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Recombinant Prion Protein rPrP27–30 from Syrian Golden Hamster Reveals Proteinase K Sensitivity

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PrP27-30 represents the protease-resistant core of the prion protein and was found to be the main component in Scrapie prion preparations. Recombinant (r) PrP27–30 corresponding to aa 90–231 from the Syrian golden hamster prion protein was expressed as a fusion with GST in *E. coli* and secreted from insect cells infected with recombinant baculoviruses. GST::rPrP27–30 isolated from either system was purified to homogenity by glutathione–Sepharose chromatography. rPrP27–30 from both systems was generated by direct cleavage of GST::rPrP27–30 in the presence of thrombin revealing a molecular weight of 17 kDa. GST::rPrP27–30 as well as the authentic protein rPrP27–30 were identified by immunoblotting employing a polyclonal antibody directed against a peptide corresponding to aa 95–110 of the Syrian golden hamster prion protein. In contrast to scrapie prion PrP27-30, the recombinant proteins GST::rPrP27-30 and rPrP27-30 were both sensitive towards proteinase K, suggesting that the molecules lack infectivity.

Proteinaceous infectious particles called prions are thought to be the causative agent of transmissible spongiform encephalopathies (TSEs) such as Scrapie, bovine spongiform encephalopathy (BSE) and Creutzfeldt-Jakob-Disease (CJD) (1). PrP27–30 lacking 67 aa at the N-terminal end is derived from PrP^{Sc} by proteinase K digestions (2–4). PrP27–30 represents the main component of prions associated in amyloid-like rods (2, 3, 5) or scrapie associated fibrils (SAF) (6). Analysis of proteinase K resistant PrP27-30 (7–9) by SDS-PAGE revealed a molecular weight between 27 and 30 kDa (5–7, 10), whereas HPLC-size exclusion chromatography resulted in a molecular weight of 19.5 kDa (3). The calculated molecular weight of the protein would be ~17 kDa in the absence of posttranslational modifications such as N-linked glycosylations at positions aa 181 and 197, respectively (11), or the carboxy-terminal glycophosphoinositol-anchor (GPI) which was proven to be present in PrP27-30 (12).

No specific nucleic acid could be detected so far in prion preparations (13) suggesting that the scrapie agent is infectious and can replicate in the absence of any nucleic acid (1). According to the ''protein-only'' hypothesis (1) exogeneous PrP^{Sc} could convert the cellular isoform PrP^{c} to PrP^{Sc}, whereas PrP^{Sc} could appear as a monomer (1) or as a nucleation or crystal seed consisting of a PrP^{Sc} oligomer (14). The *in vitro* conversion of PrP^{c} to PrP^{Sc} in the presence of exogeneous PrP^{Sc} was recently demonstrated (15–17). PrP^{Sc} can also be produced in cell culture by the posttranslational conversion of PrP^{c}(18, 19)

PrP^{c} differs from PrP27-30 with respect to its secondary structure: the α-helical and β-sheet contents of PrP^{c} are 42 and 3%, respectively(20). In contrast, the α-helical and β-sheet contents of PrP27-30 were proven to be 21 and 54%, respectively (20). Caughey et al. (18) found similar ratios for the secondary structures of PrP^{c} and PrP27-30. Thus the conversion of PrP^{c} to PrP^{Sc} most likely

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Abbreviations: BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt-Jakob disease; FCS, fetal calf serum; GST, glutathione *S*-transferase; MOI, multiplicity of infection; PBS, phosphate-buffered saline; PFU, plaque forming units; prion, proteinaceous infectious particle; PrP, prion protein; PrP^{c}, cellular isoform of the prion protein; PrP^{Sc}, scrapie isoform of the prion protein; PrP27-30, proteinase resistant core of PrP^{Sc}; rPrP27-30, recombinant isoform of PrP27-30; SAF, scrapie associated fibrils; TSE, transmissible spongiform encephalopathies.

