Complement Protein C1q Recognizes a Conformationally Modified Form of the Prion Protein†

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ABSTRACT: Several studies have suggested the implication of the classical complement pathway in the early stages of prion disease pathogenesis. To explore this hypothesis, surface plasmon resonance spectroscopy was used to test the ability of human C1q to recognize mouse PrP immobilized on a sensor chip. In this configuration, C1q bound avidly to PrP, with a K_D of 5.4 nM ($k_{on} = 2.4 \times 10^5$ M^{-1} s^{-1}; $k_{off} = 1.3 \times 10^{-3}$ s^{-1}). The isolated C1q globular domain also bound to immobilized PrP, although with a higher K_D (238 nM), due to a decreased k_{on} (4.2×10^3 M^{-1} s^{-1}). Interaction was strongly enhanced by Cu^{2+} ions, with a 10-fold increase in overall binding in the presence of 10 μM $CuSO_4$, without significant modification of the kinetic parameters. In contrast, using the same technique, no interaction was detected between immobilized C1q and soluble PrP. Likewise, gel filtration and chemical cross-linking analyses yielded no evidence for an interaction between these proteins in solution. Comparative analysis of the antigenic reactivity of soluble and immobilized PrP was performed by ELISA and surface plasmon resonance spectroscopy, respectively, using anti-PrP monoclonal antibodies. This analysis provides evidence that immobilized PrP undergoes a major conformational change in the sequence stretch 141GND-WEDRYYRENMYRYPNQ159 located in its C-terminal globular domain. It is concluded that immobilized PrP undergoes structural modifications that possibly mimic the conformational changes occurring during conversion to the pathological isoform and that C1q represents a natural sensor of these changes. Pathological implications of this recognition property are discussed in light of recent reports.

Transmissible spongiform encephalopathy (TSE)[1] diseases are infectious neurodegenerative disorders characterized by the aggregation and accumulation in the central nervous system of a protein termed prion or PrP^{sc}, corresponding to an abnormal structural isoform of PrP^c, an endogenous glycoprotein mainly expressed at the surface of neurons and other cells (*1*). The C-terminal domain of PrP^c is attached to the membrane through a glycosylphosphatidylinositol anchor and has a globular structure (*2*). In contrast, its N-terminal moiety is flexible and comprises a repeated sequence of eight amino acid residues with the ability to bind various divalent metal ions including Cu, Ni, Zn, and Mn (*3*). Owing to its higher affinity for Cu^{2+} ions (*4*), PrP^c is thought to function as a carrier for this metal, which is present at a concentration of 10−20 μM at the synapse, a PrP^c-rich area (*5*).

The prevalent hypothesis in TSE pathogenesis is that PrP^{sc} is the infectious principle and propagates by imparting its abnormal conformation onto host-borne PrP^c. This protein-only hypothesis (*6*) appears fully consistent with the demonstration that PrP^c-deficient mice are alive and resistant to prion diseases (*7*, *8*) and recently gained strong support with the generation of infectious particles from a synthetic prion protein obtained in vitro (*9*). It cannot be excluded, however, that the generation and/or propagation of PrP^{sc} requires additional cellular or molecular cofactors, and many studies have been focusing on this issue (*10*). Complement is a major element of innate immunity, providing a first line of defense against infection by pathogens. Besides this protective role, there is increasing evidence that C1, the complex that triggers the classical pathway, has the ability to recognize abnormal self structures. Thus, C1q, the binding subunit of C1, recognizes apoptotic cells and thereby activates complement, a mechanism that is likely crucial for the maintenance of immune tolerance (*11*). Several studies also provide a strong indication that the classical pathway of complement is involved in the pathogenesis of Alzheimer's disease (*12*), a hypothesis that is sustained by the demonstration that β-amyloid fibrils activate complement in vitro through direct recognition by C1q (*13*, *14*).

With respect to prion diseases, it has been shown that proteins of the classical pathway are present in amyloid plaques characteristic of Creutzfeldt−Jakob disease (*15*, *16*) and that C1q is one of the 19 proteins exhibiting increased synthesis in scrapie-infected brain tissues (*17*, *18*). Indeed, two concordant studies (*19*, *20*) demonstrate that C1q-deficient mice and, to a lesser extent, C3-deficient or -depleted mice exhibit protection against intraperitoneal

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[1] Abbreviations: TSE, transmissible spongiform encephalopathy.

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injection of limiting doses of PrP^{sc}, providing strong support to an implication of complement in the pathogenesis of prion diseases. The latter data strongly suggest that PrP^{sc} activates complement through the classical pathway, hence inducing increased localization of PrP^{sc} in follicular dendritic cells, possibly through opsonization by C3 and/or C4, a process that would facilitate subsequent recognition by the corresponding complement receptors. The aim of the present study was to verify this hypothesis by investigating at the molecular level the ability of C1q to recognize PrP.

EXPERIMENTAL PROCEDURES

Reagents and Proteins. Rabbit IgG was purified from serum by sodium sulfate precipitation as described previously (*21*). Human PTX3 was a gift from Prof. A. Mantovani (Mario Negri Institute for Pharmacological Research, Milan).

