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Prion Protein PrP^c Interacts with Molecular Chaperones of the Hsp60 Family

FRANK EDENHOFER, ROMAN RIEGER, MICHAEL FAMULOK, WOLFGANG WENDLER, STEFAN WEISS, AND ERNST-LUDWIG WINNACKER*

Laboratorium für Molekulare Biologie-Genzentrum-Institut für Biochemie der Ludwig-Maximilians-Universität München, 81375 Munich, Germany

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Prions mediate the pathogenesis of certain neurodegenerative diseases, including bovine spongiform encephalopathy in cattle and Creutzfeldt-Jakob disease in humans. The prion particle consists mainly, if not entirely, of PrP^{Sc}, a posttranslationally modified isoform of the cellular host-encoded prion protein (PrP^c). It has been suggested that additional cellular factors might be involved in the physiological function of PrP^c and in the propagation of PrP^{Sc}. Here we employ a *Saccharomyces cerevisiae* two-hybrid screen to search for proteins which interact specifically with the Syrian golden hamster prion protein. Screening of a HeLa cDNA library identified heat shock protein 60 (Hsp60), a cellular chaperone as a major interactor for PrP^c. The specificity of the interaction was confirmed in vitro for the recombinant proteins PrP^c23-231 and rPrP27-30 fused to glutathione *S*-transferase with recombinant human Hsp60 as well as the bacterial GroEL. The interaction site for recombinant Hsp60 and GroEL proteins was mapped between amino acids 180 and 210 of the prion protein by screening with a set of recombinant PrP^c fragments. The binding of Hsp60 and GroEL occurs within a region which contains parts of the putative α-helical domains H3 and H4 of the prion protein.

Transmissible spongiform encephalopathies are neurodegenerative diseases such as scrapie in sheep, bovine spongiform encephalopathy in cattle, and Creutzfeldt-Jakob disease in humans (34). Infectious preparations derived from infected brains are resistant to UV and ionizing radiation as well as other procedures which inactivate nucleic acids, indicating that nucleic acids may not be required for infectivity. Purification of infectious preparations from brains revealed the presence of a protein required for infectivity (33). These experimental observations led to the protein only hypothesis, which proposes that proteinaceous infectious particles (prions) are responsible for the transmission of transmissible spongiform encephalopathies (2, 3, 33). Prions consist mainly of a protease resistant protein designated PrP^{Sc} (PrP for prion protein and Sc for scrapie), a posttranslationally modified form of the proteinase K-sensitive host-encoded PrP^c (c for cellular) (8, 9, 11, 34). Both isoforms share the same amino acid sequence but differ in their secondary structures (31, 42). Circular dichroism and Fourier transform infrared spectroscopy revealed a significantly large β-sheet content in PrP^{Sc} compared with a large α-helix content in PrP^c (17, 31, 38). Structural predictions of PrP^c led to a model which proposed that four domains between amino acids (aa) 109 to 122, 129 to 141, 178 to 191, and 202 to 218 form α-helices (24). It has been suggested that prion propagation involves the conversion of α-helical domains in PrP^c into β-sheets in PrP^{Sc} (26, 30, 31). The in vitro conversion of PrP^c into PrP^{Sc} was demonstrated by a proteinase K resistance assay (27). A modified model was recently suggested in which PrP^c must be partially unfolded and refolded into PrP^{Sc} under the direction of an oligomeric PrP^{Sc} seed (29). This model provides explanations for scrapie species barriers (28) and strain-specific properties of prions (7). In addition, experiments employing transgenic mice led to the proposal that prion propagation requires a species-specific macromolecule designated protein X (43). In order to identify proteins capable of interacting with PrP^c, we employed a two-hybrid screen in *Saccharomyces cerevisiae* (15, 19, 21). The specificity of the observed in vivo interactions between Hsp60 and PrP^c was confirmed by in vitro binding studies with recombinant prion proteins. Mapping of the interaction site between the molecular chaperone and PrP^c was done with recombinant prion glutathione *S*-transferase (GST)-fusion peptides.