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occurs concomitant with extreme alterations in the secondary structure of the prion protein. Synthetic peptides harboring predicted α-helical domains formed spontaneously amyloids (21) and induced α-helix to β-sheet transitions (22) supporting the notion that transitions of α-helices into β-sheets are involved in the PrP conversion.

The production of recombinant rPrP27-30 is a prerequisite for detailed biochemical investigations involving conversion experiments concomitant with alterations affecting secondary structure and infectivity. Recently, we could overexpress the cellular isoform of the prion protein (PrP^c23-231) from the Syrian golden hamster in heterologous systems (23). In this communication we report the expression of recombinant rPrP27-30 in *E. coli* and insect cells infected with recombinant baculoviruses. We demonstrate that rPrP27-30 is recognized by a PrP-specific antibody and reveals proteinase K sensitivity.

MATERIALS AND METHODS

Plasmid Constructions.

Cloning experiments were performed as described (24). The amino terminus of Syrian golden hamster PrP27-30 starts at position aa 90 (3, 4). The carboxy terminus of PrP27-30 terminates at aa position 231 (4). The cloning strategy was to subclone the PrP27-30 open reading frame into appropriate expression vectors for the synthesis of PrP27-30 as a translational fusion with glutathione *S*-transferase in *E.coli* and insect cells.

Construction of the E. coli expression vector pGEX2T::rPrP27-30. The 447 bp cDNA fragment encoding Syrian golden hamster PrP27-30 (aa 90–231) was amplified by PCR (25) from expression vector pAcSG2T:: PrP^c23–231 (23). The fragment was subcloned via *Bam*HI and *Eco*RI restriction sites into expression vector pGEX2T (26) resulting in the *E. coli* expression vector pGEX2T::rPrP27-30.

Construction of the baculovirus expression vector pAcSG2T::rPrP27-30. The 447 bp cDNA fragment encoding PrP27-30 was subcloned into baculovirus transfer vector pAcSG2T (27) via *Bam*HI and *Eco*RI restriction sites. The resulting construct pAcSG2T::rPrP27-30 contained the signal sequence of gp67, the baculovirus major glycoprotein (28), followed by the amino terminus of GST. This vector enables secretion of foreign proteins into the tissue culture medium. pGEX-2T::rPrP27-30 and pAcSG2T::rPrP27-30 were both confirmed by didesoxy sequencing (29). The introduction of three adjacent TGA termination codons should result in efficient termination of translation.

Cell and Virus Culture

Recombinant baculovirus infections of cultures of *Spodoptera frugiperda* (Sf9) were performed as described (30, 31). Co-transfections employing 2 μg of pAcSG2T::rPrP27-30 and 0.5 μg of linearized baculovirus DNA and recombinant virus preparations were handled as reported recently (23). Purified plaques were used to infect Sf9 cultures and assayed for the expression of GST::rPrP27-30 by SDS-PAGE and by immunoblotting. Recombinant virus stocks were amplified by picking plaques followed by reinfections of adherent Sf9 cultures in SF900II in the absence of FCS. After several passages the recombinant virus stocks reached 10^7-10^8 PFU/ml. Infections for the synthesis of recombinant protein were performed as described (23). For protein synthesis as well as for virus stock amplification a multiplicity of infection (MOI) of 1 was used.

Synthesis of GST::rPrP27-30 in the E. coli System

Saturated overnight cultures of *E. coli* DH5α were transformed with pGEX2T::rPrP27-30. Protein expression was performed as described (23).

Purification of GST::rPrP27-30 in the E. coli and Baculovirus System

Purification of GST::rPrP27-30 expressed in either system by glutathione-Sepharose 4B chromatography was done as described (23) with the following alterations: (i) Binding of GST::rPrP27-30 synthesized in *E. coli* was done in the presence of 4% Triton-X-100. Purification was also performed in the absence of any detergent. (ii) GST::rPrP27-30 synthesized in both expression systems was competed from glutathione-Sepharose 4B beads by the addition of 18.3 mM free reduced glutathione.

