Identification of interaction domains of the prion protein with its 37-kDa/67-kDa laminin receptor

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Cell-binding and internalization studies on neuronal and non-neuronal cells have demonstrated that the 37-kDa/67-kDa laminin receptor (LRP/LR) acts as the receptor for the cellular prion protein (PrP). Here we identify direct and heparan sulfate proteoglycan (HSPG)-dependent interaction sites mediating the binding of the cellular PrP to its receptor, which we demonstrated *in vitro* on recombinant proteins. Mapping analyses in the yeast two-hybrid system and cell-binding assays identified PrPLRPbd1 [amino acids (aa) 144–179] as a direct and PrPLRPbd2 (aa 53–93) as an indirect HSPG-dependent laminin receptor precursor (LRP)-binding site on PrP. The yeast two-hybrid system localized the direct PrP-binding domain on LRP between aa 161 and 179. Expression of an LRP mutant lacking the direct PrP-binding domain in wild-type and mutant HSPG-deficient Chinese hamster ovary cells by the Semliki Forest virus system demonstrates a second HSPG-dependent PrP-binding site on LRP. Considering the absence of LRP homodimerization and the direct and indirect LRP–PrP interaction sites, we propose a comprehensive model for the LRP–PrP–HSPG complex.

Keywords: heparan sulfate proteoglycans/*in vitro* interaction/37-kDa laminin receptor precursor/67-kDa high-affinity laminin receptor/LRP–PrP-binding domains

Introduction

We recently identified the 37-kDa laminin receptor precursor (LRP) as an interactor for the prion protein (PrP) (Rieger *et al.*, 1997; for reviews see Rieger *et al.*, 1999; Gauczynski *et al.*, 2001a). Employing a series of neuronal and non-neuronal cells, we proved that the 37-kDa LRP/67-kDa high-affinity laminin receptor (LR) acts

as the receptor for the cellular PrP (Gauczynski *et al.*, 2001b). In the present manuscript we used the yeast two-hybrid system and cell-binding studies on neuronal as well as non-neuronal cells involving the Semliki Forest virus (SFV) system (for reviews see Liljestrom and Garoff, 1991; Tubulekas *et al.*, 1997) to identify domains on the PrP and the LRP involved in the PrP–LRP interaction on the cell surface. We identified two binding domains for LRP on PrP termed PrPLRPbd1 and PrPLRPbd2. The first one binds directly to LRP, whereas the second one depends on the presence of heparan sulfate proteoglycans (HSPGs) on the cell surface. The yeast two-hybrid system and cell-binding assays on wild-type and mutant HSPG-deficient Chinese hamster ovary (CHO) cells also identified two binding domains for PrP on LRP.

The relationship between 37-kDa LRP and 67-kDa LR is not yet fully understood and has been explained with homodimerization of 37-kDa LRP (Landowski *et al.*, 1995) or an additional factor, such as a polypeptide (Castronovo *et al.*, 1991), which might bind to 37-kDa LRP to form the 67-kDa form of the receptor. The 67-kDa heterodimer might be stabilized by hydrophobic interactions mediated by fatty acids such as palmitate, oleate and stearate bound to 37-kDa LRP and to a galectin-3 (gal-3) cross reacting polypeptide (Landowski *et al.*, 1995; Buto *et al.*, 1998). However, we recently proved that the β -galactoside lectin gal-3 is not present on the surface of neuronal or non-neuronal cells used for PrP-binding/internalization studies (Gauczynski *et al.*, 2001b) and anti-gal-3 antibodies failed to compete for the 37-kDa LRP/67-kDa LR-mediated binding and internalization of the cellular PrP (Gauczynski *et al.*, 2001b), suggesting that gal-3 is not a partner of the 37-kDa LRP in this context. In this study we investigated by a yeast two-hybrid system analysis whether gal-3 interacts with 37-kDa LRP and/or the cellular PrP. In addition, we investigated whether 37-kDa LRP interacts with itself in the yeast two-hybrid and analysed the monomer/dimer status of the receptor by size-exclusion chromatography. Both PrP (Gabizon *et al.*, 1993; Caughey *et al.*, 1994; Chen, S.G. *et al.*, 1995; Brimacombe *et al.*, 1999) and the 37-kDa/67-kDa LR (Guo *et al.*, 1992; Kazmin *et al.*, 2000) bind to heparan sulfates. HSPGs are required for the binding of the fibroblast growth factor (FGF) to its FGFR receptor (Yayon *et al.*, 1991; Spivak *et al.*, 1994; Venkataraman *et al.*, 1999) and act as initial attachment receptors for bacteria (Chen, T. *et al.*, 1995) and viruses including alphaviruses (Byrnes and Griffin, 1998), human immunodeficiency virus (HIV) type 1 (Mondor *et al.*, 1998) and vaccinia virus (Chung *et al.*, 1998). Heparan sulfates are components of amyloid plaques in prion diseases (Gabizon *et al.*, 1993). We investigated the role of HSPGs as possible co-factors for 37-kDa LRP mediating PrP binding. We also constructed recombinant (rec.) SFV vectors

leading to the expression of an LRP mutant termed LRPdelBD::FLAG lacking the direct-binding domain for PrP in wild-type and mutant HSPG-deficient CHO cells. We compared the PrP-binding capacity of these cells with wild-type and mutant CHO cells hyperexpressing wild-type LRP::FLAG. In light of our findings that 37-kDa LRP fails to form homodimers, and that HSPGs mediate the binding of PrP to 37-kDa LRP, the relationship between 37-kDa LRP and 67-kDa LR might be explained by the association of LRP with HSPGs as outlined in a proposed model for the LRP–PrP–HSPG complex on the cell surface.