* Corresponding author. Mailing address: Laboratorium für Molekulare Biologie-Genzentrum-Institut für Biochemie der Ludwig-Maximilians-Universität, Würmtalstrasse 221, D-81375 München, Germany. Phone: 49-89-74017-400. Fax: 49-89-74017-448. Electronic mail address: elw@lmb.uni-muenchen.de.

MATERIALS AND METHODS

Construction of yeast vectors. Cloning procedures were performed as described previously unless otherwise stated (40). The shuttle vectors pSH2-1 and pEG202, which direct the synthesis of different LexA hybrids (aa 1 to 87 and 1 to 202) (19, 21), were used to construct the LexA fusion baits.

(i) Construction of pSH2-1/pEG202-GST. A 666-bp DNA fragment coding for GST was amplified by PCR (39) from the cDNA clone pAcSG2T::PrP^c23-231 (47). The fragment was subcloned into plasmids pSH2-1 and pEG202 using the *Eco*RI-*Bam*HI restriction sites, resulting in the vectors pSH2-1/pEG202-GST.

(ii) Construction of pSH2-1/pEG202-PrP^c. A 646-bp DNA fragment containing nucleotides coding for aa 23 to 231 of the Syrian golden hamster PrP^c protein was amplified by PCR from the cDNA clone pAcSG2T::PrP^c23-231. The PrP^c cassette was cloned via *Eco*RI-*Bam*HI restriction sites into vectors pSH2-1 and pEG202, yielding pSH2-1/pEG202-PrP^c.

(iii) Construction of pSH2-1/pEG202-GST-PrP^c. A 646-bp DNA fragment coding for aa 23 to 231 of the PrP^c protein was amplified by PCR from the cDNA clone pAcSG2T::PrP^c23-231. The PrP^c fragment was cloned via *Bam*HI-*Sal*I restriction sites into vector pSH2-1-GST, yielding pSH2-1-GST-PrP^c. The GST-PrP^c cassette was excised from this vector with the *Eco*RI and *Sal*I restriction sites and cloned into pEG202, resulting in pEG202-GST-PrP^c.

(iv) Construction of pSH2-1-PrP^c-GST. The PrP^c23-231 cassette was amplified by PCR from pAcSG2T::PrP^c23-231 and cloned into pSH2-1-GST via the *Eco*RI restriction site resulting in pSH2-1-PrP^c-GST.

The correct orientation, reading frames, and sequences of the PCR-amplified fragments were confirmed by dideoxy sequencing (41).

Two-hybrid screen. Detailed procedures for using the yeast two-hybrid system have been described previously (4, 6, 19, 21). *S. cerevisiae* EGY48 (*MAT***a** *ura3 his3 trp1 LEU2*::*LexAop6-LEU2*), which carries a chromosomal insertion of LexA binding sites upstream of the *LEU2* gene, was used as the recipient host (19, 21). In brief, yeast strain EGY48 was transformed with the reported plasmid pSH18-34 containing a LexA-controlled *lacZ* gene as a second reporter. *S. cerevisiae* cells were cotransformed with the bait-plasmid and pJG4-5 containing a HeLa cDNA library fused to the acidic B42 transactivation domain (19, 21).

The cDNA insert of the pJG4-5 plasmid is controlled by a galactose-inducible promotor. Therefore, interaction between the two hybrids occurs only in the presence of galactose. Colonies able to grow in the absence of leucine (first reporter gene) were dotted onto galactose plates supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and screened for β-galactosidase production (blue color, second reporter gene).

cDNAs of positive clones were recovered from 5 ml of *S. cerevisiae* cell cultures. Cells were incubated at 30°C for 2 days, harvested by centrifugation (3,000 rpm, 10 min at 4°C), and washed (1 M sorbitol, 0.1 M EDTA [pH 8.0]). After resuspension in SCE (1 M sorbitol, 0.1 M sodium citrate [pH 5.8], 10 mM EDTA, 0.1 M β-mercaptoethanol), the cells were incubated with 40 μl of Lyticase (5 U/μl; Sigma) for 1 h at 37°C. After centrifugation, the cells were resuspended in TE lysis buffer (50 mM Tris-HCl [pH 7.4], 20 mM EDTA containing 1% sodium dodecyl sulfate [SDS]) and incubated for 30 min at 65°C. The lysate was phenol extracted, and the DNA was ethanol precipitated and resuspended in TE. The DNA was transformed in *Escherichia coli* KC8, which enables the selection of pJG4-5 plasmids by ampicillin resistance and complementation of its tryptophan auxotrophy (19, 21). As control experiments, the plasmids were retransformed in EGY48 and the transformants were tested for β-galactosidase production and for the Leu$^+$ phenotype. At least five retransformants were dotted onto corresponding plates and incubated for 5 days at 30°C.

