunizing recombinant bovine PrP and is purported to be specific to $PrP^{Sc}$, reacted with three PrP segments, residues 141–147, 161–269, and 213–215 of MoPrP in pepspots analysis (Korth et al., 1997). Involvement of the extreme C-terminus in the epitope for mAb 15B3 is intriguing; however, mAb 6H10 did not react with any PrP peptides in pepspots analysis (data not shown). Thus, the epitope for mAb 6H10 seems to differ from that for mAb 15B3. Recently, mAb 15B3 is reported to react with infectious and non-infectious PrP

aggregates (Biasini et al., 2008), therefore, we analyzed the reactivity of mAb 6H10 to aggregated rMoPrP (Fig. 4). Although a weak non-specific reaction to aggregated rMoPrP was observed, mAb 6H10 did not show any specific reaction to aggregated rMoPrP, suggesting mAb 6H10 is more specific to $PrP^{Sc}$ generated in brains of prion-infected animals.

Some mAbs against either PrP or non-PrP molecules have been reported to immunoprecipitate $PrP^{Sc}$ via an epitope-independent reaction when mAbs were bound to a solid phase such as magnetic beads, as binding of immunoglobulins on the limited area of the solid surface increases the concentration of immunoglobulins in that area and thus a low-affinity interaction between $PrP^{Sc}$ and immunoglobulins on the beads may occur (Morel et al., 2004). We considered that this possibility is unlikely in the case of mAb 6H10 for several reasons. First, bead-free mAb 6H10 reacted with $PrP^{Sc}$ in ELISA and histoblot analysis (Figs. 1 and 5). In addition, a direct interaction of fluorescent dye-labeled mAb 6H10 with non-denatured $PrP^{Sc}$ in brain homogenates of mice infected with prions can be detected in solution by fluorescent correlation spectroscopy (K. S. and M.H., in preparation). Secondly, a Fab' fragment of mAb 6H10 still reacted with native purified $PrP^{Sc}$ in ELISA (data not shown). However, a weak non-specific reaction to aggregated rMoPrP would be expected depending on experimental conditions (Fig. 4), careful experimental design, such as the use of negative control mAb and comparison between prion-infected and mock-infected materials, will be required to ensure specific reactivity of mAb 6H10 to $PrP^{Sc}$.

The reactivity of mAb 6H10 to $PrP^{Sc}$ in biochemical analysis strongly suggests that mAb 6H10 discriminates $PrP^{Sc}$ from $PrP^{C}$. However, in histoblot analysis, the mAb showed weak reaction to certain host molecule in *Prnp*$^{-/-}$ mice. This raises two possibilities. First, the mAb may recognize a host molecule other than PrP with an epitope similar to that for mAb 6H10 on $PrP^{Sc}$. The presence of third abundant phage clone (ph#98) that selected by mAb 6H10, aa sequence of which showed no apparent homology to PrP, may clue the identification of such host molecule. However, no protein or peptide domain was found by homology search using protein blast and tblastn or domain search using Conserved Domain Database. Secondly, the mAb may recognize a host factor tightly associated with $PrP^{Sc}$ in the prion-infected mouse brain, rather than reacting with $PrP^{Sc}$ itself. The latter possibility suggests that the positive reaction to PK-treated $PrP^{Sc}$ in ELISA was due to a reaction to a PK-resistant molecule tightly associated with $PrP^{Sc}$, which seems unlikely because the $PrP^{Sc}$ fraction used in this study was estimated to be nearly 90% pure after PK treatment (Kim et al., 2004a). However, this possibility cannot be completely ruled out at present, particularly as several macromolecules, including glycosaminoglycans (Snow et al., 1989), ubiquitin (Lowe et al., 1990), apolipoprotein E (Namba et al., 1991) have been shown to co-localize with $PrP^{Sc}$ in brains of prion-infected animals and human. In addition, nucleic acids (Aiken et al., 1990; Sklaviadis et al., 1993) and sphingolipid (Klein et al., 1998) have been co-purified with $PrP^{Sc}$.