Results

Identification of a direct LRP interaction domain on PrP by the yeast two-hybrid system

To determine the domains of PrP interacting directly with LRP, we employed a yeast two-hybrid analysis with truncated PrP molecules in the bait position and LRP44–295 in the prey position. Only truncated PrP retaining the regions amino acids (aa) 144–179 (Figure 1A, rows 6 and 7) interacted with LRP. This region contains domains corresponding to the first α -helix (aa 144–154), the second β -strand (aa 161–164) and the first amino acid of the second α -helix (aa 179–193) of the human PrP (Zahn *et al.*, 2000). Regions from aa 23 to 143 of the human PrP are not sufficient for binding to LRP (Figure 1A, rows 1–5). Regions from aa 180 to 230 of human PrP (row 8) are not required for the direct interaction between PrP and LRP. We termed this LRP interaction domain on PrP PrPLRPbd1.

Retrenchment of the direct PrP-binding domain on LRP by the yeast two-hybrid system

Recently, we mapped a direct PrP-binding domain on LRP between aa 157 and 180 (Rieger *et al.*, 1997) employing N-terminally truncated LRP molecules. In order to retrench this binding domain precisely we co-expressed the C-terminally truncated LRP molecules LRP44–101 and LRP44–160, respectively, together with full-length PrP in the yeast two-hybrid system. Both truncations failed to interact with PrP (Figure 1B) confirming that this direct PrP-binding site coincides with the laminin-binding domain (aa 161–180). Expression of an LRP mutant lacking this direct PrP-binding domain (LRPdelBD161–180) in CHO cells (Figure 4K) showed that LRPdelBD161–180::FLAG was still able to bind to PrP, indicating the presence of a second binding site for PrP on LRP, which locates either between aa 101 and 160 or 181 and 295 of LRP.

PrP144–179 interacts directly with LRP161–179 in the yeast two-hybrid system

In order to prove a direct interaction between the PrP and the 37-kDa/67-kDa LRP/LR via PrP144–179 and LRP161–179, we co-expressed both protein domains in bait and prey position, respectively, resulting in a strong interaction (Figure 1C, row 1). In contrast, huPrP144–179 failed to interact with LRP180–295 (row 2) or LRP44–160 (row 3).

β -galactoside gal-3 does not interact with PrP or LRP in the yeast two-hybrid system

The association of gal-3 with the LRP has been suggested (Landowski *et al.*, 1995; Buto *et al.*, 1998). However, gal-

3 antibodies do not influence the LRP-dependent binding/internalization of PrP on the cell surface, suggesting that this molecule does not act as a co-receptor for LRP (Gauczynski *et al.*, 2001b). For confirmation that gal-3 does not interact with PrP, we expressed gal-3 in bait and PrP in prey position of the yeast two-hybrid system resulting in no interaction between the two proteins (Figure 2A, row 2). Gal-3 also failed to interact with LRP in the yeast two-hybrid system (Figure 2A, row 3).

37-kDa LRP fails to interact with itself in the yeast two-hybrid system and appears monomeric by size-exclusion chromatography

The polymorphism of the LR is still unclear. In order to test whether homodimerization of LRP could account for this 37-kDa/67-kDa polymorphism and to understand better the configuration of the LRP–PrP-binding complex, we cloned the cDNA encoding for LRP in the bait and prey position of the yeast two-hybrid system. LRP fails to interact with itself (Figure 2B, row 2), suggesting that LRP is unable to directly form homodimers. For confirmation we purified LRP::FLAG from SFV RNA-LRP::FLAG-transfected BHK cells by anti-FLAG antibody chromatography to homogeneity and analysed the native protein by SDS–PAGE and size-exclusion chromatography. The protein migrated as a 37-kDa protein on an SDS–polyacrylamide gel (Figure 2C, lane 1) and eluted as a 40-kDa protein from a native size-exclusion column (Figure 2D), confirming that 37-kDa LRP is monomeric under native conditions. Thus, the 67-kDa form of the LR may result from the association of the LRP with other molecules such as HSPGs.

