The Expanded Octarepeat Domain Selectively Binds Prions and Disrupts Homomeric Prion Protein Interactions*

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Insertion of additional octarepeats into the prion protein gene has been genetically linked to familial Creutzfeldt Jakob disease and hence to de novo generation of infectious prions. The pivotal event during prion formation is the conversion of the normal prion protein (PrP\(^\text{C}\)) into the pathogenic conformer PrP\(^\text{Sc}\), which subsequently induces further conversion in an autocatalytic manner. Apparently, an expanded octarepeat domain directs folding of PrP\(^\text{C}\) into the pathogenic conformer PrP\(^\text{Sc}\), which subsequently becomes transmissible (1, 2). This phenomenon has not provided a model on how altered molecular interactions between wild-type and mutant PrP set the stage for familial Creutzfeldt Jakob disease with octarepeat insertions. First, we showed that wild-type octarepeat domains interact in a copper-dependent and reversible manner, a “copper switch.” This interaction becomes irreversible upon domain expansion, possibly reflecting a loss of function. Second, expanded octarepeat domains of increasing length gradually form homogenous globular multimers of 11–21 nm in the absence of copper ions when expressed as soluble glutathione S-transferase fusion proteins. Third, octarepeat domain expansion causes a gain of function with at least 10 repeats selectively binding PrP\(^\text{Sc}\) in a denaturant-resistant complex in the absence of copper ions. Thus, the combination of both a loss and gain of function profoundly influences homomeric interaction behavior of PrP with an expanded octarepeat domain. A multimeric cluster of prion proteins carrying expanded octarepeat domains may therefore capture and incorporate spontaneously arising short-lived PrP\(^\text{Sc}\)-like conformers, thereby providing a matrix for their conversion.

Prion diseases include scrapie of sheep and goats, bovine spongiform encephalopathy of cattle, and chronic wasting disease of American mule deer and elk. Transmissibility between species is limited and regulated by a species barrier that is determined by genetic differences in the PRNP gene and eventually by other genes (4, 5). In contrast to the seemingly exclusive occurrence of genetic prion disease in humans, polymorphisms in PRNP are known to occur in many species and to influence prion infection susceptibility (3).

The essential molecular component of prions is PrP\(^\text{Sc}\), a pathological conformation of the prion protein that replicates without the need for nucleic acids (1). Once initiated, the prion replication mechanism is characterized by the conformational conversion of the cellular (“normal”) isoform of the prion protein (PrP\(^\text{C}\)) into PrP\(^\text{Sc}\), which in turn induces further conversion of PrP\(^\text{C}\), thus propagating the PrP\(^\text{Sc}\) conformation (1). Currently, 55 pathogenic mutations have been identified that cause inherited CJD in humans. Of those, 24 are missense mutations and 27 are insertion mutations consisting of up to 9 additional 24-bp repeats and corresponding to an increase (“expansion”) in the number of octarepeats, of which there are normally four consecutive copies (3). Interestingly, the clinical phenotype of iCJD with insertion mutations can mimic that of Huntington disease in the early phases of the disease (6).

Attempts at rebuilding genetic mutations that cause fCJD in cell or animal models in order to reproduce de novo prion genev has not been successful so far (7), suggesting that either unknown factors in the human genetic background or lifespan contribute to genetic prion formation. Prion initiation, meaning de novo generation of infectivity by spontaneous conversion of PrP\(^\text{C}\) to PrP\(^\text{Sc}\) without template, and prion propagation, i.e. conversion of PrP\(^\text{C}\) to PrP\(^\text{Sc}\) in the presence of PrP\(^\text{Sc}\) template, are likely to involve two different molecular mechanisms, both remaining as yet unresolved. Although it has long been possible to maintain prion propagation continuously in animals (8) and in cell culture (9, 10), only recently have there been significant advances in reproducing both prion initiation and propagation in vitro (11, 12).