Production of Recombinant Murine PrP. The cDNA sequence coding for residues 23−232 of murine PrP was cloned in a pET-17b expression vector (Novagen, Madison, WI) and expressed in the cytoplasm of *Escherichia coli* BL21 Codon Plus (Novagen). The recombinant protein was recovered from inclusion bodies solubilized in 5 M guanidinium hydrochloride, pH 7.0, purified using affinity chromatography on Ni−chelating Sepharose (Quiagen, Hilden Germany) and gel filtration chromatography on a Hi Prep 26/10 column (Amersham Pharmacia Biotech), and then concentrated using centricon on a Ultrafree Biomax 5K membrane (Millipore). Details of the expression and purification procedures will be described elsewhere (Vendrely, C., Valadié, H., Cardin, L., Bednarova, L., Pasdeloup, M., Cappadoro, J., and Jamin, M., unpublished experiments). The concentration of the purified protein was determined by UV spectroscopy using a molar extinction coefficient at 280 nm of 62 400, calculated from the amino acid content (*22*), and a predicted molecular mass of 23 061 Da.

Purification of Human C1q and Its Domains. C1q was purified from human plasma as described in ref *23*, and its concentration was determined spectrophotometrically using a value of *A* (1%, 1 cm) at 280 nm of 6.8 and a molecular mass of 459 300 Da (*23*). Its globular domain was obtained after digestion of the collagenous portion of C1q with collagenase and purified by ion exchange chromatography as described previously (*24*). The collagen-like fragment of C1q was prepared essentially as described in ref *25*, except that residual uncleaved C1q molecules were removed by an additional affinity chromatography step on Con A-Sepharose. The concentrations of the purified globular domain and collagen-like fragment were determined using values of *A* (1%, 1 cm) at 280 nm equal to 7.0 and 2.1 and molecular masses of 48 000 and 189 900 Da, respectively (*14*).

Anti-PrP Antibodies. The monoclonal antibodies used in this study recognize the sequence segments GGGWGQPHGGGWGQG (Saf 32, Saf 37, 4F2), SQWNKP (8G8), and GNDWEDRYYRENMNRYPNQ (Saf 60, Saf 61, Saf 69, Saf 70), corresponding to amino acid residues 77−91, 96−101, and 141−159 of murine PrP. Antibodies 4F2 and 8G8 were raised against human recombinant PrP (*26*). The ≪Saf≫ antibodies were raised against a preparation of scrapie-associated fibrils from infected hamster brain (*27*, *28*) and were provided by Dr. Jacques Grassi (CEA Saclay, France). The rabbit polyclonal antibody P45−66 was raised by immunization of rabbits with a synthetic peptide encompassing mouse PrP residues 45−66 (*29*) and was provided by Dr. Sylvain Lehmann (CNRS, Montpellier, France).

Surface Plasmon Resonance Spectroscopy. Interactions between mouse PrP and various ligands including human C1q, its globular domain and collagen-like fragment, and monoclonal anti-PrP antibodies were analyzed by surface plasmon resonance spectroscopy using a BIAcore X apparatus (BIAcore AB, Uppsala, Sweden). The running buffer for protein immobilization was 150 mM NaCl, 0.005% surfactant P20, 10 mM HEPES, pH 7.4 (HBS-P buffer, BIAcore AB). Recombinant murine PrP was diluted to 10 μg/mL in 20 mM Na acetate, pH 3.6, and immobilized on the carboxymethyl groups of a CM4 (F1) sensor chip (BIAcore AB) using the amine coupling chemistry (BIAcore AB amine coupling kit). The same methodology was applied to immobilize C1q (30 μg/mL in 10 mM Na acetate, pH 4.5) and bovine serum albumin (40 μg/mL in 10 mM Na formate, pH 3.0), the latter protein being used as a reference for nonspecific binding.

Binding of the soluble analytes was measured at 25 °C over 700−900 RU of immobilized PrP or 12 000 RU of immobilized C1q in the HBS-P buffer, containing in some cases 3.4 mM EDTA, as indicated. To study the effect of divalent metals on the interaction between the C1q or its globular domain and PrP, $CuSO_4$, $NiSO_4$, $ZnSO_4$, $MnSO_4$, or $MgSO_4$ were added individually at a concentration of 40 μM to the analyte sample prior injection into the flow cell. For experiments designed to perform antigenic mapping of immobilized PrP, increasing concentrations of anti-PrP monoclonal antibodies were injected at a flow rate of 50 μL/min. Association and dissociation curves were recorded for 4 or 5 min each. Regeneration of the surfaces was achieved by injection of at least 3 μL of 0.1 M NaOH, followed by 20 μL of 10 mM EGTA for those analyses performed in the presence of divalent metals. The specific binding signal shown was obtained by subtracting the background signal, routinely obtained by injection of the protein sample over immobilized bovine serum albumin. To determine the kinetic constants, the binding data were analyzed by global fitting of both association and dissociation phases for several concentrations simultaneously, using the BIAevaluation 3.1 software (BIAcore AB). The apparent equilibrium dissociation constants (K_D) were calculated from the ratio of the dissociation and association rate constants (k_{off}/k_{on}). Maximal binding capacities (R_{max}) were determined using the same model. The data shown correspond to a representative series of binding studies performed on the same sensor chip. In each case, similar results were reproduced from at least two independent experiments, using different sensor chips.