Identification of the PrP interaction domains PrPLRPbd1 and PrPLRPbd2 by binding assays with prion peptides to NT2 and N2a cells

The yeast two-hybrid system identified the domain aa 144–179 of PrP as a direct binding site for LRP termed PrPLRPbd1. To identify other domains of PrP, which might bind indirectly to LRP, we exposed NT2 and N2a cells to glutathione *S*-transferase (GST)-fused PrP peptides covering the entire PrP sequence. Besides peptide 129–175 encompassing the direct binding domain PrPLRPbd1, GST::PrP53–93 bound to the cells in an LRP-dependent fashion. The binding of GST::PrP53–93 (Figure 3A) and GST::PrP129–175 (Figure 3C) is shown on NT2 cells in comparison with GST::PrP90–109 (Figure 3B). This binding can be inhibited by addition of the LRP antibody W3 (insets in Figure 3A and C). The binding properties of the whole array of peptides are also shown on N2a cells (Figure 3D–J). Only GST::PrP53–93 (Figure 3E) and GST::huPrP129–175 (Figure 3H) bound to the cells dependent on LRP–LR (LRP antibody competition is shown in the bottom insets of Figure 3). The integrin laminin receptor VLA6 does not co-localize with PrP or LRP–LR on the surface of neuroblastoma cells (Gauczynski *et al.*, 2001b). The addition of an anti-VLA6 antibody, failed also to compete for the binding of GST::PrP53–93 or GST::PrP129–175 to N2a cells (Figure 3E and H, top insets), confirming that VLA6 does not act as a receptor for PrP. We termed the indirect binding domain PrPLRPbd2. As the two binding domains are located N- and C-terminally of the proteinase K

cleavage site of PrPres, we tested longer peptides in our binding assay corresponding to the two fragments that result from proteolytic cleavage of PrP, i.e. PrP23–89 and PrP90–230. Both peptides bound to both cell types in an LRP-dependent manner (Table I). Combining these data with the results from the yeast two-hybrid system (Figure 1) we conclude that two binding sites on PrP for LRP termed PrPLRPbd1 (aa 144–179) and PrPLRPbd2 (aa 53–93) do exist. Results of the PrP peptide binding studies to N2a and NT2 cells including antibody competitions are summarized in Table I.

Binding of PrP to LRP via PrPLRPbd2 is dependent on HSPGs

The cell-binding assay led to the identification of an additional binding domain for LRP on PrP which was not identified in the yeast two-hybrid system, indicating that a third molecule is necessary to mediate the binding of LRP to PrPLRPbd2. It has been reported that 60% of the binding of rec. chicken PrP to CHO cells depends on the presence of endogenous heparan sulfates (Shyng *et al.*, 1994). Mutant CHO cells (S745) are severely deficient in HSPGs because of an altered xylose transferase activity, the first enzyme required for glycosylaminoglycan (GAG) synthesis (Esko *et al.*, 1985) and, therefore, represent an appropriate model system to investigate whether HSPGs might represent the third interactor in the binding of PrPLRPbd2 to LRP. First, we proved that the binding of rec. GST::huPrP23–230 to wild-type CHO cells (Figure 4A) and to the mutant CHO cells (Figure 4B) was LRP–LR-dependent (Figure 4C and D). We then saturated selectively PrPLRPbd1 by incubating GST::huPrP23–230 with a monoclonal PrP antibody directed against the domain 140–180 of PrP. Obstructing PrPLRPbd1 inhibited the binding of the rec. PrP to HSPG-deficient (Figure 4F) but not to wild-type CHO cells (Figure 4E), demonstrating that binding of PrP to LRP via PrPLRPbd2 needs the presence of HSPGs. The peptide GST::PrP53–93 corresponding to PrPLRPbd2, which bound to normal cells (Figure 4G), did not bind to HSPG-deficient cells (Figure 4H). However, the binding was restored in a dose-dependent manner after addition of soluble HSPGs (Figure 4I and J), confirming that the interaction of PrP to LRP via PrPLRPbd2 is HSPG dependent.

Identification of an HSPG-dependent second binding site for PrP on LRP

The two binding sites on PrP, one of which is direct and the other indirect, suggested that two ‘acceptor’ sites might also exist on LRP. To test this hypothesis we adapted our SFV expression system to CHO cells. We then expressed LRP::FLAG and a mutant LRP lacking the direct PrP-binding domain (aa 161–180), termed LRPdelIBD::FLAG,

in wild-type CHO cells and the mutant CHO-S745 cell line lacking HSPGs (Figure 4K, lower panels). In wild-type CHO cells (Figure 4K, lanes 1–6) the binding of GST::huPrP was enhanced, when LRP::FLAG was hyperexpressed (Figure 4K, lanes 2 versus 6). Hyperexpression of LRPdelIBD::FLAG did not reduce the amount of the bound GST::huPrP (Figure 4K, lane 4),

Fig. 2. LRP fails to interact with itself in the yeast two-hybrid system and appears monomeric by native size-exclusion chromatography. Gal-3 fails to interact with PrP and LRP. (A) huPrP23–230 and LRP fail to interact with the β -galactoside lectin gal-3 in the yeast two-hybrid system. huPrP23–230 fused to GST in bait position was co-expressed with LRP44–295 in prey position (row 1), gal-3 in bait position was co-expressed with GST::huPrP23–230 (row 2) and LRP44–295 (row 3) in prey position. (B) LRP fails to interact with itself in the yeast two-hybrid system. GST (row 1), LRP44–295 (row 2) and GST::huPrP23–230 (row 3) were expressed in bait and GST (row 1) and LRP44–295 (row 2 and 3) in prey position of the yeast two-hybrid system. Detection (A and B) by the β -galactosidase reporter system. (C) Analysis of rec. LRP::FLAG on SDS–PAGE. One microgram of rec. LRP::FLAG purified under native conditions from the SFV system was analysed on a 12.5% SDS–PA gel stained with silver (lane 1). Marker proteins are indicated. (D) Analysis of rec. native LRP::FLAG by size-exclusion chromatography. Homogeneous LRP::FLAG (2.2 μ g) were analysed by size-exclusion chromatography on a Superose 12PC3.2/30 column (Amersham Pharmacia). Marker proteins are indicated.