DNA sequencing and computation. cDNA inserts were sequenced with the T7-sequencing kit (Pharmacia) by the dideoxy method (41). Homology searches for the cDNA sequences were performed at the National Center for Biotechnology Information with the BLAST network service (http://www.ncbi.nlm.nih.gov/Recipon/bs_seq.html).

Proteins and antibodies. GST, GST::PrP^{c}23-231, and the GST::PrP^c fragments GST::P23-52, GST::P53-93, GST::P90-109, GST::P129-175, GST::P180-210, and GST::P218-231 were prepared as described previously (47). GST::rPrP27-30 (aa 90 to 231 of the Syrian golden hamster prion protein) was expressed in and purified from *E. coli* and from the baculovirus expression system (48). GroEL and anti-rabbit immunoglobulin G POD as well as anti-mouse immunoglobulin G POD were obtained from Boehringer Mannheim. Recombinant human Hsp60 was provided by StressGen, and the monoclonal mouse anti-Hsp60 antibody was obtained from Sigma.

SDS-PAGE and immunoblotting. Protein samples were analyzed on 12.5% SDS Phastgels (Pharmacia) as described previously (47). A rainbow marker (RPN 756; Amersham) was used as a size standard. Following polyacrylamide gel electrophoresis (PAGE), gels were blotted onto nitrocellulose (Schleicher & Schuell) or polyvinylidene difluoride membranes (Millipore) (40 min at 70°C). The blots were incubated with a polyclonal anti-GroEL or anti-PrP antibody at a 1:800 or 1:400 dilution, respectively. Incubation steps were performed as described previously (44). Antibody detection was performed by chemiluminescence (ECL system; Amersham) or in the presence of DAB (Sigma).

In vitro binding of GST fusion proteins. Pull-down assays were performed by equilibrating glutathione-Sepharose 4B beads (Pharmacia) loaded with GST or the GST fusion protein in refolding buffer (32) including 0.5% Triton X-100. The equilibrated beads were incubated with an up-to-10-fold molar excess of GroEL or Hsp60 (monomer) at room temperature in the presence of refolding buffer including 0.5% Triton X-100. After centrifugation (2,500 rpm, 10 min) the beads were washed with refolding buffer, analyzed on a 12.5% SDS Phastgel, blotted, and probed for the presence of GroEL or Hsp60.

RESULTS

Expression of the bait protein LexA-GST-PrP^c in *S. cerevisiae* EGY48. For two-hybrid screening, we tested a fusion protein bait consisting of the bacterial repressor LexA binding domain (19, 21) and the Syrian golden hamster prion protein PrP^{c}23-231 (aa 23 to 231, referred to as PrP^c) (5) fused to GST. As reported recently, fusion with GST significantly enhances the solubility and stability of recombinant PrP^c (47, 48). Cells of the yeast strain EGY48 were cotransformed with the reported plasmid pSH18-34 and the bait plasmids and tested for their intrinsic ability to activate the reporter system. The pSH-GST-PrP^c construct showed a low level of intrinsic activation. Expression of the LexA-GST-PrP^c fusion protein in *S. cerevisiae* was confirmed by immunoblotting with a polyclonal anti-PrP antibody (20) directed against aa 95 to 110 (data not shown).