Chemical Cross-Linking. Recombinant murine PrP (1.4 μM) was incubated with increasing amounts of either C1q or its globular domain (PrP:C1q or PrP:C1q globular domain molar ratios ranging from 4:1 to 1:4) in the presence of 6 mM 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (BIAcore AB) and 15 mM *N*-hydroxy-succinimide (BIAcore AB) in 0.1 M NaCl, 20 μM CuS04, 0.1 M MES, pH 6.0, for 1 h at 25 °C. The reaction mixtures were analyzed by SDS−PAGE.

Size-Exclusion Chromatography. Analyses were performed on a 10 mm × 30 cm Superose 12 HR gel filtration column

(Amersham Pharmacia Biotech) attached to an FPLC system (Amersham Pharmacia Biotech). The column was loaded with 60 μL of the proteins (murine PrP, C1q, and its globular domain) alone or in mixtures, each at a concentration of 15 μM. Elution was carried out in the HBS-P buffer containing 20 μM CuS04 at a flow rate of 0.5 mL/min, and proteins were monitored by absorbance at 280 nm.

Sandwich ELISA. The wells of a microtiter plate (Polysorb, Nunc) were coated overnight at 4 °C with 100 μL of purified anti-PrP monoclonal antibodies diluted to 10 μg/mL in 0.2 M Na carbonate-bicarbonate buffer, pH 9.3, 0.15 M NaCl. The coated plates were washed with PBS (Dulbecco's phosphate-buffered saline, Gibco) containing 0.05% Tween 20 and incubated with 1% w/v bovine serum albumin in PBS for 2 h at room temperature to prevent nonspecific binding. Recombinant PrP (from 0.1 to 5 μg/mL) in PBS/Tween 20 was then added and allowed to incubate for 90 min at room temperature. After addition of the rabbit polyclonal anti-PrP antibody diluted 1:2000 in PBS/Tween 20 and incubation for 90 min at room temperature, bound antigen was measured using a peroxidase-conjugated anti-rabbit immunoglobulin (Sigma) diluted 1:40 000. Binding was measured by optical density at 450 nm using 3,3′-5,5′tetramethylbenzidine (Sigma) as a substrate. The reaction was stopped after a maximum of 10 min by sulfuric acid being added. To analyze the data, the change in the absorbance signal was plotted versus the concentration of anti-PrP antibody added to the wells. The apparent dissociation constant for each anti-PrP antibody was deduced from the corresponding saturation curve.

RESULTS

C1q Recognizes Immobilized Murine PrP through Its Globular Domain. The interaction between C1q and PrP was investigated using a model system, mainly chosen on the basis of safety considerations, consisting of human C1q purified from plasma and recombinant murine PrP23−232 expressed in *E. coli*. Although this system is heterologous, it should be stressed that murine PrP23−232 shares 91% amino acid sequence homology with its human counterpart. In addition, most of the acidic residues (11/14), which are potential targets for C1q, are conserved in the two species. Surface plasmon resonance spectroscopy was used initially to analyze the interaction between C1q and murine PrP, using PrP as the immobilized ligand and C1q as the soluble analyte (i.e., a model mimicking the in vivo configuration). As illustrated in Figure 1A, which shows the association and dissociation curves for a representative series of analyses performed with five different C1q concentrations, C1q readily bound to immobilized PrP in a dose-dependent manner. Complete elution of the bound protein could be achieved in all cases by a pulse injection of 0.1 M NaOH. Analysis of the association and dissociation curves (see Experimental Procedures) yielded k_{on} and k_{off} values of $2.4 \pm 1.1 \times 10^5$ M^{-1} s^{-1} and $1.3 \pm 0.4 \times 10^{-3}$ s^{-1}, respectively, and a resulting K_D value of 5.4 ± 1.5 nM, indicative of high affinity.

To identify the region of the C1q molecule involved in PrP recognition, additional binding experiments were carried out using the isolated C1q globular domain as the soluble analyte. Again, this domain was found to interact with immobilized PrP in a dose-dependent fashion (Figure 1C).