Thrombin Cleavage of GST::rPrP27-30 Immobilized on Glutathione–Sepharose 4B

Cleavage reactions were performed in thrombin cleavage-buffer (23) containing 1–2% Triton-X-100 to prevent unspecific binding of rPrP27-30 to the glutathione-beads. 2 μl of human thrombin (0.2 u; Sigma) were added to 2 μg of immobilized GST::rPrP27-30 and the reaction mixture was shaken for 4 h at 25°C. Beads and supernatant were separated by centrifugation and analyzed by SDS-PAGE and Western Blotting.

Proteinase K Assays

Authentic or GST tagged rPrP27-30 were incubated in 10 mM Tris-HCl pH 7.0 at 25°C, 130 mM NaCl and 1 mM $CaCl_2$ and in the absence and presence of proteinase K (Sigma). Reactions were terminated at individual periods of time by the addition of SDS-sample buffer, incubated for 10 min at 94°C and analyzed by SDS-PAGE and/or Western Blotting.

SDS-PAGE/Western Blotting/Determination of Protein Concentrations

Protein samples were analyzed on 12.5% or 20% SDS Phastgels (Phastsystem). Conditions for 12.5% gels were performed as described (23), 20% Phastgels were run at 250 V, 1 mA, and 3 W for 1 V·h (step 1) and at 250 V, 10 mA, and 3 W for 95 V·h (step 2). Blotting and $AgNO_3$ staining was performed as described (23). A rabbit polyclonal antibody was raised against a peptide corresponding to a region comprising aa 95 to 110 of the Syrian golden hamster prion protein (32). Protein concentrations were determined as described (23).

RESULTS

Expression of GST::rPrP27-30 in E.coli and insect cells infected with recombinant baculoviruses. GST::rPrP27-30 was efficiently expressed in insect cells infected with AcSG2T::rPrP27-30 and in *E. coli* DH5α transformed with pGEX2T::rPrP27-30. As demonstrated in Figure 1A, lanes 1 and 3, GST::rPrP27-30 synthesized from both systems was purified to homogenity by glutathione *S*-transferase chromatography. Both recombinant proteins were specifically recognized by a polyclonal antibody directed against a peptide corresponding to aa 95–110 from the Syrian golden hamster (Figure 1*B*, lanes 1 and 3). GST::rPrP27-30 from both systems reveals a molecular weight of 38 kDa compared to GST::PrP^c23–231 (23) with a molecular weight of 45 kDa (Figure 1*A/B*, lanes 2 and 4).

Yields of soluble homogeneous GST::rPrP27-30 reached about 3 mg per liter of culture medium both in the *E. coli* and baculovirus system. Thrombin cleavages of the fusion proteins yielded up to 0.1 mg of authentic protein per liter of culture medium produced from either system.

Proteinase K sensitivity of GST::rPrP27-30 synthesized in the E. coli and baculovirus system. Isolated PrP27-30 differs from PrP^c23-231 in several biochemical features. One main difference is the sensitivity of both proteins toward proteinase K (33). Native PrP27-30 is resistant towards proteinase K, whereas recombinant PrP^c (23) or PrP^c isolated from hamster brains (33, 34) display proteinase K sensitivity. To investigate the proteinase K status of GST::rPrP27-30 synthesized in the recombinant expression systems, we incubated both proteins in the absence and presence of proteinase K. Figure 2*A* demonstrates that GST::rPrP27-30 synthesized in insect cells (lane 1) and *E. coli* (lane 3) is sensitive towards proteinase K (lanes 2 and 4, respectively). Additional bands