Identification of PrP^c-Hsp60 interaction by the two-hybrid screen. Yeast cells expressing the LexA-GST-PrP^c bait were cotransformed with the reporter plasmid pSH18-34 and a plasmid encoding a HeLa cDNA library fused to the acidic transactivation domain B42 (19, 21). The resulting pool of approximately 6×10^5 transformants was screened for a Leu$^+$ phenotype, resulting in approximately 2,000 positive colonies. A total of 300 colonies were screened for β-galactosidase expression. The cDNAs of 55 positive clones were recovered, retransformed in *E. coli*, and sequenced. Approximately 20% (corresponding to nine cDNAs) encoded Hsp60. The isolated Hsp60 cDNAs encode three N-terminally-truncated proteins with different lengths starting at position aa 146, aa 228, and aa 298, respectively (EMBL M34664). All of them contain parts of the putative peptide binding domain of Hsp60 (14).

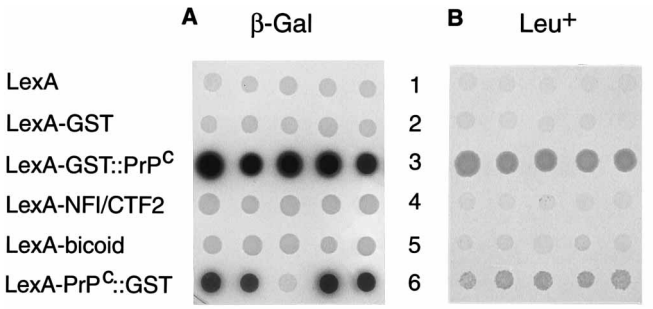

FIG. 1. Identification of PrP^{c}23-231-Hsp60 interaction with the two-hybrid system. Two different phenotypes confirmed this interaction. Yeast cells containing the reporter plasmid pSH18-34 were cotransformed with the pJG4-5 plasmid carrying the cDNA clone encoding Hsp60 (aa 146 to 544) and the bait plasmids pSH2-1 (row 1), pSH2-1-GST (row 2), pSH2-1-GST-PrP^c (row 3), pSH2-1-NFI/CTF2 (row 4) (49), pEG202-bicoid (row 5) (21), and pSH2-1-PrP^c-GST (row 6). Each of five transformants were resuspended in TE, dotted onto galactose plates that were either supplemented with X-Gal (A) or leucine deficient (B), and incubated at 30°C for 5 days. β-Gal, β-galactosidase.

Test for specificity of PrP^c-Hsp60 interaction. Specificity of the observed in vivo interaction between PrP^c and Hsp60 (Fig. 1, row 3) was demonstrated by several recloning experiments. In particular, we showed that the inverse fusion PrP^c::GST (Fig. 1, row 6), as well as authentic PrP^c lacking GST (data not shown), strongly interacts with Hsp60. In contrast, LexA-GST (Fig. 1, row 2), authentic LexA (Fig. 1, row 1), and the two false-baits LexA-NFI/CTF2 (49) (Fig. 1, row 4) and LexA-bicoid (21) (Fig. 1, row 5) showed no interaction with Hsp60.

Recombinant Hsp60 binds specifically to PrP^{c}23-231 and rPrP27-30 in vitro. PrP^{c}23-231 represents the mature form of the cellular prion protein. Scrapie prion isolates consist mainly of the protease-resistant core, which is 27 to 30 kDa in size (referred to as PrP27-30) (35, 42), comprising amino acids 90 to 231. We employed recombinant GST fusion proteins bound to glutathione-Sepharose beads to confirm the interaction with recombinant full-length Hsp60 in vitro. GST as well as PrP^{c}23-231 and rPrP27-30 fused to GST (47, 48) was immobilized and incubated with Hsp60. Hsp60 was detected in the presence of GST-PrP^c (Fig. 2A, lane 2) and GST-rPrP27-30 (Fig. 2A, lane 3) but not with GST alone (Fig. 2A, lane 4). Another human chaperone, Hsp70, did not interact with any of these proteins (data not shown), demonstrating that the interaction of PrP^c with Hsp60 is highly specific.

Binding of bacterial GroEL to PrP^{c}23-231 and rPrP27-30 in vitro. To investigate whether GroEL, the prokaryotic homolog of the Hsp60 family is also capable of binding to PrP^c, we employed recombinant GroEL in corresponding in vitro binding experiments. GroEL was found to exhibit similar specificity in the interaction with PrP^c (Fig. 2B, lane 3) and rPrP27-30 (Fig. 2C, lane 2) fused to GST whereas no binding occurs in the presence of GST alone (Fig. 2B, lane 4 and Fig. 2C, lane 3).