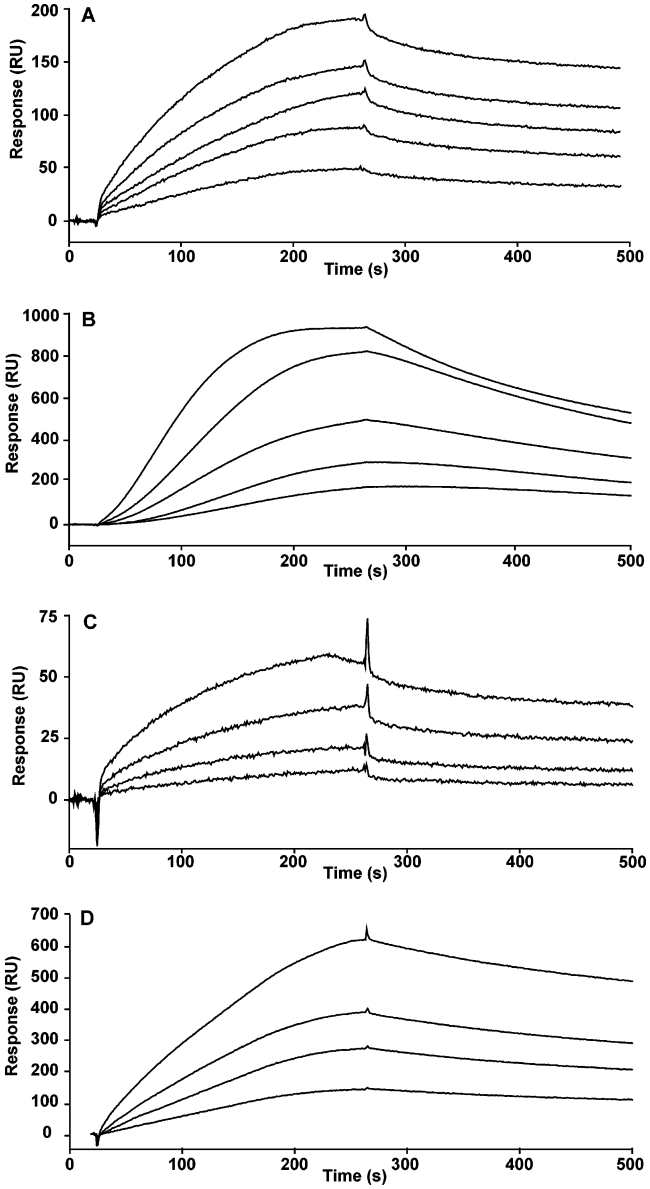

FIGURE 1: Analysis by surface plasmon resonance spectroscopy of the interaction between C1q or its globular domain and immobilized PrP. (A) Interaction with C1q. (B) Interaction with C1q in the presence of 10 μM $CuS0_4$. (C) Interaction with the C1q globular domain. (D) Interaction with the C1q globular domain in the presence of 40 μM $CuS0_4$. Recombinant murine PrP (800 RU) was immobilized on the surface of a sensor chip as described under the Experimental Procedures. Intact C1q was injected at concentrations of 5, 10, 15, 20, and 30 nM (A) and 1, 2, 3, 5, and 15 nM (B). The C1q globular domain was injected at concentrations of 0.2, 0.5, 1.0, and 2 μM in both cases (C and D). Flow rates of 10 and 20 μL/min were used for C1q and its globular domain, respectively. After each analysis, the surface was regenerated until the resonance signal was back to the basal level. The data shown are representative of at least two separate experiments and were obtained by subtracting the background signal observed when injecting each sample over immobilized bovine serum albumin.

Analysis of the binding data at different analyte concentrations yielded a k_{off} value of $1.0 \pm 0.1 \times 10^{-3}$ s^{-1}, similar to that determined for intact C1q, but a much lower k_{on} value ($4.2 \pm 0.7 \times 10^3$ M^{-1} s^{-1}). As a result, as compared to intact C1q, K_D was increased approximately 45-fold (Table 1). Consistent with the large difference in molecular weight between C1q and its globular domain, the maximal binding capacity (R_{max}) of the latter was lower (Table 1). Further

Table 1: Kinetic and Dissociation Constants for the Interaction between Immobilized PrP and C1q or Its Globular Domain

	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (nM)	R_{max}
C1q	$2.4 \pm 1.1 \times 10^5$	$1.3 \pm 0.4 \times 10^{-3}$	5.4 ± 1.5	332 ± 42
globular domain	$4.2 \pm 0.7 \times 10^3$	$1.0 \pm 0.1 \times 10^{-3}$	238 ± 10	97 ± 40
globular domain (Cu^{2+} ions)[a]	$1.8 \pm 0.3 \times 10^3$	$1.4 \pm 0.1 \times 10^{-3}$	778 ± 213	1428 ± 327

[a] Values determined in the presence of 40 μM Cu^{2+}.

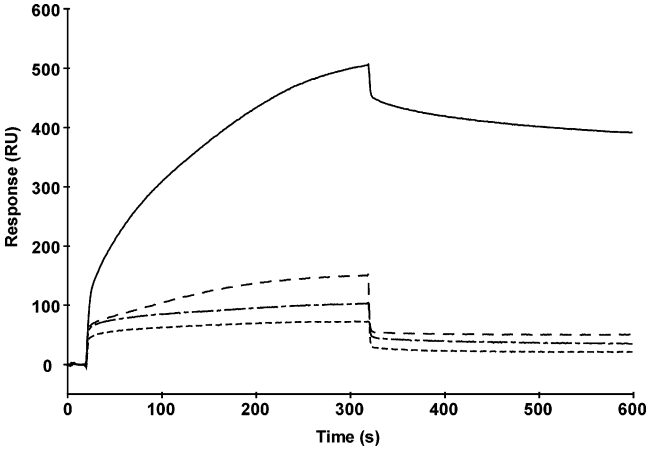

FIGURE 2: Effect of EDTA on the interaction between C1q or its globular domain and immobilized PrP. Recombinant murine PrP (900 RU) was immobilized on the surface of a sensor chip as described under Experimental Procedures. Intact C1q or its globular domain was allowed to bind at concentrations of 24 nM and 0.5 μM, respectively, in the presence or absence of 3.4 mM EDTA. From top to bottom: binding curves for C1q, C1q + EDTA, C1q globular domain, and C1q globular domain + EDTA.