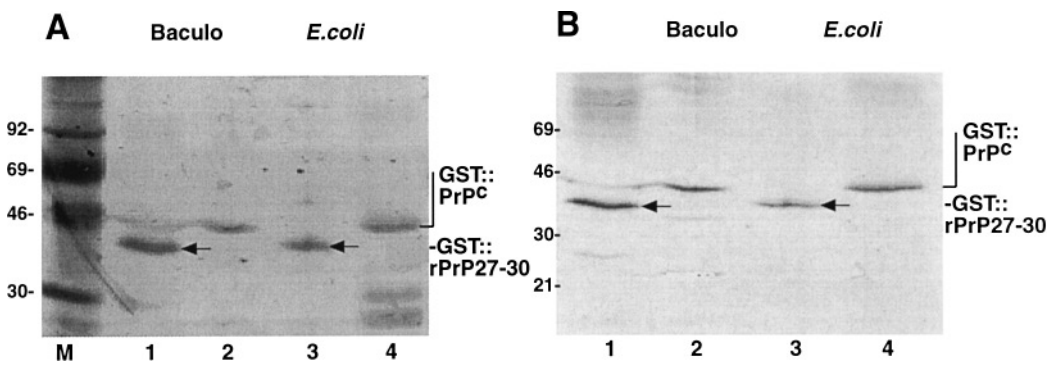

FIG. 1. Heterologous expression of GST::rPrP27-30 in *E. coli* and in Sf9 cells infected with recombinant baculoviruses. **Panel A,** 0.5 μg each of the individual marker proteins (rainbow marker RPN 756; Amersham) (lane M), 0.5 μg each of GST::rPrP27-30 (lane 1) and GST::PrP^c23-231 (lane 2; 23) expressed in the baculovirus system as well as GST::rPrP27-30 (lane 3) and GST::PrP^c23-231 (lane 4; 23) synthesized in the *E. coli* system were analyzed on a 12.5% SDS gel stained with $AgNO_3$. **Panel B,** Western Blots of proteins from Panel A using a polyclonal antibody directed against a peptide corresponding to a region comprising aa 95 to 110 of the Syrian golden hamster PrP^c (32).

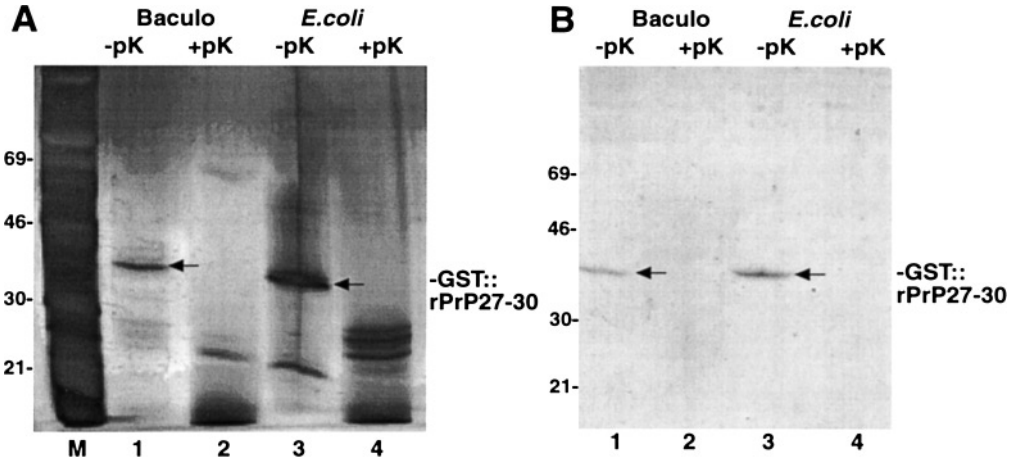

FIG. 2. Proteinase K analysis of GST::rPrP27-30 expressed in the *E. coli* and baculovirus system. **Panel A,** 0.8 μg each of the individual marker proteins (rainbow marker RPN 756; Amersham) (lane M), 0.3 μg of GST::rPrP27-30 synthesized in baculovirus infected insect cells in the absence (lane 1) and presence (lane 2) of 0.6 μg proteinase K, and 0.6 μg of GST::rPrP27-30 expressed in *E. coli* in the absence (lane 3) and presence of 2 μg proteinase K (lane 4) were analyzed on a 12.5% SDS gel followed by $AgNO_3$-staining. Samples were incubated with proteinase K for 30 minutes. **Panel B,** Western blot analysis of Panel A employing the polyclonal PrP antibody.