Mapping of interaction site for Hsp60 and GroEL within PrP. To obtain a comprehensive map of the PrP^c binding site on the molecular chaperones, we employed six fragments of

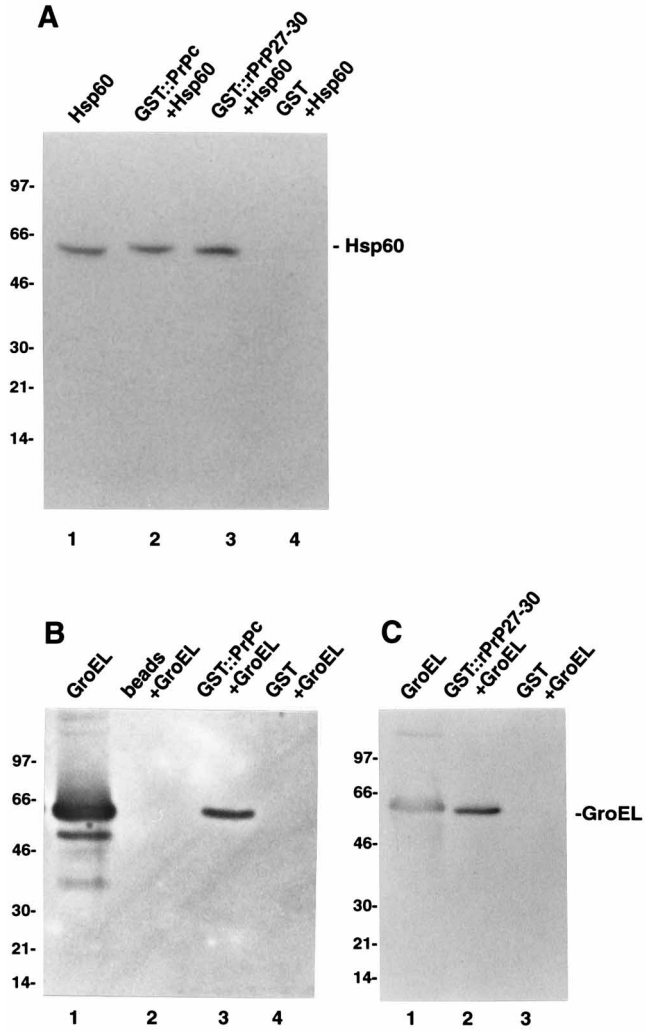

FIG. 2. Immunoblot analysis of pull-down assays to demonstrate the in vitro interaction of Hsp60 and GroEL in the presence of PrP fused to GST. Numbers on the left indicate size in kilodaltons. (A) Recombinant GST (1 μg), GST-rPrP27-30 (2 μg), and GST-PrP^c (2 μg) immobilized on glutathione-Sepharose were incubated with 10 μg of Hsp60. After centrifugation, the beads were washed and resuspended in sample buffer. GST-PrP^c (lane 2), GST-rPrP27-30 (lane 3), and GST (lane 4) (4 μl each) as well as 200 ng of Hsp60 as a control (lane 1) were analyzed by SDS-PAGE (12.5%) and immunoblotting (polyvinylidene difluoride membranes) with a monoclonal mouse anti-Hsp60 antibody and chemiluminescence detection. (B) Recombinant GST (1 μg) and GST-PrP^c (2 μg) immobilized on glutathione-Sepharose as well as glutathione-Sepharose alone were incubated with 25 μg GroEL. After the beads were washed, they were resuspended in sample buffer. A 1:1 slurry of beads (lane 2), GST-PrP^c (lane 3), and GST (lane 4) (4 μl each) as well as 2 μg of GroEL as a control (lane 1) were analyzed on a 12.5% SDS gel and blotted onto a nitrocellulose membrane. Protein detection was done with an anti-GroEL antibody and chemiluminescence. (C) Recombinant GST (1 μg) and GST-rPrP27-30 (2 μg) immobilized on glutathione-Sepharose were incubated with 25 μg of GroEL. After the beads were washed, they were resuspended in sample buffer. GST-rPrP27-30 (lane 2) and GST (lane 3) (4 μl each) as well as 1 μg of GroEL as a control (lane 1) were analyzed by SDS-PAGE and immunoblotting with an anti-GroEL antibody and chemiluminescence.