Elucidating the NMR structure of the recombinant prion protein produced in Escherichia coli has been instrumental in determining the structural effects of disease-linked amino acid changes (13, 14). The mature prion protein (residues 23–231) can be divided into an N-terminal (23–120) and a C-terminal domain (121–231) (13). Whereas the C terminus adopts a mainly α-helical globular conformation, the N terminus is largely disordered (14), although it may adopt a non-random conformation at physiological pH (15). The most prevalent missense mutations causing fCJD are localized in the C-terminal domain and clustered at the edges of α-helical structures. However, recombinant PrP carrying disease-linked amino acid substitutions is not thermodynamically destabilized (16), pointing to a disease mechanism more complex than mere misfolding.

The N-terminal domain contains four highly conserved copper binding octarepeats (ORs) of the sequence PHGGGWGQ (single letter

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2 The abbreviations used are: CJD, Creutzfeldt-Jakob disease; fCJD, familial CJD; DLS, dynamic light scattering; GST, glutathione S-transferase; NHA, normal non-infected hamster brain; OR, octarepeat; PK, proteinase K; PrP, prion protein; PRNP, prion protein gene; ScHa, scrapie-infected hamster brain; SEC, size exclusion chromatography; BSA, bovine serum albumin; NTA, nitritotriacetic acid; MES, 4-morpholineethanesulfonic acid.

3268 JOURNAL OF BIOLOGICAL CHEMISTRY

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amino acid code; residues 60–91). These are flanked by one nonarepeat (residues 51–59; PQGGGTTWGQ) and one partial repeat (residues 92–98; GGGTHNQ) that could bind copper as well (17). The OR domain binds copper in a cooperative manner at physiological pH and undergoes a distinct conformational change as a result, whereas copper affinity is abolished below pH 6 (18, 19).

The N-terminal domain of PrP, including the OR domain, is of little importance for prion propagation because removal of the N terminus from PrPSc by partial protease digestion does not significantly alter infectivity titers (20). Likewise, transgenic mice expressing PrP constructs with a deleted OR domain on the PrP knock-out background can still produce infectious prions, albeit with increased incubation times and reduced prion titers when inoculated with full-length prions (21–24). The redundancy of the OR domain for prion propagation stands in contrast to its genetic linkage to FCD when the OR domain is expanded (25–27), indicating that the OR region does play a decisive role in prion initiation (28).

Our goal was to investigate the pathogenesis of FCD by determining how OR domain expansion, being the result of an insertional mutation, starts the PrP misfolding pathway and ultimately leads to the formation of infectious prions. In a series of biochemical and biophysical experiments, we demonstrated how the OR domain mediates copper-dependent and -independent homomeric interactions between PrP molecules. OR domain expansion changes these properties in such a way that binding between OR domains is no longer fully reversible and binding to PrPSc instead of PrPSc is favored. Thus, by preferentially interacting with PrPSc, PrPSc with an expanded OR domain may have a higher likelihood of undergoing conversion, thereby facilitating development of FCD.

**EXPERIMENTAL PROCEDURES**

**Cloning**

SyHaPrP-(8OR, -10OR, and -16OR) fragments were assembled from the following oligonucleotides: 1) phos-5′-GGCGCGCGGCAGCCCCATGGTGGTG-3′, 2) phos-5′-CTGCTCCCATACCCAGTGGGG-3′, 3) phos-5′-GGATCCCGGGGCAGCGC-3′, 4) phos-5′-GGCTGCGGGAGTTAGAAGATTGC-3′, 5) 5′-GCGCGGGGATCCCCCATGGTGGTG-3′, and 6) TCTTTCTCTCGAATTCTTATCA. First, we ligated oligonucleotides 2, 3, and 5 (10 μM each in 10 μl) using Taq ligase (New England Biolabs; 45 min, 45 °C). This ligation mix was then diluted 1/50 (mol/mol) in a mix of primers 1 and 2 (20 μM each, in 10 μl), followed by further ligation with Taq ligase (40 °C). We then added primers 4 and 6 (250 nmol each) and ligated with T4 ligase (16 °C). The resulting mixture was separated on 1.5% agarose gel. Several OR peptides each (data not shown).