Fig. 1. Identification of direct PrP–LRP-interaction domains. (A) Identification of the direct PrP–LRP interaction domain on PrP. HuPrP23–93 (row 1), huPrP23–118 (row 2), huPrP23–127 (row 3), huPrP23–131 (row 4), huPrP23–143 (row 5), huPrP23–154 (row 6), huPrP23–181 (row 7) and huPrP180–230 (row 8) were co-expressed in fusion with GST in the bait position together with LRP in prey position of the yeast two-hybrid system. (B) Retrenchment of the direct PrP–LRP interaction domain on LRP (Rieger *et al.*, 1997). LRP44–101 (row 1), LRP44–160 (row 2) and LRP44–295 (row 3) were co-expressed in prey position together with huPrP23–230 fused to GST in bait position of the yeast two-hybrid system. *Interactions between LRP157–295 and LRP180–295 (in prey position) versus huPrP23–230 fused to GST (in bait position) have been investigated previously (Rieger *et al.*, 1997). (C) PrP144–179 interacts directly with LRP161–179 in the yeast two-hybrid system. PrP144–179 fused to GST in bait position was co-expressed with LRP161–179 (row 1), LRP180–295 (row 2) and LRP44–160 (row 3) in prey position of the yeast two-hybrid system. (A–C) All interactions were monitored by the β -galactosidase reporter system.

Fig. 3. Identification of PrP-interaction domains for LRP/LR by binding assays with rec. prion peptides on NT2 and N2a cells. (A–C) Binding to NT2 cells. NT2 cells were incubated with PrP peptides fused to GST in the absence (A–C) and presence (insets in A–C) of the pre-incubated pAb LRP W3 (dilution 1:50). The following peptides (4 µg/ml) were used: GST::PrP53–93 (A), GST::PrP90–109 (B) and GST::PrP129–175 (C). Immunofluorescence analysis was performed by triple labelling involving actin staining (phalloidin, red), nuclear staining [4',6-diamidino-2-phenylindole (DAPI), blue] and GST staining (sec. Ab FITC, green) (magnification ×630). (D–J) Binding to N2a cells. N2a cells were incubated with PrP peptides fused to GST in the absence (D–J) or presence of either pre-incubated pAb LRP (dilution 1:50) (bottom insets in D–J) or pre-incubated mAb VLA6 (dilution 1:50) (top inset in E and H). The following peptides (4 µg/ml) were used: GST::PrP23–52 (D), GST::PrP53–93 (E), GST::PrP90–109 (F), GST::PrP110–128 (G), GST::PrP129–175 (H), GST::PrP180–210 (I) and GST::PrP218–230 (J). Immunofluorescence was performed with mAb GST, sec. Ab Texas Red, DAPI staining (magnification ×400).

Table I. Summary of the binding behaviour of individual GST-fused PrP peptides to NT2 and N2a cells including LRP–LR and VLA6 antibody competition

Peptide (aa)	Binding to N2a cells	LRP–LR antibody competition	Binding to NT2 cells	LRP–LR antibody competition
PrP53–93	+++	+++ ^a	+++	+++
PrP90–109	–	–	–	–
PrP110–128	–	–	–	–
PrP129–175	+++	+++ ^a	+++	+++
PrP180–210	–	–	–	–
PrP218–230	–	–	–	–
PrP23–89	+++	+++	+++	+++
PrP90–230	+++	+++	+++	+++

^aNo competition with a VLA6 antibody.
+++, strong binding/competition.
–, no binding/competition.

indicating that the binding was HSPG mediated. In mutant CHO-S745 cells lacking HSPGs (Figure 4K, lanes 7–13) the amount of bound GST::huPrP was also enhanced in LRP::FLAG hyperexpressing cells compared with

non-transfected cells (Figure 4K, lanes 8 and 13). CHO-S745 cells expressing LRPdelBD::FLAG, however, showed a reduced binding of GST::huPrP (Figure 4K, lane 10), similar to non-transfected cells (Figure 4K, lane 13) suggesting that the second indirect PrP-binding site was not functioning in the absence of HSPGs. In order to confirm that HSPGs are responsible for the binding of PrP to the second binding domain on LRP, we added HSPGs to cells overexpressing LRPdelBD::FLAG resulting in a total restoration of the PrP binding (Figure 4K, lane 11). HSPGs failed to increase the binding of GST::huPrP to CHO wild-type (Figure 4L, lanes 2 and 3) and CHO-S745 cells (Figure 4L, lanes 5 and 6) due to the presence of the direct binding domains on PrP and LRP. We conclude from these data that two binding sites for PrP on LRP exist: a direct one, which is located from aa 161–179, and a second indirect one, which resides either between aa 101 and 160 or between aa 180 and 295 of LRP.