In this study, we showed the possibility that the C-terminal region forms a $PrP^{Sc}$-specific epitope. Recently, immunization of β-form recombinant human PrP could induce production of mAbs that react with the N-terminal region of $PrP^{Sc}$ (aa91–110; Khalili-Shirazi et al., 2007). The immunization of aggregated synthetic peptide corresponding to aa106–126 of PrP also generated a mAb that discriminates $PrP^{Sc}$ from $PrP^{C}$ (Jones et al., 2009). These results suggest that $PrP^{Sc}$-specific epitopes are present at various regions on $PrP^{Sc}$. A fusion protein of PrP and Fc-region of immunoglobulin and PrP-peptide-grafted antibodies have been reported to recognize $PrP^{Sc}$ (Meier et al., 2003; Moroncini et al., 2004, 2006; Solforosi et al., 2007; Lau et al., 2007). In addition, there are nucleotide aptamers that possess higher affinity to $PrP^{Sc}$ than to $PrP^{C}$ (Rhie et al., 2003), and small chemicals such as 9-aminoacridine, streptomycin, luminescent conjugated polymers, polyionic polymers Seprion (Microsens bio-technologies) and others, may also act as $PrP^{Sc}$-specific probes (Moussa et al., 2006; Phuan et al., 2007; Sigurdson et al., 2007). Despite technical difficulties in manipulating $PrP^{Sc}$ due to its aggregation-prone propensity and heterogeneity, $PrP^{Sc}$-specific molecular probes are gradually accumulating. As the availability of a panel of anti-PrP mAbs has greatly contributed to characterization of the biochemical properties of PrP, a panel of $PrP^{Sc}$-specific molecular probes appears to be indispensable tools for analyzing the biochemical and biological properties of native $PrP^{Sc}$ with which prion infectivity is believed to be associated.

## Materials and methods

### *Purification of $PrP^{Sc}$ and production of mAbs*

The purification $PrP^{Sc}$ from brains of mice infected with prion Obihiro strain and purity of $PrP^{Sc}$ were reported elsewhere (Kim et al., 2004a). Immunization of $PrP^{Sc}$ and production of mAbs were carried out as described (Kim et al., 2004a).

### *Antibodies*

The following mAbs against mouse PrP molecules were used: 31C6 (IgG1, epitope: aa 143–149), 44B1 (IgG 2a, epitope: aa 155–231), 72 (IgG1, epitope: aa 89–231), and 110 (IgG2b, epitope: aa 59–89) (Kim et al., 2004a). Anti-keyhole limpet hemocyanin (KLH) mAb (IgG2a) and anti-parvovirus mAb P2-168 (IgG2b) (Horiuchi et al., 1997) were used as negative controls. Rabbit antiserum raised against bovine PrP synthetic peptide 103-121 (pAb B103) was also used (Horiuchi et al., 1995). Purification of mAbs was carried out as described elsewhere (Kim et al., 2004a). Conjugation of mAbs with horseradish peroxidase (HRP) was carried out as follows: purified mAbs were digested with pepsin and reduced by 2-mercapthoethanolamine (MEA) to generate Fab' fragments. After removal of MEA with a PD-10 size exclusion column (GE Healthcare), the Fab' fragments were mixed with HRP coupled with the bi-directional cross-linker GMBS (Dojin).

### *ELISA*

Ninety-six well plates (MaxiSorp, Nunc) were coated overnight at 4 °C with either 200 ng purified $PrP^{Sc}$ or 100 ng rMoPrP in 50 μl of 20 mM phosphate buffer (pH 7.0). After adsorption, wells were blocked with 5% fetal bovine serum (FBS) in PBS containing 0.1% Tween 20 (PBST) for 2 h at room temperature (r.t.), and incubated with antibodies diluted with 1% FBS in PBST for 1 h. After washing with PBST, wells were incubated with HRP-conjugated secondary antibodies for 1 h. The antigen–antibody complexes were visualized with 2,2′-azino-bis(3-etht-benzthiazoline-6-sulfonic acid), 0.04% $H_2O_2$ in 50 mM citrate–phosphate buffer, pH 4.0, and the absorbance at 405 nm was measured with a microplate reader. In some cases, $PrP^{Sc}$ adsorbed to the well was digested with various concentrations of proteinase K (PK) in Tris-buffered saline (TBS; 50 mM Tris–HCl [pH 8.0] and 150 mM NaCl) at 37 °C for 45 min. After terminating PK activity by adding Pefabloc (Roche Diagnostic) to a final concentration at 2 mM, the plates were subjected to the immune reaction.

### *Immunoblotting*

SDS-PAGE and immunoblotting of proteins on an Immobilon-P Transfer Membrane (Millipore) were carried out as described elsewhere (Kim et al., 2004a, 2004b). Membranes were blocked with 5% skim milk in PBST for 1 h and then incubated with HRP-conjugated Fab fragment of anti-PrP mAbs (direct staining) or anti-PrP mAb followed by incubation with HRP-conjugated secondary antibodies (indirect staining). ECL Western blotting detection reagents (GE Healthcare) and X-ray film were used for visualization.

*Immunoprecipitation*

Protein G-coupled magnetic beads (Dynabeads) were blocked with blocking buffer containing 5% skim milk and 50% Sea Block (Pierce) in PBS. Brain homogenates (2.5%) from prion-infected or uninfected animals were prepared with PBS containing 0.5% I-Block (Applied Biosystem), and were incubated with 10 μg of mAb and protein G magnetic beads for 45 min at 37 °C. The magnetic beads were washed four times with PBS containing 2% Triton X-100 using a magnetic separator. After washing, the beads were suspended with 50 μl of TBS and divided into two tubes. An equal volume of 2 sample buffer (8 M urea, 10% SDS, 8% *b*-mercaptoethanol, 125 mM Tris–HCl [pH 6.8], 6 mM EDTA, 10% glycerol, and 0.04% bromophenol blue) was added to one tube to make samples without PK treatment. The other was treated with 40 μg/ml of PK for 30 min at 37 °C. The reaction with PK was stopped with 2 mM Pefabloc before adding 2 sample buffer.