**Recombinant Protein Expression and Purification**

Free GST (vector only), GST-HD20, GST-HD51, and GST-OR fusion proteins were expressed in BL21 (DE3) according to standard methods. Following lysozyme lysis, the suspension was brought to 50 mM Tris, pH 7.5, 10 mM EDTA (2 h, room temperature) at a protein concentration of 0.25 mg/ml and centrifuged at 10,000 × g for 10 min. The supernatant was dialyzed against 50 mM Tris, pH 8, 150 mM NaCl, 20 mM EDTA, 1% Triton X-100, and 0.2% sarkosyl, cleared (20 min, 20,000 × g), and affinity purified on glutathione-Sepharose (Amersham Biosciences). After elution, all proteins were directly treated with iodoacetamide (50 mM, 30 min, room temperature) to block free Cys residues on the GST moiety. The GST-OR fusions were further purified on Zn2+-nitriol-triacetic acid (NTA)-agarose (Nanogen). All proteins were then extensively dialyzed against 10 mM KPO4, pH 7.5, 0.1 mM EDTA. SDS-PAGE analysis confirmed that batches of all GST-OR fusion proteins were consistently purified to homogeneity and migrated at their expected molecular masses (Table 1).

**Covalent Coupling of GST-OR Fusions to Sepharose and OR Peptides to BSA**

**Coupling to Sepharose**—GST, GST-HD20, GST-HD51, and GST-OR fusions were covalently coupled to N-hydroxysulfosuccinimide-activated Sepharose (Amersham Biosciences) in 50 mM KPO4, pH 7.5, 0.3% sarkosyl, 50 μM EDTA (2 h, room temperature) at a protein concentration of 0.5 mg/ml and a coupling density of 5 mg/ml.

**Peptide Synthesis**—Peptides corresponding to SyHaPrP-(55–67) (1OR) and SyHaPrP-(55–98) (4OR) were synthesized by the Biomedizinisches Forschungszentrum at the University of Düsseldorf.

**Bovine Serum Albumin**—1OR and 4OR peptides were linked to succinimidyl-acetylthioacetate (Sigma) and then combined with BSA (Bio-Rad) derivatized with succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Molecular Probes) to a 1/10 (mol/mol) ratio in 50 mM NaPO4, pH 8, 50 μM hydrazide (2 h, room temperature). SDS-PAGE analysis showed that BSA-1OR/4OR conjugates carried several OR peptides each (data not shown).

**Pulldown of PrPSc and PrPSc from Brain Extracts with Immobilized GST-OR Fusions**

**Capture of PrPSc and PrPSc**—Normal hamster (NHa) or scrapie-infected hamster (ScHa; 263 K strain) brain homogenates (20% w/v stock in 50 mM HEPES, pH 7.5, 100 KAc, 250 mM sucrose, 5 mM MgCl2, 5× protease inhibitors (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride) were diluted to 0.5% (NHa) or 1% (ScHa) in binding buffer, pH 7.5 (50 mM HEPES, 10 mM Tris, pH 7.5, 300 mM NaCl, 0.6% Nonidet P-40, 0.3% sarkosyl) or binding buffer, pH 5.5 (100 mM NaAc, pH 5.5, 300 mM NaCl, 0.6% Nonidet P-40, 0.3% sarkosyl), both containing 1× protease inhibitors and either 50–200 μM CuSO4/ZnSO4 or 5 mM EDTA, and then cleared (5 min, 10,000 × g). Sepharose beads coated with GST, GST-HD20, GST-HD51, or GST-OR (20 μl) were combined with 0.5 ml of NHa or 1 ml of ScHa extract and incubated overnight at 4 °C. Beads incubated with NHa extract were washed and then boiled in 2× SDS-PAGE sample buffer. Beads incubated with ScHa extract were first split, i.e. half was boiled directly, while the other half was digested with 20 μg/ml PK (Merck) for 1 h at 37 °C in binding buffer, pH 7.5, plus 5 mM EDTA (stopped with 5 mM phenylmethylsulfonyl fluoride) prior to boiling. Samples were run on 12.5% SDS-PAGE, and blots were developed with a PrP monoclonal antibody 3F4 (29).