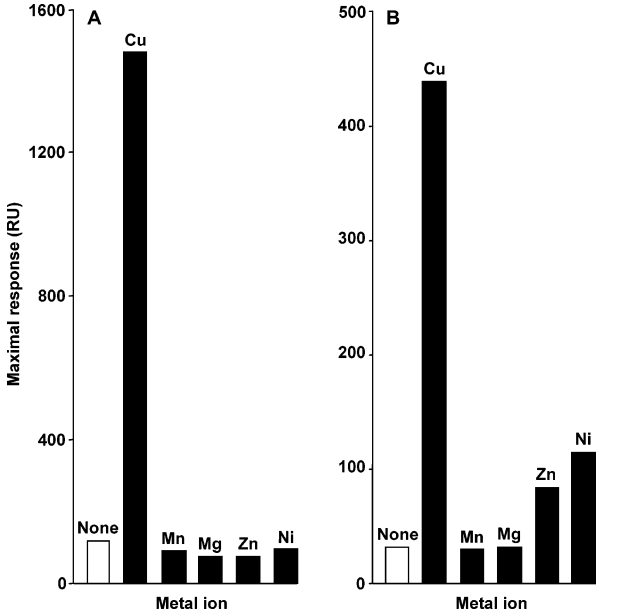

FIGURE 3: Cu^{2+} ions specifically enhance binding of C1q and its globular domain to immobilized PrP. The interaction between C1q (A) or its globular domain (B) and immobilized PrP (800 RU) was analyzed using surface plasmon resonance spectroscopy, in the absence or presence of $CuS0_4$, $MnS0_4$, $MgS0_4$, $ZnS0_4$, or $NiS0_4$, each at 40 μM. The concentrations of C1q and its globular domain were 20 nM and 1 μM, respectively. The maximal binding value reached at the end of the association phase was determined in each case.

binding assays were carried out using the collagen-like fragment of C1q as the soluble analyte, at concentrations of 0.1−0.4 μM. Although a dose-dependent binding was observed within this range, this was nonsaturable, with a linear increase during the association phase and no decrease of the bound material during the dissociation phase (data not shown), resulting in virtually infinite K_A and R_{max} values. This behavior, strikingly different from that of the parent C1q molecule, led us to the conclusion that the observed binding resulted from nonspecific interactions due to the removal of the globular domains.

C1q-PrP Interaction Is Cu^{2+}-Dependent. To investigate whether the observed interaction between C1q and PrP was dependent on divalent cations, C1q and its globular domain were allowed to bind to immobilized PrP either in the absence or in the presence of EDTA. As shown in Figure 2, EDTA led to a dramatic decrease (80−90%) in the binding of C1q, suggesting a cation-dependent process. Binding of the C1q globular domain was also inhibited by EDTA, although to a lesser extent (40−50%).

To identify the divalent cation(s) involved in the interaction, binding of C1q and its globular domain was carried out in the presence of different metal ions, each at a concentration of 40 μM. As illustrated in Figure 3A, interaction between C1q and PrP was strongly enhanced (~10-fold) in the presence of Cu^{2+} ions, whereas the Mn^{2+}, Mg^{2+}, Zn^{2+}, and Ni^{2+} ions had no significant effects. Again, in the case of the C1q globular domain, a similar 10-fold increase of the binding was observed in the presence of the Cu^{2+} ions. In addition, in contrast with intact C1q, slight but significant enhancing effects were also observed in the presence of Zn^{2+} and Ni^{2+} ions (Figure 3B).

To check whether Cu^{2+} ions had an effect on the binding parameters, C1q (5−20 nM) was allowed to bind to immobilized PrP in the presence of Cu^{2+}. Surprisingly, Cu^{2+} ions not only increased overall binding but also strikingly modified the shape of the binding curve, which became sigmoidal at all C1q concentrations tested (Figure 1B). Consequently, it was not possible to determine the kinetic constants of the interaction using a simple 1:1 Langmuir interaction model. We next analyzed the binding of the C1q globular domain at different concentrations (0.2−2 μM) in the presence of Cu^{2+} ions. In contrast to intact C1q, the binding curves had a shape similar to that obtained in the absence of Cu^{2+} ions (Figure 1B,C). Comparison of the kinetic parameters revealed that k_{off} was practically unchanged in the presence of Cu^{2+}, k_{on} being slightly diminished, resulting in a 3-fold increase in the K_D value (Table 1). Consistent with the overall enhancing effect previously observed (Figure 3B), Cu^{2+} ions strongly increased (~15-fold) the R_{max} value of the binding. Taken together, these data confirmed that the interaction between C1q and immobilized PrP was Cu^{2+}-dependent and indicated that Cu^{2+} ions did not significantly modify the kinetic constants of the binding, at least at the level of the individual globular domains.