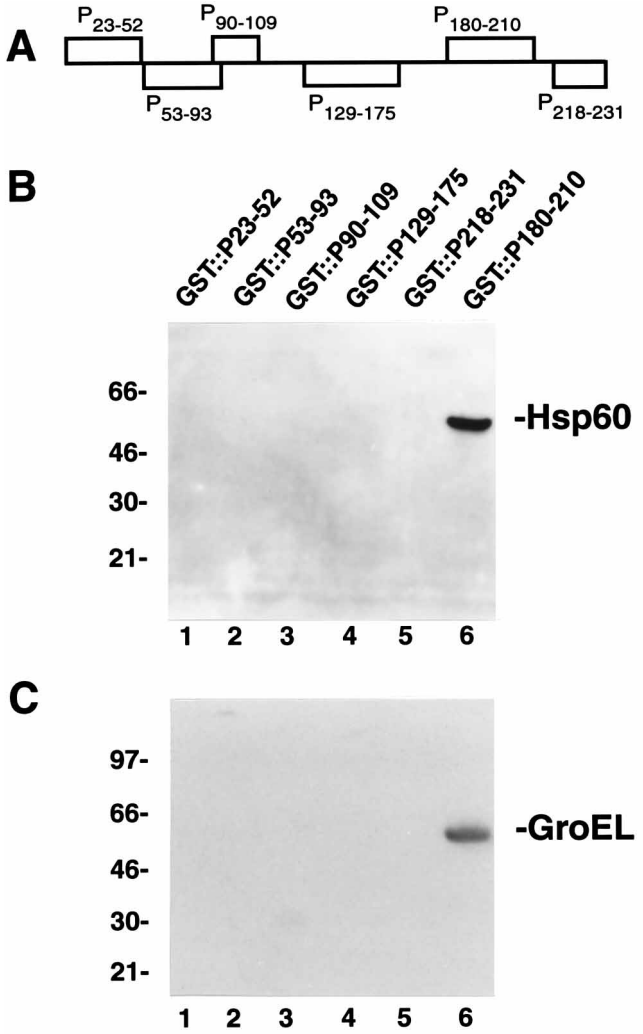

FIG. 3. Mapping the PrP^c-Hsp60/GroEL interaction site with fragments of PrP^c as fusions with GST. (A) Six fragments of PrP^c were designed on the basis of biochemical predictions such as hydrophilicity, antigenicity, and secondary structures and represent aa 23 to 52, 53 to 93, 90 to 109, 129 to 175, 180 to 210, and 218 to 231 (47). (B) Mapping analysis of the PrP-Hsp60 interaction site with the six GST fusion PrP fragments. The fragments (2 μg each) bound to glutathione-Sepharose were incubated with 10 μg of Hsp60. The beads were washed and resuspended in sample buffer. Each (4 μl) of the fragments was analyzed on a 12.5% SDS gel, blotted onto a polyvinylidene difluoride membrane, developed with an anti-Hsp60 antibody and chemiluminescence. (C) Mapping analysis of PrP-GroEL interaction site. The fragments (2 μg each) bound to glutathione-Sepharose were incubated with 10 μg of GroEL. The beads were washed and resuspended in sample buffer. Each (4 μl) of the fragments were analyzed on a 12.5% SDS gel and blotted (polyvinylidene difluoride membranes). GroEL was detected by chemiluminescense with an anti-GroEL antibody. Molecular sizes (in kilodaltons) are indicated on the left.

PrP fused to GST and tested their ability to bind Hsp60 and GroEL. These peptides were designed on the basis of biochemical predictions regarding hydrophilicity, antigenicity, and secondary structure (47) and represent aa 23 to 52, 53 to 93, 90 to 109, 129 to 175, 180 to 210, and 218 to 231 (Fig. 3A). The immobilized peptides were incubated with Hsp60 and GroEL individually. This mapping analysis identified Hsp60 (Fig. 3B, lane 6) and GroEL (Fig. 3C, lane 6) only in the presence of GST::P180-210, demonstrating that it is only the PrP region represented by amino acids 180 to 210 which interacts with the molecular chaperone.