**Removal of PrPSc from PrPSc**—Sepharose beads coated with GST-16OR (20 μl) were incubated with ScHa extract (buffer, pH 7.5, plus 5 mM EDTA) obtained from infected hamsters in the terminal stage (low PrPSc/PrPSc ratio) or 42 days after infection (high PrPSc/PrPSc ratio). Beads were washed and then eluted with 50 ml of 20 mM HEPES, pH 7.5, 1 ml EDTA, 0.25–1.5% SDS (10 min, room temperature). After collection of the eluate, beads were washed with a further 1 ml of SDS buffer and then boiled.

**HaPrP Enzyme-linked Immunosorbent Assay**—For calibration, a stock solution of 0.5 mg/ml recHaPrP-(23–231) (30) was freshly diluted to 50–0.5 ng/ml in 100 mM NaHCO3, pH 8.3, 7 mM guanidinium HCl (GuHCl buffer) and coated onto Maxisorp plates (Nunc) overnight at room temperature. After blocking, wells were probed with monoclonal antibody 3F4 in 50 mM Tris, pH 8, 150 mM NaCl, 1 mM EDTA, 1% BSA, 0.1% Tween 20, 0.1% Nonidet P-40 (2 h, room temperature) and then with peroxidase-labeled anti-mouse IgG (Pierce) in the same buffer (1 h, room temperature). Plates were developed with TMB substrate.
**Prion Protein Octarepeat Domain Expansion**

**TABLE 1**

Description and measured particle sizes of GST-OR fusion proteins

| Construct         | Calculated molecular mass (kDa) | Measured $D_h$ and molecular mass at pH 7.5 (nm/kDa) | Measured $D_h$ and molecular mass at pH 5.5 (nm/kDa) |
|-------------------|---------------------------------|--------------------------------------------------|---------------------------------------------------|
| GST               | 26.2                            | 5.4 ± 0.2/34 ± 4                                 |                                                   |
| GST-SyHaPrP-(23–98) (4OR) | 33.7                          | 6.8 ± 0.2/59 ± 4                                 |                                                   |
| GST-SyHaPrP-(52–98) (4OR) | 30.7                          | 6.0 ± 0.2/44 ± 4                                 |                                                   |
| GST-16OR          | 32.5                            | 10.6 ± 0.6/170 ± 25                               |                                                   |
| GST-10OR          | 34.1                            | 15.8 ± 0.4/420 ± 30                               | 7.8 ± 0.3/79 ± 8                                 |
| GST-16OR          | 38.7                            | 21.2 ± 0.6/840 ± 60                               | 10.6 ± 0.6/170 ± 25                               |

(Pharmingen; 15 min, room temperature) according to the manufacturer’s protocol.

Quantification of GST-16OR-bound PrPSc by Sequential Pulldown—1 ml of 1% ScHa extract was sequentially incubated with two batches of 150 μg of Sepharose-linked GST-16OR (overnight, 4 °C). In parallel, we incubated ScHa homogenate (diluted), the pellet thereof (resuspended in 1 ml of binding buffer, pH 7.5), and extract without Sepharose beads. These samples, in parallel with the extract after GST-16OR pulldown, were digested with PK (20 μg/ml, 1 h at 37 °C), after which PrPSc was pelleted (1 h, 120,000 × g) and washed once with 1 ml of 100 mM NaHCO₃, pH 8.3. Pellets were then taken up in 200 μl of GuHCl buffer. Following pulldown, beads were washed with binding buffer, pH 7.5, and 100 mM NaHCO₃, pH 8.3, and then PK digested and subsequently extracted with 200 μl of GuHCl buffer. The PrP content of all GuHCl samples was determined by enzyme-linked immunosorbent assay as described above.

**Size Exclusion Chromatography**

Purified GST-4OR, GST-10OR, and GST-16OR (3 mg/ml) were fractionated on a HiPrep 16/60 Sephacryl S-200 HR column (Amersham Biosciences) at 0.5 ml/min in binding buffer, pH 7.5, plus 5 mM EDTA or in 20 mM NaAc, pH 5.5. Fractions were analyzed on 4–20% SDS-PAGE gels (Bio-Rad), stained overnight with SYPRO Ruby (Bio-Rad).