Interaction of PrP and LRP in vitro

The direct binding domains on LRP (aa 161–179) and PrP (aa 144–179) should allow the two proteins to interact with

each other *in vitro*. GST-fused LRP (Rieger *et al.*, 1997) and immobilized FLAG::huPrP (Figure 4M, lanes 1 and 2) were able to interact with each other *in vitro* as shown in the pull down assay depicted in Figure 4M. As already observed on wild-type CHO and CHO-S745 cells, HSPGs did not influence the interaction due to the presence of the direct interaction domains (Figure 4M, lanes 2 and 3).

However, HSPGs did affect the LRP-PrP53-93 interaction (HSPG-dependent binding domain on PrP) and the LRPdelBD-PrP interaction (lacking the direct binding domain on LRP) in CHO-S745 cells (Figure 4G-J and K, respectively).

Discussion

Cell-binding and internalization studies proved that the 37-kDa LRP/67-kDa LR acts as the receptor for the cellular PrP, PrP^C, on the cell surface (Gauczynski *et al.*, 2001b). In order to investigate the interaction domains on PrP and LRP-LR mediating the binding of these two proteins a series of interaction studies employing the yeast two-hybrid system as well as PrP-binding assays with neuronal and non-neuronal cells including the SFV system have been performed.

Fig. 4. Influence of HSPGs on the LRP-LR-PrP-binding reaction analysed by wild-type and mutant HSPG-deficient CHO cells; use of an LRP deletion mutant lacking the direct PrP-binding domain on LRP; PrP-LRP *in vitro* interaction studies. (A-F) Binding of GST::huPrP23-230 to CHO cells and to HSPG-deficient CHO cells (S745) (Esko *et al.*, 1985) in the absence and presence of antibodies. Binding of 4 µg/ml GST::huPrP23-230 to CHO wild-type cells in the absence of any antibody (A), in the presence of the pAb LRP W3 (1:50) (C), mAb SAF 70 (aa 140-180 of PrP) (E). Binding of GST::huPrP23-230 to CHO-S745 cells in the absence of any antibody (B), in the presence of the pAb LRP W3 (D) and in the presence of the mAb SAF 70 (F). The mAb SAF70 was used at a 1:1000 dilution to saturate GST::huPrP23-230 while pAb LRP was pre-incubated with the cells at a 1:50 dilution prior to addition of the rec. PrP. Immunofluorescence analysis: pAb LRP (sec. Ab FITC), mAb 3F4 (sec. Ab Texas Red; DAPI staining; magnification $\times 400$). (G-J) Binding of GST::PrP53-93 to CHO wild-type and CHO-S745 cells. Binding of 4 µg/ml GST::huPrP53-93 to CHO wild-type (G) and CHO-S745 cells in the absence of HSPGs (H), and in the presence of 10 (I) and 40 µg/ml (J) HSPGs, respectively. Immunofluorescence analysis: mAb GST (sec. Ab Texas Red; DAPI staining; magnification $\times 400$). (K) Binding of PrP by wild-type CHO and mutant HSPG-deficient CHO-S745 cells hyperexpressing LRP::FLAG or LRPdelBD::FLAG (lacking aa 161-180). CHO cells (lanes 1-6) either hyperexpressing LRP::FLAG (lanes 1 and 2), LRPdelBD::FLAG (lanes 3 and 4) by the SFV system or non-transfected (lanes 5 and 6) were incubated with 5 µg/ml GST::huPrP (lanes 2, 4 and 6). CHO-S745 cells (lanes 7-13) either hyperexpressing LRP::FLAG (lanes 7 and 8), LRPdelBD::FLAG (lanes 9-11) by the SFV system or non-transfected (lanes 12 and 13) were incubated with 5 µg/ml GST::huPrP (lanes 8, 10, 11 and 13). HSPGs (40 µg/ml) were added simultaneously with GST::huPrP to the CHO-S745 cells overexpressing LRPdelBD::FLAG (lane 11). Total cell extracts were analysed by western blotting employing the mAb 3B5 (upper panels) or pAb LRP W3 (lower panels). (L) Binding of PrP by non-transfected wild-type CHO and mutant HSPG-deficient CHO-S745 cells. Non-transfected CHO wild-type cells (lanes 1-3) and non-transfected CHO-S745 cells (lanes 4-6) were incubated with 5 µg/ml GST::huPrP (lanes 2, 3, 5 and 6). HSPGs (40 µg/ml) were added simultaneously with GST::huPrP to both cell types (lanes 3 and 6, respectively). Total cell extracts were analysed as described in (K). Interaction of rec. FLAG::PrP and rec. GST::LRP *in vitro* (M). FLAG::huPrP23-230 (0.5 µg) immobilized on anti-FLAG Sepharose beads analysed on a 12% SDS-PA gel stained with silver (lane 1) and by western blotting employing the pAb JB007 (lane 2) were incubated with 1 µg of GST::LRP in the absence (lane 4) or in the presence of 1.5 µg/µl HSPGs (lane 3), 0.5 µg of GST in the absence (lane 6) or the presence of 1.5 µg/µl HSPGs (lane 5). Unloaded beads were incubated with 1 µg of GST::LRP in the presence of 1.5 µg/µl HSPGs (lane 7). Beads after washing were analysed by western blotting on a 12% SDS-PA gel employing mAb GST (sec. antibody POD).