observed after an incubation period of 30 minutes in the presence of 2 μg (lane 2) and 6 μg proteinase K (lane 4) correspond to proteinase K as demonstrated by SDS-PAGE analysis of proteinase K (data not shown). Western Blot analysis of this digest (Figure 2*B*) reveals that GST::rPrP27-30 expressed in *E. coli* (lane 1) and insect cells (lane 3) was not recognized by a PrP-specific polyclonal antibody after proteinase K digestion (lanes 2 and 4, respectively) demonstrating that the GST::rPrP27-30 is entirely proteinase K sensitive.

Synthesis of recombinant rPrP27-30 by thrombin cleavage. The fusion protein GST::rPrP27-30 contains a thrombin cleavage site between its GST and rPrP27-30 domains. Thrombin cleavage of the fusion protein should generate rPrP27-30 carrying two additional amino acids (Gly and Ser) at the amino terminus. GST::rPrP27-30 synthesized in insect cells was incubated in the absence (Figure 3*A*, lane 1) and presence (lanes 2–3) of thrombin. Analysis of the beads after thrombin digestion showed that GST was still immobilized on the beads (Figure 3*A*, lane 2). Analysis of a supernatant fraction revealed three bands. A comparison with authentic thrombin (Figure 3*A*, lane 4), showed that small amounts of thrombin are present in the supernatant (lane 3). In addition, soluble GST was also detectable in the supernatant together with rPrP27-30 displaying a molecular weight of 17 kDa. The rPrP27-30 protein was recognized by a polyclonal PrP^c antibody (Figure 3*B*, lane 3). Recombinant GST::rPrP27-30 from *E. coli* (Figure 3*C*, lane 1) was also susceptible towards thrombin resulting in rPrP27-30 (Figure 3*C*, lane 2) revealing a molecular weight which is comparable to rPrP27-30 synthesized in the baculovirus system (Figure 3*A*, lane 3). *E. coli* expressed rPrP27-30 was also recognized by the polyclonal PrP^c antibody (Figure 3*D*, lane 2).

rPrP27-30 reveals proteinase K sensitivity. To investigate whether recombinant rPrP27-30 reveals proteinase K sensitivity, we incubated rPrP27-30 synthesized in the baculovirus system in the presence of proteinase K. As demonstrated in Figure 4, rPrP27-30 (lane 1) was completely degraded after 60 minutes (lane 4), demonstrating that a proteinase K sensitive form of the PrP27-30 was produced. Likewise, rPrP27-30 expressed in the *E. coli* system was sensitive towards proteinase K (data not shown).

DISCUSSION

PrP27-30 represents the main component of prions from scrapie-infected brains and can be isolated from amyloid-like rods (5). PrP27-30 is extremely unsoluble (3, 5, 10, 35), resistant