**Dynamic Light Scattering**

Dynamic light scattering (DLS) measurements were performed on a DynaPro-M5/X machine (Protein Solutions). BSA, BSA-1OR, and BSA-4OR were diluted to 30–250 μg/ml in 50 mM HEPES, pH 7.5, 150 mM NaCl, supplemented with either 200 μM CuSO₄ or 2 mM EDTA. GST and GST-OR fusions were measured at dilutions of 100–1000 μg/ml in 50 mM HEPES, 5 mM EDTA, or in 100 mM NaAc, pH 5.5.

**Scanning Force Microscopy**

GST-OR fusions were deposited on a freshly cleaved mica surface in 5 mM HEPES/MES, pH 7.9/5.5, 3 mM KCl, 5.5 mM MgCl₂, air-dried, and analyzed as described (31).

**Animal Inoculations**

Syrian Gold hamsters (6–8 weeks old) were inoculated intracerebrally using a 24-gauge needle (four or five hamsters each group) with the following material. (A), starting material: 1 ml of 1% ScHa brain extract (263 K) in binding buffer, from which PrPSc was collected by ultracentrifugation (45 min, 100,000 × g in an Optima table ultracentrifuge (BeckmanCoulter)) and subsequently washed twice with 70% ethanol and twice with sterile PBS. (B), beads coated with GST-16OR (see above) that had been incubated overnight in ScHa extract produced as in (A) and then washed three times in binding buffer, twice with 70% ethanol, and twice with sterile PBS. (C), 1 ml 1% ScHa brain extract in binding buffer after pull down with GST-16OR prepared as described in (A). (D), as a negative control, GST-4OR beads were prepared as in (B). (E) As another negative control, GST beads were prepared as for (B). For (B), (D), and (E), we choose to inoculate the whole bead fraction in order to investigate all infectivity captured and to avoid manipulating infectivity by elution procedures. Animals were examined daily for standard neurological symptoms and were sacrificed because of animal protection aspects when severe clinical symptoms were observed. The animal experimentation protocol had been approved to Lothar Stitz.

**RESULTS**

The Wild-type Prion Protein OR Domain Is a Reversible, Copper-dependent Self-association Domain—First, we established the copper-dependent mode of the homomeric interactions between OR domains. Glutathione S-transferase (GST) fusion proteins, in which GST was linked N-terminal to SyHaPrP N-terminal fragments with different OR lengths (Table 1) were used to circumvent poor solubility of both wild-type and expanded OR domains when present as free polypeptides or within full-length PrP (data not shown). Because the octarepeat sequences of human and hamster PrP are identical, we considered these constructs to be valid models for investigating biochemical characteristics of the OR domain in human PrP. We covalently coupled GST alone, GST with four ORs (GST-4OR), and GST with sixteen consecutive ORs (GST-16OR) to Sepharose via amine linkage, thus ensuring that only the GST moiety was bound to the solid support. GST-16OR was used as a model protein for expanded OR domains as occurring in iCJD, where the maximum number of ORs reported so far is 14. When incubated with brain extract from normal, non-infected hamsters (NHa) in sarkosyl-containing buffer (0.3%), both the GST-4OR and GST-16OR captured PrPSc in the presence of copper ions at pH 7.5 with a half-maximal effect between 75 and 125 μM (Fig. 1A). However, unlike GST-4OR, GST-16OR still retained PrPSc even in the absence of copper ions (Fig. 1B), suggesting partial loss of copper-dependent reversibility for PrP binding. Under the conditions used here, the full N-terminal fragment PrP-(23–98) expressed as a fusion protein to GST (GST-SyHaPrP-(23–98)) showed essentially the same effect as GST-4OR (data not shown), demonstrating that the OR domain alone is sufficient for PrPSc binding. Experiments performed with zinc yielded the same results as copper over the same concentration range.