Mapping of LRP interaction sites on PrP

First, we aimed to determine which part of the PrP interacts with LRP. We performed a yeast two-hybrid analysis with a series of PrP deletion variants and employed a series of PrP peptides covering the entire PrP in various cell-binding assays.

In the yeast two-hybrid system, we identified the PrP domain aa 144–179 as a direct LRP-binding domain, termed PrPLRPbd1. This binding domain was confirmed in cell-binding assays in N2a and NT2 cells with PrP peptides encompassing the entire PrP sequence. As already observed with full-length PrP (Gauczynski *et al.*, 2001b), the staining pattern with the PrP peptides to N2a and NT2 cells is also punctuate due to either receptor clustering or PrP–PrP peptide aggregation, or both. Receptor clustering was observed with a variety of other cell-surface receptors such as the FGF receptor (Utton *et al.*, 2001), the muscle nicotinic acetylcholine receptor (AChR) (Hoch *et al.*, 2001) or the tumour necrosis factor receptor (TNFR55) (De Wilde *et al.*, 2001). In addition to the yeast two-hybrid assay, which identified the direct PrP–LRP–LR interaction domain PrPLRPbd1, the cell-binding assay identified a second binding domain between aa 53 and 93 of PrP, termed PrPLRPbd2. This domain was not functional in the yeast two-hybrid system, indicating that an additional factor lacking in the yeast cell nucleus is required for mediating the interaction between LRP and PrP through PrPLRPbd2. Employing a mutant HSPG-deficient CHO cell line, the binding assay revealed that PrPLRPbd2 binds to LRP via HSPGs. This finding was confirmed by using the PrP peptide 53–93 corresponding to PrPLRPbd2. PrP53–93 failed to bind to the mutant CHO cells but the binding was restored in a dose-dependent manner by the addition of soluble HSPGs.

Our mapping data, which resulted in the identification of two LRP-binding sites on PrP, are consistent with the results obtained with PrP knock-out mice expressing PrPs with N-proximal deletions, suggesting that PrP could bind to its natural ligand, termed Lprp, which could represent a PrP receptor, in a region C-terminal to aa 134 of the PrP (Shmerling *et al.*, 1998). In line with this study, PrPLRPbd1 would correspond to the Lprp-binding domain. The same authors hypothesized the existence of a second domain located more N-terminally initiating a signal transduction necessary to maintain normal cellular functions. Recently, a signal transduction activity of the PrP by activation of the tyrosine kinase Fyn was described (Mouillet-Richard, 2000). As PrP resides as a GPI-anchored protein outside the cell, whereas the tyrosine kinase Fyn locates to the inner plasma membrane inside the cell, transmembrane orientated LRP–LR might be a reasonable candidate mediating the intracellular signal transduction between PrP^c and Fyn. The cytosolic domain of N-syndecans (syndecan-3), one of a family of four transmembrane cell surface HSPGs, has recently been shown to bind to complexes containing c-Src and Fyn kinases (Kinnunen *et al.*, 1998). This activity is related to neurite outgrowth (Kinnunen *et al.*, 1998). Hence, it is also conceivable that HSPGs might be necessary for the so far hypothetical LRP–LR-mediated signal transduction between PrP^c and Fyn. A possible intercellular role of the PrP–LRP–LR interaction involving HSPGs which might result in signalling or cell attachment (for review see

Gauczynski *et al.*, 2001a) will be further investigated in detailed cell–cell interaction assays.

Recently, studies on the transmission of human prions to transgenic mice suggested that a so far unidentified protein X may participate in the formation of PrPres. Substitution of residues 167, 171 and 218 prevented PrPres formation suggesting that protein X may interact with those residues of the PrP (Kaneko *et al.*, 1997). As aa 167 and 171 reside within the direct PrP–LRP interaction domain termed PrPLRPbd1 it cannot be excluded that LRP–LR might function as protein X. Application of LRP–LR in PrP oligo/multimerization processes may enlighten a possible role of the 37-kDa/67-kDa LR in PrPres formation.