Soluble PrP Is Not Recognized by C1q. To investigate whether the ability of murine PrP to bind C1q was a property

Table 2: Dissociation Constants[a] for the Interaction between Different Monoclonal Antibodies and the Soluble and Immobilized Forms of PrP

antibody	4F2	Saf32	Saf37	8G8	Saf60	Saf61	Saf69	Saf70
soluble PrP	9.8 ± 1.5	9.8 ± 1.5	25.0 ± 10.8	19.6 ± 9.2	30.2 ± 0.3	18.5 ± 4.6	19.6 ± 3.1	40.2 ± 4.6
immobilized PrP	2.2 ± 0.2	1.1 ± 0.1	0.5 ± 0.02	0.80 ± 0.01	10.3 ± 0.3	0.02 ± 0.001	0.11 ± 0.01	0.7 ± 0.02

[a] All K_D values are expressed in nM.

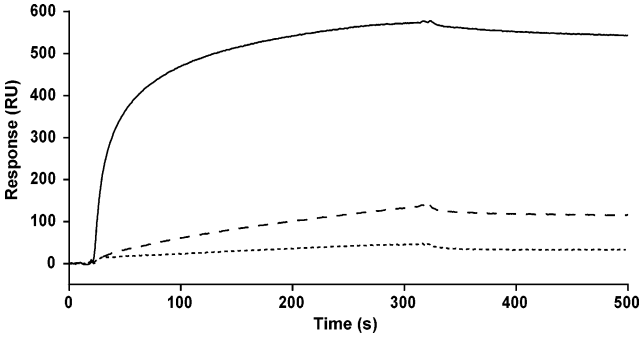

FIGURE 4: Analysis by surface plasmon resonance spectroscopy of the interaction between immobilized C1q and soluble PrP. C1q (12 000 RU) was immobilized on the surface of a sensor chip as described under the Experimental Procedures and allowed to interact with three different soluble proteins: rabbit IgG (1.5 μM), human PTX3 (0.1 μM), and recombinant murine PrP (1 μM). Interaction with PrP was measured in the presence of 10 μM $CuS0_4$. From top to bottom: binding curves of PTX3, IgG, and PrP.

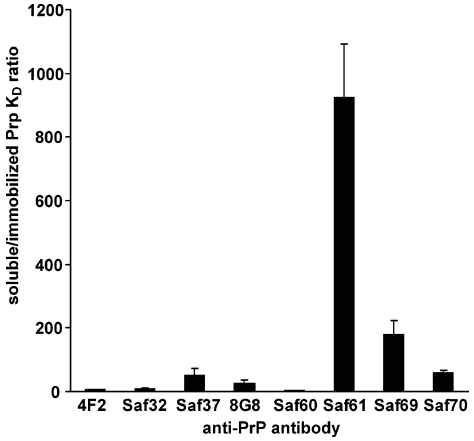

FIGURE 5: Comparative analysis of the antigenic reactivity of immobilized and soluble murine PrP. A panel of eight monoclonal antibodies was allowed to react with immobilized PrP using surface plasmon resonance spectroscopy and with soluble PrP using a sandwich ELISA, as described under the Experimental Procedures. The dissociation constants were derived in each case from experiments using at least four antibody concentrations. The ratios of the K_D values determined in both configurations (K_D for soluble PrP/K_D for immobilized PrP) are indicated for each monoclonal antibody.

inherent in the recombinant material used, or was a consequence of its immobilization on the surface of a sensor chip, several methods were used to analyze the interaction between C1q and soluble PrP. Murine PrP was first incubated with varying amounts of either C1q or its globular domain under conditions compatible with the interaction observed by surface plasmon resonance spectroscopy, in the presence of the cross-linking reagent 1-ethyl-3-[3-dimethylamino)propyl]-carbodiimide. Analysis of the reaction mixtures by SDS−PAGE (data not shown) revealed that, in all instances, the electrophoretic behavior of PrP, C1q, and its globular domain was unchanged as compared to control samples and provided no evidence for the formation of a complex between PrP and either protein. Further analyses were carried out using size-exclusion chromatography. Again, the elution positions of the three proteins were similar whether these were injected individually or as PrP + C1q or PrP + C1q globular domain equimolar mixtures (data not shown). In the same way, no extra peak corresponding to a complex could be detected in either case.

This question was further investigated by surface plasmon resonance spectroscopy, using C1q as the immobilized ligand and murine PrP as the soluble analyte. To check that immobilized C1q retained its recognition properties, two of the known C1q ligands, IgG and the long pentraxin PTX3 (*30*), were used as positive controls. As shown in Figure 4, both proteins bound to immobilized C1q, indicating that the globular domains of the molecule were still available for interaction. The binding ability of soluble PrP was tested in the same configuration, using varying concentrations of PrP. As shown in Figure 4, a very low binding signal, close to the background level, was observed at the highest PrP concentration tested (1 μM), and no significant interaction could be detected at lower concentrations, providing strong support that C1q was unable to recognize soluble PrP.

PrP Undergoes Conformational Changes upon Immobilization. The previous data led us to hypothesize that the observed ability of C1q to recognize immobilized PrP resulted from conformational changes induced by the immobilization process. To check this hypothesis, a series of monoclonal antibodies was used to compare the antigenic reactivity of PrP in its immobilized and soluble forms. In the first case, reactivity was monitored by surface plasmon resonance spectroscopy, using soluble antibodies and immobilized PrP. Attempts to use the same technique in the reverse configuration proved unsatisfactory, some of the antibodies losing their reactivity upon immobilization. A sandwich ELISA was therefore used in this case, using coated antibodies and soluble PrP (see Experimental Procedures).