To establish whether OR domains could interact directly in solution and to analyze the critical OR length needed for such an interaction, we covalently linked synthetic 1OR (residues 55–67) and 4OR (residues 55–98) peptides via amine linkage to BSA and analyzed copper-dependent OR–OR interactions in vitro by DLS. In the absence of copper ions (2 mM EDTA), the hydrodynamic diameters ($D_h$) of BSA alone, BSA-1OR,
and BSA-4OR were 7.5 ± 0.4 nm (70 ± 10 kDa), indicating that all three conjugates were essentially monomeric. Adding copper ions (200 μM CuSO₄) caused BSA-4OR, but not BSA or BSA-1OR, to associate into large, heterogeneous particles (DₚH = 85 nm), indicating binding between BSA-4OR conjugates, each carrying several peptides. These results demonstrated that 4OR, but not 1OR, peptides directly self-assemble in the presence of copper ions, presumably because of the conformation-inducing effect of copper binding on the OR domain (19). We were unable to determine the effect of copper on the size distribution of GST-OR proteins by DLS as GST itself was no longer monodisperse in the presence of copper, thus prohibiting reliable data collection.

The Expanded OR Domain as in fCJD Leads to the Formation of Distinct Multimeric Complexes—When we examined GST-16OR by DLS in the absence of copper ions, we found that it was present as a monodisperse multimeric complex with a DₚH of 21.2 ± 0.6 nm, corresponding to 850 ± 50 kDa at pH 7.5 (Table 1). Under the same conditions, GST alone and GST-4OR were measured to be essentially monomeric (Table 1). These findings demonstrate that OR domain expansion brings about new homomeric interactions that are copper independent and ordered in nature. Interestingly, GST-OR proteins with intermediate OR lengths also formed particles of intermediate size: GST-8OR and GST-10OR had diameters of 10.6 ± 0.6 nm (160 ± 30 kDa) and 15.8 ± 0.4 nm (420 ± 30 kDa), respectively, demonstrating a gradual effect of OR length on multimerization. Upon lowering the pH to 5.5, multimeric GST-16OR readily dissolved into lower molecular mass complexes with a DₚH of 10.7 ± 0.6 nm (170 ± 30 kDa). Likewise, GST-10OR multimers converted to monomer- or dimer-like particles (Table 1), confirming that higher order multimerization by the expanded OR domain is a phenomenon that only occurs at physiological pH.

Our DLS findings on multimerization of expanded OR domains were confirmed by scanning force microscopy and SEC (Fig. 2, A and B, respectively). Scanning force microscopy analysis demonstrated that, compared with GST-4OR, all (detergent-free) GST-10OR and GST-16OR multimers appeared as essentially homogenous, spherical particles and not as, for instance, fibrillar species (Fig. 2A). Indeed, purified GST-16OR did not bind thioflavin T, indicating that these multimers were not amyloid like (data not shown). Quantitative analysis of scanning force microscopy images showed that GST-16OR multimers had a diameter of 46.3 ± 9.8 nm (Fig. 2A); this apparent discrepancy with the multimer size determined by DLS (~21 nm) was most likely because of tip convolution effects. Furthermore, SEC analysis demonstrated that GST-16OR multimers, but not those of GST-10OR, were stable in 0.3% sarkosyl and that GST-16OR multimers converted to oligomers at pH 5.5 (Fig. 2B) in a manner that was consistent with our DLS measurements. Taken together, our results demonstrate that OR domains containing at least 8 repeats can form homogenous multimeric complexes of distinct size under physiologically relevant conditions, indicating that increasing the number of ORs favors the formation of stable homomeric complexes of PrPSc.

The Mutant Expanded, but Not Wild-type, OR Domain Binds PrPSc. We went on to investigate whether, in parallel to multimerization, wild-type and expanded OR domains differed in their interaction with PrPSc.
which could indicate that the expanded OR domain stabilizes this pathological conformation. When we incubated Sepharose-immobilized GST-OR fusion proteins with brain extract from ScHa in the presence of sarkosyl-containing buffer, we observed that only GST-16OR captured PrPSc at pH 7.5 in the absence of copper ions (Fig. 3, A and B), demonstrating selective interaction of the expanded OR domain with PrPSc. Adding copper or zinc (200 μM CuSO4/ZnSO4) or lowering the pH to 5.5 during incubation essentially abolished PrPSc binding. As controls, we verified that both GST-HD20 and GST-HD51, GST fusion proteins with the huntingtin exon-1 polypeptide containing a sequence of 20 or 51 glutamine residues, respectively (32), did not bind PrPSc (Fig. 3A for GST-HD51, GST-HD20 data not shown), thereby ruling out nonspecific interactions with PrPSc. At pH 5.5, GST-16OR did not bind protease-resistant PrPSc (Fig. 3B), but both the GST-4OR and, especially, GST-16OR did bind PK-sensitive PrP, possibly PrPc.