Mapping of the PrP interaction sites on LRP

A yeast two-hybrid analysis with C-terminal LRP truncations extended a previous analysis (Rieger *et al.*, 1997) and retrenched the direct PrP-binding domain to aa 161–179 of LRP. As the binding domain PrPLRPbd2 on PrP is HSPG dependent, we hypothesized that a second indirect binding domain for PrP may also exist on LRP. To test this hypothesis, we analysed PrP-binding to wild-type CHO cells, to mutant HSPG-deficient CHO cells and to both cell types hyperexpressing either the full-length LRP::FLAG or an LRP mutant lacking the direct PrP-binding domain (aa 161–180), termed LRPdelBD::FLAG. HSPGs failed to increase the binding of GST::huPrP to CHO wild-type and CHO-S745 cells due to the presence of the direct binding domains on PrP and LRP. CHO and CHO-S745 cells transfected with recombinant SFV RNAs further revealed the role of HSPGs in the binding of PrP to LRP. Hyperexpression of LRP::FLAG or LRPdelBD::FLAG in wild-type CHO cells resulted in approximately the same increase of GST::huPrP-binding when compared with non-transfected cells. This indicates that binding of PrP to LRP::FLAG can occur via a second PrP-interaction domain present in LRPdelBD::FLAG. In HSPG-deficient CHO cells, however, PrP-binding to cells hyperexpressing LRPdelBD::FLAG was similar to non-transfected cells, indicating that the second interaction domain on LRP was not functioning. In contrast, LRP::FLAG hyperexpressing HSPG-deficient CHO cells showed an increased binding of GST::huPrP due to the presence of the direct HSPG-independent PrP-binding domain (aa 161–179). The addition of HSPGs to LRPdelBD::FLAG hyperexpressing HSPG-deficient CHO cells restored GST::huPrP binding to levels achieved with LRP::FLAG hyperexpressing cells. The data demonstrate the existence of a second HSPG-dependent PrP-binding domain on LRP, residing either between aa 101 and 160 or aa 181 and 285 of LRP. The identification of a HSPG-binding domain on LRP–LR which resides between aa 205 and 229 of LRP–LR (Kazmin *et al.*, 2000) suggests that this domain may represent the HSPG-dependent-binding site for PrP. A monoclonal antibody directed against aa 167–243 of LRP reduces PrP-binding to neuronal cells (Gauczynski *et al.*, 2001b) suggesting that the indirect binding domain may reside between aa 180 and 285 rather than aa 101–160 of LRP.

Interaction of PrP and LRP *in vitro*

Rec. purified PrP and LRP interact with each other *in vitro* due to the presence of the direct PrP–LRP interaction

Fig. 5. Model for the function of LRP-LR as the receptor for PrP. The PrP molecule binds to LRP-LR via PrPLRPbd1 and PrPLRPbd2. PrPLRPbd2 (aa 53–93) is dependent on the presence of a heparan sulfate arm of a HSPG molecule whereas PrPLRPbd1 (aa 144–179) interacts directly with LRP-LR (as shown in the yeast two-hybrid system). The simultaneous presence of both PrPLRPbd1 and PrPLRPbd2 would stabilize considerably the binding of the entire PrP molecule to its receptor. Direct binding of LRP-LR to PrP occurs via the direct binding site located between aa 161–179 of LRP-LR (Figure 1). The indirect HSPG-dependent binding domain might locate between aa 101 and 160 or between aa 180 and 285 [presumably aa 205 and 229 of LRP (Kazmin *et al.*, 2000)]. The association of LRP-LR with HSPGs might explain the relationship between 37-kDa LRP and 67-kDa LR.

domains. The influence of HSPGs on the indirect PrP-LRP interaction domains, however, was only detectable on the HSPG-deficient CHO-S745 cells employing PrP53–93 (representing the indirect binding domain on PrP) and LRPdelBD (lacking the direct binding domain on LRP).

The relationship between 37-kDa LRP and 67-kDa LR

Attempts to isolate the gene for the 67-kDa LR revealed a cDNA fragment encoding the 37-kDa LRP (Rao *et al.*, 1983; Yow *et al.*, 1988; Grosso *et al.*, 1991). Pulse-chase experiments with 37-kDa LRP specific antibodies demonstrated that 37-kDa LRP is the precursor of 67-kDa LR (Rao *et al.*, 1989; Castronovo *et al.*, 1991). The 37-kDa molecule encoded by the full-length gene identified for the LR, is virtually identical to the ribosomal protein p40. The 37LRP/p40 evolved from a ribosomal protein essential for protein synthesis lacking any laminin-binding abilities, to a laminin-binding cell surface receptor (for review see Ardini *et al.*, 1998). The molecular structure of the 37-kDa LRP/67-kDa LR and the mechanism by which the 37-kDa LRP forms the mature 67-kDa LR remains unclear. Our data from a yeast two-hybrid analysis show that LRP fails to interact with itself, an argument against the hypothesis of a direct homodimerization. In addition, homogeneous rec. LRP::FLAG appears to be monomeric as analysed by size-exclusion chromatography. Although we only used 0.1% Triton X-100 in the purification procedure, we cannot exclude that this small amount of non-ionic detergent may disrupt a native dimeric state of the receptor. A recent study suggested that acylation of LRP would allow it to associate with a heterologous molecule (Buto *et al.*, 1998). In the light of our results, the relationship between the 37-kDa LRP and 67-kDa LR

might be explained by the association of an LRP molecule with heparan sulfates.