To examine the reactivity of mAbs to non-infectious PrP aggregates, rMoPrP23-231 or rMoPrP89-231 (Kim et al., 2004a) were diluted to 20 μg/ml with 1 ml of 1% Triton-X 100 in PBS (PBS-Triton, pH 7.2) and kept for 30 min at 20 °C. Then samples were centrifuged at 100,000×*g* for 45 min at 20 °C. The supernatant was recovered and used as non-aggregated rMoPrP, while the resulting precipitate was resuspended with 1 ml of PBS-Triton by sonication and used as aggregated rMoPrP. Protein G-coupled magnetic beads (100 μl) were incubated with 20 μg of mAbs and then blocked with 5% skim milk and 5% N102 blocking reagent (NOF Corporation, Japan) in PBS-Triton for 1 h at r.t. After washing with PBS-Triton once, the beads were mixed with 300 μl of non-aggregated or aggregated rMoPrP for 45 min at r.t. The beads were washed four times with 1 ml of 2% Triton X-100 in PBS and finally washed with PBS. Proteins bound to the magnetic beads were eluted with 100 μl of 1× sample buffer.

*Histoblot analysis*

Histoblot analysis was carried out as described by Taraboulos et al. (1992). Immobilon-P Transfer Membranes were activated with methanol and then equilibrated with lysis buffer (0.5% Nonidet P-40, 0.5% sodium deoxycholate, 100 mM NaCl, 10 mM EDTA, 10 mM Tris–HCl [pH 7.8]). Mouse brain cryosections (8 μm) were prepared and placed on glass slides. The glass slides carrying the sections were immediately pressed onto membranes on layers of filter paper saturated with lysis buffer for 1 min. The membranes were thoroughly air-dried and stored at -80 °C until use. Before immunostaining, the membranes were rehydrated in PBST for 1 h at r.t. and then immunostaining was carried out as described above for immunoblotting.

*Neutralization of prion infectivity*

Fifteen micrograms of purified $PrP^{Sc}$ were incubated with 36 μg of mAb in 300 μl of PBS containing 0.1% Zwittergent 3–12 for 2 h at r.t. The mixture (20 μl) containing 1 μg $PrP^{Sc}$ was then inoculated intracerebrally into 4-week-old female slc:ICR mice. For dose–infectivity correlation, 20 μl each of serially diluted brain homogenates from mice infected with the Obihiro strain were inoculated intracerebrally to 4-week-old female slc:ICR mice.

*Peptide phage display*

The Ph.D.-12™ Phage Display Library Kit (New England Biolabs), a combinatorial library which expresses random 12-mer-peptides at the N-terminus of a minor coat protein of M13 phage, was used according to the supplier's instructions. Polystyrene Petri dishes (60×15 mm) were coated overnight at 4 °C with 1.5 ml of 100 μg/ml antibodies in 0.1 M $NaHCO_3$ (pH 8.6). After blocking with 0.5% FBS in PBS for 1 h, dishes were rinsed six times with PBST and then inoculated with $4\times10^{10}$ of the phage library. After 1 h incubation at r.t., the dishes were washed 10 times with PBS and then bound phage was eluted by addition of 1 ml 0.2 M glycine–HCl (pH 2.2) for 10 min. The eluate was immediately neutralized with 150 μl of 1 M Tris–HCl (pH 9.1). In total, 1 ml of the eluate was used for amplification of the selected phage pool and the amplified phage stock was subsequently used for the next panning.

After three to five rounds of panning, phage DNA encoding the selected random peptide sequences was amplified directly from individual plaques by PCR using primers PD12F (5′-TCAAGCTGTT-TAAGAAATTCACC-3′) and PD12R (5′-TAAAGTTTTGTCGTCTTTCCA-GAC-3′). The PCR products were purified by S-300 HR spin column (GE Healthcare) and used as templates for DNA sequencing. DNA sequences were determined with an automated DNA sequencer (ABI-373A, Applied Biosystems) and using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit.

Binding of antibodies to selected peptides was confirmed by a captured ELISA. Briefly, 96-well plates coated with antibodies (20 ng/well) were incubated with four-fold serial dilutions of the phage stock for 1 h at r.t. After washing with PBST, plates were incubated with HRP-conjugated anti-M13 antibody to detect the phage captured by the antibodies. Phage–antibody complexes were detected as described for ELISA.

*Pepspots analysis*

Pepspots analysis was carried out as described elsewhere (Kim et al., 2004a).

*Statistical analysis*

Statistical analysis was done with JMP software (SAS Institute Inc.).

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