A Threshold of 10 OR in the Expanded OR Domain Establishes a PrPSc Binding Site—To determine how many consecutive ORs were needed for the emergence of the PrPSc binding site in the expanded OR domain, we performed pulldown experiments from ScHa brain extracts with GST-OR proteins of different OR lengths. We observed a clear threshold effect, namely a complete switch from no to full PrPSc binding between eight and ten ORs (Fig. 3C). Remarkably, ten ORs has previously been reported to be the minimum number of OR to be required for transmissibility in ICJD with expanded OR (28). As with GST-16OR, the presence of copper ions inhibited binding of PrPSc to GST-10OR.

Having shown by SEC analysis that GST-10OR was not multimeric under binding conditions used here, we conclude that it is an intrinsic conformational change of the expanded OR domain that creates a PrPSc binding site rather than its multimerization. Resistance to Denaturing Buffer Conditions Demonstrates Tight Binding between PrPSc and the Mutant, Expanded OR Domain—Because GST-16OR bound both PrPc and PrPSc at physiological pH and in the absence of copper ions, both forms were invariably retained during a pulldown experiment from ScHa extract (Fig. 3A). To investigate differences between PrPc and PrPSc binding to GST-16OR and to define conditions where GST-16OR could select between the two PrP isoforms, we tested a range of washing buffers for their ability to remove PrPSc while...
Prion Protein Octarepeat Domain Expansion

retaining PrPSc. We found that PrPSc could be removed by washing with at least 0.5% SDS, while leaving PrPSc bound to GST-16OR (Fig. 4A). Attempts at achieving the same kind of separation using sarkosyl (5%), urea (10 m), high ionic strength (1 M NaCl), low pH (10% acetic acid), or copper ions (up to 200 μM) were unsuccessful (data not shown). By means of the SDS washing technique, we were able to detect a small amount of PrPSc in ScHa extract even at a high PrPSc/PrPC ratio, namely in brain homogenates from asymptomatic scrapie-infected Syrian hamsters (culled at day 42 after inoculation of a 60-day incubation period; Fig. 4B). These results clearly show how effectively PrPSc is captured by the expanded OR domain even when relatively low levels of PrPSc are present in early stages of disease.

The Expanded OR Domain Recognizes a Distinct Subpopulation of PrPSc Molecules—To investigate how efficient recruitment of PrPSc by expanded octarepeats was, we quantified the amount of PrPSc that we could pull down from ScHa brain extracts. Surprisingly, only a small fraction of the total amount of available PK-resistant PrPSc was pulled down (Fig. 5). When the supernatant of the first pulldown was again probed with GST-16OR, no additional PK-resistant PrPSc was bound, indicating that the first round had depleted the brain homogenate of a particular PrPSc species present in the “total” PrPSc population under the experimental conditions used here (Fig. 5). Quantification of the pulled down fraction by enzyme-linked immunosorbent assay demonstrated that this GST-16OR-specific PrPSc species made up ~4% of the total amount of PK-resistant PrPSc present in the extract that itself contained 70% of total PK-resistant PrPSc in ScHa brain. The PrPSc species pulled down consisted of full-length PrPSc that was primarily double glycosylated, although other PrP glycoforms were also pulled down (see Figs. 3, 4, and 5). On undigested pulled down samples, no PrP fragments could be detected, indicating that the subpopulation of PrPSc pulled down consisted mostly of full-length PrP. When that material was protease digested, a shift in PrP immunoreactivity with an electrophoretic mobility similar to that of the starting material was observed (see Fig. 5) and there was no decrease in signal intensity, demonstrating that all pulled down material consisted of protease-resistant full-length PrPSc. Thus, the pulled down PrPSc fraction probably corresponded to a particular conformation within a seemingly heterogenous population of PrPSc.