As listed in Table 2, all monoclonal antibodies used recognized PrP in both configurations, with K_D values of 21 pM to 10.3 nM in the case of immobilized PrP, significantly lower than those determined for the soluble form (9.8−40 nM). As illustrated by the ratio of the K_D values determined in each case (Figure 5), the antibodies directed against the N-terminal part of PrP (4F2, Saf32, Saf37, 8G8) had similar affinities for the soluble and immobilized forms of the protein, with only a modest increase in the affinity for immobilized PrP in the case of Saf37 and 8G8. In contrast, although Saf60 had almost the same affinity for both forms, and Saf70 only exhibited a slightly better affinity for immobilized PrP, the other two antibodies directed against amino acid residues 141−159 of the C-terminal domain both showed strongly increased affinity for immobilized PrP, with a ratio close to 1000 in the case of Saf61. These data were fully consistent with the occurrence of conformational

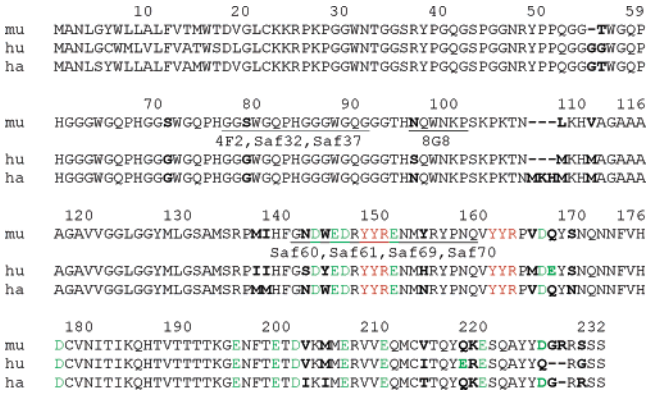

FIGURE 6: Amino acid sequences of murine, human, and hamster PrP. The amino acid numbering shown is that of murine PrP. The sequence segments used to generate the anti-PrP monoclonal antibodies used in this study are underlined. Tyr-Tyr-Arg motifs are shown in red, acidic residues are shown in green, and residues not conserved in the three proteins are shown in bold characters.

changes in PrP upon immobilization on the sensor chip surface, yielding strong indication that a major modification had occurred in the sequence stretch 141−159 of the C-terminal domain and providing a structural basis for the observed ability of C1q to recognize immobilized PrP.

DISCUSSION

Using surface plasmon resonance spectroscopy, we have obtained evidence that human C1q binds to recombinant murine PrP when this protein is immobilized on the surface of a sensor chip. The interaction is characterized by a very high affinity and is also reproduced using the C-terminal globular domain of C1q, providing strong support that this domain, which is known to mediate recognition of most of the C1q ligands (*31*), is responsible for the interaction. In this respect, the observation that the globular domain binds immobilized PrP with a significantly decreased affinity (about 45-fold) as compared to intact C1q is fully consistent with the fact that, whereas the latter is hexameric, the former is monomeric and therefore lacks the binding avidity inherent in the whole C1q molecule. Interestingly, the decrease in affinity only resulted from a decrease in the k_{on} value, the k_{off} remaining unchanged. Again, this observation appears consistent with an involvement of the globular domain since a similar effect was observed using other C1q ligands known to be recognized through this domain, such as gC1q-R (*32*) or heparin sulfate (unpublished data).

On the other hand, analysis by the same technique of the interaction between the collagen-like fragment of C1q and immobilized PrP reveals a nonsaturable binding characterized by a virtually infinite apparent K_A value, strongly suggestive of a nonspecific interaction. It may be hypothesized from this behavior that removal of the six globular heads from the C1q molecule leaves a truncated structure with deeply modified physicochemical and binding properties as compared to the corresponding collagen-like moiety of intact C1q. This may explain why, in addition to the globular domain, interaction with the collagen-like fragment has been reported for certain nonimmune C1q ligands (*33*). Indeed, it appears likely that some of these interactions may have resulted from a nonspecific binding similar to the one observed here in the case of PrP.

In addition to the previous observations, the fact that Cu^{2+} ions strikingly enhance binding of both intact C1q and its isolated globular domain also provides support that recognition of immobilized PrP is mediated by this domain. With respect to Cu^{2+} binding, there are, however, some differences between C1q and its isolated globular domain. Thus, whereas binding of intact C1q is sensitive to Cu^{2+} ions only, slight but significant stimulating effects are also observed with Zn^{2+} and Ni^{2+} ions in the case of the globular domain. Moreover, in addition to its enhancing effect, Cu^{2+} also modifies the kinetics of the interaction in the case of C1q, with sigmoidal binding curves indicative of cooperative binding. A likely hypothesis is that saturation of immobilized PrP with Cu^{2+} ions strongly increases the number of PrP molecules competent for binding to C1q, thereby enhancing overall binding, hence the stimulating effect observed with both C1q and its globular domain. In addition, the increased density of competent PrP molecules is expected to favor clustering of C1q molecules on the sensor chip surface and thereby possibly to induce nonspecific aggregation through the collagen-like moiety of the protein. Indeed, this hypothesis appears fully consistent with the observations that (i) the intensity of C1q binding in the presence of Cu^{2+} did not increase linearly when increasing C1q concentration (Figure 1B), suggesting saturation of the nonspecific component of the interaction at high C1q concentrations and (ii) decreasing PrP density on the sensor chip surface by decreasing the amount of immobilized molecules clearly resulted in attenuation of the sigmoidal shape of the binding curves (data not shown).