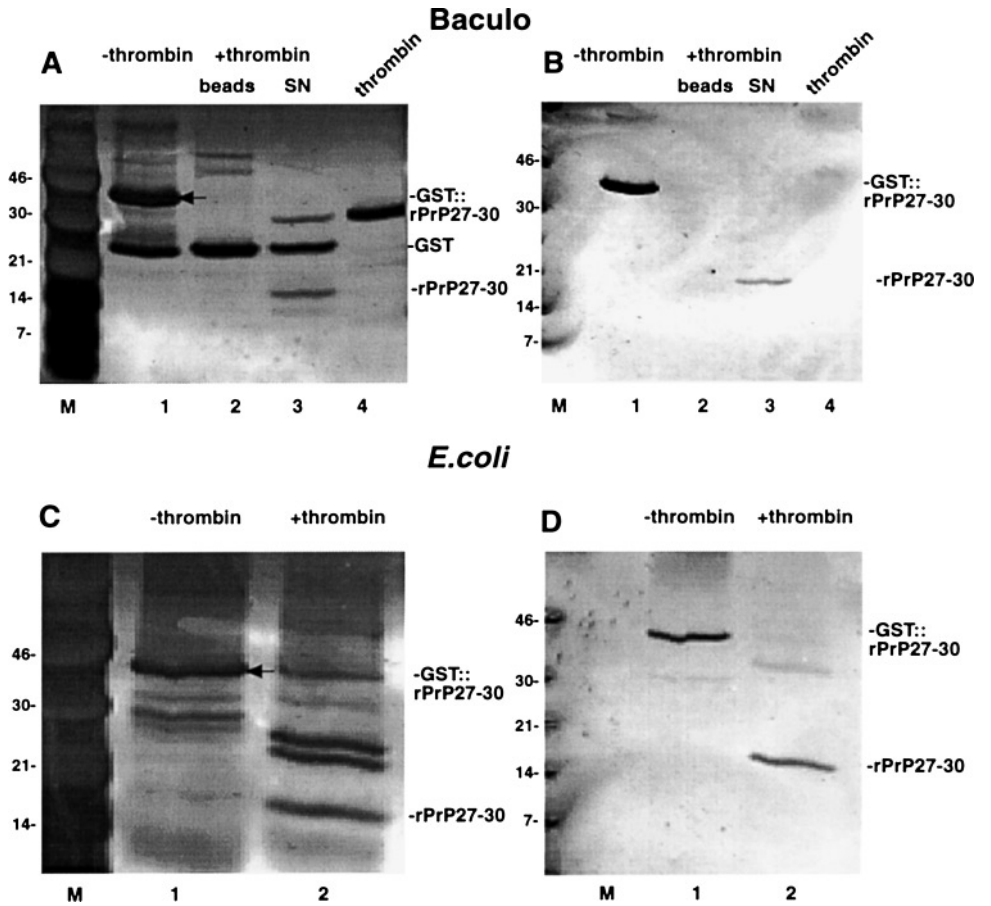

FIG. 3. Analysis of rPrP27-30 generated by thrombin cleavage from GST::rPrP27-30 synthesized in the *E. coli* and baculovirus systems. **Panel A,** 0.8 μg each of the individual marker proteins (rainbow marker RPN 755; Amersham) (lane M), 0.8 μg of the bead fraction of GST::rPrP27-30 synthesized in insect cells infected with AcSG2T::rPrP27-30 in the absence (lane 1) and presence (lane 2) of thrombin. 0.8 μg of proteins from the supernatant after thrombin cleavage (lane 3) and authentic thrombin (lane 4) were analyzed on a 12.5% SDS gel stained with $AgNO_3$. **Panel B,** Western Blot of **Panel A** using the PrP antibody (32). **Panel C,** 0.8 μg each of the individual marker proteins (rainbow marker RPN 755; Amersham) (lane M), 0.5 μg of GST::rPrP27-30 synthesized in *E. coli* in the absence (lane 1) and presence (lane 2) of thrombin were analyzed on a 12.5% SDS gel stained with $AgNO_3$. **Panel D,** Western Blots of *Panel C* employing the polyclonal PrP antibody.

towards proteinase K digestions (7, 8) and reproducibly copurifies with infectivity (7, 35). For a detailed biochemical and biophysical characterization we expressed PrP27-30 in high yields in heterologous systems. To enhance solubility and stability of PrP27-30 we designed the protein as a fusion with glutathione *S*-transferase (GST). Furthermore GST acts as a purification aid enabling to purify the protein by a one-step glutathione-Sepharose affinity chromatography. The presence of a thrombin cleavage site enables isolation of authentic rPrP27-30.