These data parallel those under “Results” (Figs. 2 and 3) where we found that an OR length-dependent conformational change in the expanded OR domain rather than multimerization of GST-OR molecules created the novel PrPSc binding site (Figs. 2 and 3). GST-16OR pulled down material inoculated into Syrian Gold hamsters demonstrated infectivity with an average time to death of 89 ± 7 days (4 of 4 hamsters dead, compared with 77 ± 5 days for starting material or material after GST-16OR extraction). Because incubation time of the GST-16OR-captured infectivity was significantly shorter than that of negative controls (GST-4OR, 98 ± 12 days to death (Student’s t-test p <0.001); GST alone, 107 ± 19 days to death (Student’s t-test p <0.001)), these experiments indicate that the PrPSc species pulled down was associated with infectivity. The presence of infectivity in negative controls was unavoidable because the beads could not be washed harshly enough without interfering with prion infectivity itself. Our results thus provide evidence for the heterogeneity of the PrPSc population. To our knowledge, GST-16OR is the first ligand described that specifically targets an infectious subpopulation of PrPSc.

DISCUSSION

Expansion of the OR domain profoundly changes the reversible, homomeric, and copper-dependent interactions that are mediated by the N-terminal OR-containing domain of PrP. Our studies identified three new features that arise from OR domain expansion, namely partial loss of reversibility of copper-dependent interaction, gain of a PrPSc binding site, and gradual multimerization ability. Although our data do not reveal how the expanded OR directs protein misfolding of PrPC to PrPSc, our results permit us to propose a model for the events preceding prion conversion in fCJD with insertional mutations (see Fig. 6). Our model addresses interactions between mutant PrP molecules and how these could favor prion conversion but does not relate to any intrinsic conformational shift toward PrPSc that might be brought about by OR domain expansion. In the presence of copper and at physiological pH, the OR domain with wild-type 4 ORs undergoes transient, reversible homomeric interactions with PrPSc but not with PrPSc (see also Fig. 3).
This interaction mode is based on a conformational change in the OR domain that is induced by copper binding (19). The expanded OR domain behaves in the same way, with the exception that it does not fully release PrPSc upon copper depletion. In the absence of copper, the wild-type OR domain loses all affinity for PrPSc, whereas the expanded domain now tightly binds PrPSc and forms distinct multimers. When these two properties act either simultaneously or consecutively, it is likely that incorporating PrPSc or transient PrPSc-like conformers into a multimeric complex forms a nucleus for further PrPSc formation by switching from no to full PrPSc binding upon going from eight to ten ORs.

The reversible, copper-dependent interactions of the wild-type four-OR repeat domain makes us think of a "copper switch." Only four ORs, but not one single OR peptide, constitute a copper switch, suggesting that the copper-induced conformational change of the OR domain as a whole rather than copper coordination alone is responsible for self association, in a manner similar to what has been reported by Viles et al. (19). Of note, it was found that PrP with nine extra ORs recombinantly expressed in cells did not undergo copper-induced endocytosis, whereas wild-type PrP did (35). This observation is consistent with a loss-of-function phenotype and with our finding that OR domain expansion interferes with the reversibility of the wild-type copper switch that might be crucial for this type of endocytosis. The ultimate purpose of reversible interactions of PrP with itself or other molecules is unclear. Nevertheless, the report that PrP with expanded OR domains, but not other missense mutations, converts PrPSc from non-mutant alleles (41) suggests that such a mechanism may be unique to fCJD with insertional mutations. Until now, animal models have failed to accurately mimic genetic prion disease. For example, a transgenic mouse strain (Tg(PG14)) expressing a nine-OR insertion homologue within the mouse genetic background.

Whether missense mutations causing other forms of fCJD could also act by recruitment of PrPSc and subsequent conversion enhancement is unclear. Nevertheless, the report that PrP with expanded OR domains, but not other missense mutations, converts PrPSc from non-mutant alleles (41) suggests that such a mechanism may be unique to fCJD with insertional mutations. Until now, animal models have failed to accurately mimic genetic prion disease. For example, a transgenic mouse strain (Tg(PG14)) expressing a nine-OR insertion homologue within the mouse genetic background.

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The Expanded Octarepeat Domain Selectively Binds Prions and Disrupts Homomeric Prion Protein Interactions
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