DISCUSSION

Our results show that a GST-PrP^c fusion protein binds specifically to Hsp60 in an *S. cerevisiae* environment as well as in vitro. The Hsp60 family is one of the best-characterized mem-

bers of the molecular chaperones which mediate ATP-dependent folding of polypeptide chains (13, 18, 22, 23) and which are widely distributed and conserved between prokaryotes and mammals. Human Hsp60 (544 aa) is proposed to form tetradecameric complexes in vivo as shown in the crystal structure of the prokaryotic homolog GroEL (10). The cDNAs isolated by the two-hybrid screen encode N-terminally-truncated proteins of 399, 317 and 246 aa in length, comprising at least in part the apical domain of the Hsp60 monomer. This apical domain contains several amino acid residues which specifically mediate peptide binding in the case of GroEL (14). Specificity of the PrP^c-Hsp60 interaction in vivo was confirmed with the false baits LexA-bicoid and LexA-NFI/CTF2 as well as authentic LexA and LexA-GST. We confirmed the interaction in vitro using recombinant GST-PrP^c and recombinant full-length Hsp60 as well as GroEL. This result indicates that the PrP^c-Hsp60 interaction does not involve additional factors. The recombinant rPrP27-30 (48) represents the proteinase K-sensitive isoform of the proteinase K-resistant core PrP27-30 isolated from scrapie preparations. The in vitro interaction between rPrP27-30 and Hsp60 appears to be at least as strong as that with PrP^{c}23-231, revealing that the core region of PrP (aa 90 to 231) is sufficient for binding to Hsp60.

Identification of the interaction site between aa 180 and 210 by mapping of PrP^c peptides showed that binding of Hsp60 to PrP^c occurs within a highly conserved region of the prion protein containing aa 180, 198, 200, and 210. Mutation of these residues segregate with inherited prion diseases in humans (36). In addition, the chaperone-binding fragment GST:: P180-210 contains, at least in part, the two putative α-helical domains H3 (aa 178 to 191) and H4 (aa 202 to 218) (24). The conversion of α-helical regions into β-sheets of PrP are thought to be responsible for PrP^{Sc} formation. There are several possibilities to suggest a possible physiological relevance of the Hsp60-PrP interaction. (i) Hsp60 might be involved in the propagation of PrP^{Sc} as has been shown for the interaction of the yeast prion-like factor (*psi*$^+$) with the molecular chaperone Hsp104 (12, 50). On the basis of studies with transgenic mice, it has been suggested recently that a species-specific macromolecule, designated protein X, participates in prion formation (43). Protein X was proposed to function as a molecular chaperone facilitating the transformation of PrP isoforms. This unknown factor X might in fact be Hsp60. (ii) Alternatively, Hsp60 could prevent aggregation of PrP^c to PrP^{Sc} amyloids, e.g., by trapping misfolded forms of PrP^c. (iii) Finally, molecular chaperones are known to protect proteins against advanced glycation (16), which is known to contribute to amyloidosis in Alzheimer's disease (46). Similarly, Hsp60 might be capable of preventing advanced glycation of PrP^c.

The observed interaction between PrP^c and Hsp60 implies that the two proteins contact each other directly in the cell. According to several reports, PrP^c and Hsp60 indeed share the same subcellular compartments. Scrapie infectivity can occur in mammalian brain mitochondria (1), the main cellular location of Hsp60. In addition, Hsp60 is found in the endoplasmic reticulum in the Golgi apparatus, in condensing vacuoles, and in secretory granules (45), as well as in membrane fractions of mammalian cells (37). Recently it has been reported that Hsp60 is also located in the cytoplasm of several mammalian tissues (25). In addition to cytosolic forms, PrP^c is also known to use the endosomal pathway via the endoplasmic reticulum and Golgi to the cell surface (9). The subcellular site(s), however, at which the PrP^c-Hsp60 interaction occurs remains unknown.

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