As judged from binding of the isolated globular domain, the kinetic constants of the interaction are not improved in the presence of Cu^{2+}, with even a slight decrease in k_{on} and, as a result, a slight increase in K_D. The most likely explanation is therefore that the overall number of PrP molecules competent for binding increases in the presence of Cu^{2+}. Thus, the low-level interaction observed in the absence of Cu^{2+} would result from the presence of trace amounts of Cu^{2+} in the buffers, allowing a small proportion of the immobilized PrP molecules to be recognized by C1q. These trace amounts would be chelated by EDTA, hence the observed inhibition (Figure 2). In contrast, addition of Cu^{2+} ions to the buffers would allow most of the PrP molecules to bind C1q. In this respect, it should be emphasized that the enhancing effect of Cu^{2+} was observed at micromolar concentrations. This is consistent with (i) the physiological concentration of Cu^{2+} ions (*5*) and (ii) the dissociation constants determined for Cu^{2+} binding to the N-terminal octarepeat region of PrP (*4*), providing strong indication that the observed ability of immobilized PrP to bind C1q requires binding of Cu^{2+} to this region.

Three different approaches were used to investigate interaction between soluble PrP and intact C1q or its globular domain, and each failed to yield evidence for formation of a complex, providing strong indication that, unlike immobilized PrP, soluble PrP is not recognized by C1q. Indeed, this appeared consistent with the fact that the recombinant murine PrP used in this study was not expected to have a pathological conformation. Therefore, unless there are major structural differences between human and murine PrP, the latter was not expected to be recognized by human C1q. Taken together, our observations strongly suggested therefore

that the observed ability of C1q to recognize immobilized PrP resulted from a conformational change in this protein.

Indeed, our comparative analysis of the antigenic reactivity of PrP in its soluble and immobilized forms provides clear evidence that the latter has a different conformation, implying that structural changes occur upon immobilization. This observation is in line with a previous report that, upon immobilization on the surface of a sensor chip, hamster PrP undergoes spontaneous rearrangement to a conformation having features in common with the infectious form (*34*). A significant difference between the two studies is that the changes described by Leclerc et al. (*34*) were found to be time-dependent and developed on a scale of several days. In contrast, no such variations were observed in our case, and comparable results were obtained consistently over time, both in terms of C1q binding and antibody reactivity. The major changes detected in our case occurred in a sequence stretch (141−159) located in α-helix 1 of the C-terminal globular domain. It should be emphasized that this stretch contains, at positions 148−150, a Tyr-Tyr-Arg sequence (i.e., a motif that was recently proposed to be selectively exposed in the pathological isoform of mouse PrP but not in the normal cellular isoform (*35*)). Although these authors consider that the best candidate for the PrP^{sc}-selective epitope is the neighboring Tyr-Tyr-Arg motif located in β-strand 2 (positions 161−163), our data are strikingly consistent with these observations and provide strong support to the hypothesis that the sequence stretch 141−163 of PrP is prone to major conformational changes. To further investigate the nature of the conformational changes observed in our case, immobilized PrP was submitted to treatment with proteinase K (data not shown). Treatment under conditions known to digest normal PrP but not PrP^{sc} (30 min at 37 °C, proteinase K:PrP molar ratio = 1:50 (*36*)) did not alter interaction with either C1q or any of the anti-PrP antibodies. In contrast, incubation under more drastic conditions (100 min at 37 °C, proteinase K:PrP molar ratio = 6:1) abolished recognition in all cases. It appears likely, therefore, that the immobilized form of PrP recognized by C1q has a conformation close to that of the pathological isoform or intermediate between the normal and pathological isoforms.

Another interesting feature is that four acidic residues (Asp143, Glu145, Asp146, and Glu151) are located in the sequence stretch recognized by antibodies Saf61, Saf69, and Saf70 (Figure 6). Given that C1q shows preferential binding for negatively charged targets, these residues are plausible candidates for recognition by C1q. However, further studies are required to identify the recognition site of C1q and confirm that this overlaps with the epitope recognized by these antibodies.

Whereas the C1q binding site is likely to be located in the C-terminal globular domain of PrP, recognition obviously requires Cu^{2+} binding to the N-terminal octarepeat region, suggesting that the conformational changes occurring upon immobilization are complex and likely require interactions between the N- and C-terminal moieties of PrP. Further investigations are obviously required to address this question. However, our observations appear consistent with previous data indicating that Cu^{2+} binding induces structural changes in the C-terminal domain of PrP (*37*). We propose that the structural modifications detected in the present study mimic the conformational changes that occur in PrP during conversion and that C1q represents a natural sensor of these changes. This is fully consistent with the reports that C1q deficiency protects mice against experimental infection by limiting doses of PrP^{sc} (*19*, *20*) and strongly supports the hypothesis of an involvement of the classical pathway of complement in the early phase of prion pathogenesis, due to the ability of C1q to recognize a conformationally modified form of PrP.

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