Recombinant (r) PrP27-30 was successfully synthesized as a fusion with GST in the baculovirus and the *E. coli* system. Yields of GST::rPrP27-30 in either system were about 3 mg/l of culture medium and therefore higher as compared to PrP^c23–231 which was also expressed in both systems (23). The fusion protein revealed a molecular weight of 38 kDa which is less than expected by molecular mass calculations; i.e. 27 kDa for GST (26) and 17 kDa for rPrP27-30. Authentic rPrP27-30 plus two additional amino acids (glycin and serin at the NH_2-terminus) was generated

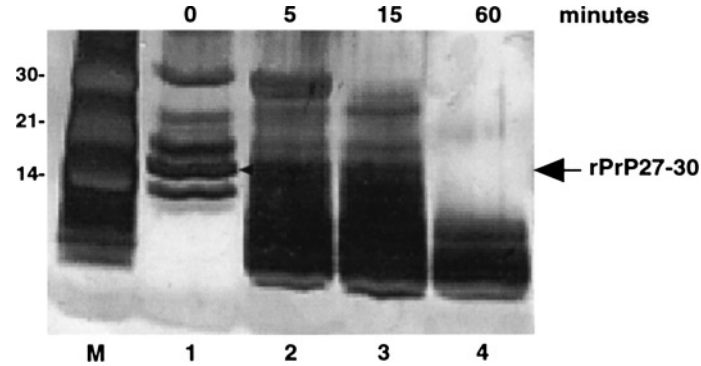

FIG. 4. Proteinase K analysis of rPrP27-30. 0.8 μg each of the individual marker proteins (rainbow marker RPN 755; Amersham), 2 μg of rPrP27-30 synthesized by thrombin cleavage from GST::rPrP27-30 synthesized in the baculovirus system was incubated in the presence of 4 μg proteinase K. Samples 0 (lane 1), 5 (lane 2), 15 (lane 3) and 60 minutes (lane 4) after the addition of proteinase K were analyzed on a 20% SDS gel stained with $AgNO_3$.

by thrombin cleavage from both fusion proteins. rPrP27-30 revealed a molecular weight of ~17 kDa as was analyzed by SDS-PAGE and migrates differently from scrapie PrP27-30 which shows a molecular weight between 27 and 30 kDa (5–7, 10) also calculated by SDS-PAGE analysis. This difference could result either from the lack of posttranslational modifications such as N-linked glycosylations and/or the absence of a GPI anchor both present in native PrP27-30 (11, 12). The fact that rPrP27-30 expressed from either systems shows the same molecular weight suggests that rPrP27-30 from the baculovirus system was not or only marginally glycosylated. In addition, a modified secondary and tertiary structure of rPrP27-30 compared to scrapie PrP27-30 could also account for the differences in the molecular weight. Different sensitivity patterns of scrapie PrP27-30 and rPrP27-30 towards proteinase K suggest different secondary structures. Scrapie PrP27-30 is proteinase K resistant (7, 8) whereas GST::rPrP27-30 and authentic rPrP27-30 synthesized in the baculovirus or the *E. coli* system reveal proteinase K sensitivity.

The proteinase K sensitive cellular PrP^c (34) shows dramatic differences regarding secondary structures compared to PrP27-30. PrP^c contains predominantly α-helical domains, whereas PrP27-30 reveals a high β-sheet content (20, 36). We assume that proteinase K sensitive rPrP27-30 shares structural homology with the cellular isoform PrP^c. In addition, proteinase K sensitivity of rPrP27-30 suggests that the molecule lacks infectivity although this conclusion is only inferred from the corresponding behaviour of PrP^c.

Authentic rPrP27-30 as well as GST::rPrP27-30 were specifically recognized by a polyclonal PrP^c antibody generated by a peptide corresponding to aa 95–110 of the Syrian golden hamster prion protein demonstrating that the molecule was immunologically active.

The proteinase K sensitive recombinant PrP27-30 molecule is a prerequisite for detailed studies of the propagation mechanism of prion-proteins. Conversion experiments of rPrP27-30 from the proteinase K sensitive to a proteinase K resistant isoform in the absence and presence of externally added PrP^{Sc} as well as other cofactors are under investigation.

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