Role of heparan sulfates in the PrP-LRP interaction process

The binding of chicken PrP to the surface of mammalian cells has been shown to depend partly on the availability of heparan sulfates expressed by these cells (Shyng *et al.*, 1995). PrP interacts with heparan sulfates (Gabizon *et al.*, 1993; Caughey *et al.*, 1994; Chen, S.G. *et al.*, 1995; Brimacombe *et al.*, 1999). Recent studies demonstrated that the binding of copper to PrP which occurs within the octarepeat region (Brown *et al.*, 1997) (corresponding to PrPLRPbd2) can be competed by the addition of HSPGs (Brimacombe *et al.*, 1999), confirming that this region of PrP binds to HSPGs. Finally, LRP-LR has also been shown to be a heparin/heparan sulfate-binding molecule (Guo *et al.*, 1992; Kazmin *et al.*, 2000). The predicted α -helical structure (aa 205–229) of LRP-LR is proposed to have heparin-binding characteristics (Kazmin *et al.*, 2000). The requirement of heparin-like molecules for the formation of a ligand-receptor complex is not unprecedented and is well illustrated by the example of the binding of the FGF to its receptor, FGFR (Yayon *et al.*, 1991; Spivak *et al.*, 1994; Venkataraman *et al.*, 1999). All these data match very well with our findings, providing a comprehensive model of the PrP-LRP-LR interaction (Figure 5). A heparan sulfate arm of a cell-surface HSPG molecule might be located between PrPLRPbd2 and the indirect binding domain of LRP to create a sandwich interaction site, whereas PrP would interact with LRP-LR (aa 161–179) directly via PrPLRPbd1. We cannot exclude that HSPGs intervene in a more indirect manner, by changing the conformation of LRP-LR as to render it amenable to its interaction with the PrPLRPbd2 region of PrP.

Components blocking the direct and indirect PrP–LRP interaction domains on PrP and LRP may represent a novel class of molecules suitable for a therapeutic intervention in prion diseases.

Materials and methods

Construction of pSFV1-LRPdelBD::FLAG-preparation of SFV mRNAs in vitro

The LRP mutant pSFV1-LRPdelBD::FLAG (deletion of the direct PrP-binding domain located between aa 161 and 180) was generated by the QuikChange™ site-directed mutagenesis method (Stratagene) using the pSFV1-LRP::FLAG plasmid DNA (Gauczynski *et al.*, 2001b) as template for PCR and confirmed by dideoxy sequencing. The rec. plasmid DNAs pSFV3-lacZ (Life Technologies), pSFV1-LRP::FLAG and pSFV1-LRPdelBD::FLAG were linearized, purified and transcribed as described (Gauczynski *et al.*, 2001b). The correct length of the transcripts was verified by agarose gel electrophoresis. RNA was stored at -20°C .

Mammalian cell culture, transfection and co-transfection studies with the SFV system

Mutant CHO cells (S745) deficient in xylose transferase (Esko *et al.*, 1985) as well as wild-type CHO (K1) were cultivated in NUT.MIX.F-12(HAM) supplemented with GLUTAMAX-I (Gibco-BRL), 10% fetal calf serum (FCS), 100 $\mu\text{g}/\text{ml}$ penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin at 37°C with 5% CO_2 . Transfections and co-transfections were carried out as described (Gauczynski *et al.*, 2001b). Transfection efficiencies as determined by transfecting SFV3-lacZ control RNA followed by X-gal staining were $\sim 80\%$ for CHO-K1 or CHO-S745 cells.

Purification of LRP::FLAG from the SFV system

Transfection of BHK cells with the rec. SFV LRP::FLAG RNA was performed as described (Gauczynski *et al.*, 2001b). The total volume of the electroporated cells was plated on 10-cm dishes containing 15 ml of complete growth medium followed by incubation for at least 48 h at 37°C . Forty-eight hours post-transfection, cells were harvested, washed once with phosphate-buffered saline (PBS) and then lysed in PBS supplemented with 0.1% Triton X-100 by repeated freezing and thawing. The crude lysate was obtained by centrifugation at 14 000 r.p.m., 4°C for 15 min and purified by the batch method using an anti-FLAG M2 affinity gel (Sigma). The FLAG-tagged protein was bound over night by rotating at 4°C , washed four times with Tris-buffered saline (TBS), eluted over night by competition with 1 ml TBS containing 100 $\mu\text{g}/\text{ml}$ FLAG peptides and dialysed against 20 mM HEPES pH 7.4. The purity and the concentration of the protein was checked by SDS–PAGE followed by silver staining of the gel.

Recombinant proteins generated in the Baculovirus and Escherichia coli system

Rec. GST, GST::huPrP23–230, GST::haPrP23–89 and GST::haPrP90–231 were expressed in Baculovirus infected Sf9 cells and purified to homogeneity as described for hamster GST::PrP fusions previously (Weiss *et al.*, 1995, 1996). GST-fused PrP-peptides (haPrP23–52, haPrP53–93, haPrP90–109, haPrP129–175, haPrP180–210, haPrP218–231) were expressed in *Escherichia coli* and purified to homogeneity as described for GST fusions (Weiss *et al.*, 1995). cDNA encoding for huPrP110–128 was cloned via *EcoRI* (5') and *BamHI* (3') into pGEX-2T, GST::huPrP110–128 was expressed in *E. coli* and purified to homogeneity as described (Weiss *et al.*, 1995). All rec. proteins were dialysed against 20 mM HEPES, pH 7.4.

PrP-binding assays followed by immunofluorescence analysis

N2a, and human NT2 cells were maintained in DMEM medium containing 10% FCS, 1% glutamine, 100 $\mu\text{g}/\text{ml}$ penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. Mutant CHO cells (S745) and wild-type CHO-K1 were cultivated as described above. For competition studies, the cells were either pre-incubated for 2 h with the individual antibody diluted in culture medium or co-incubated with rec. protein and antibody (inoculum saturation). In case of pre-incubation, medium was replaced and cells were incubated overnight with 4 $\mu\text{g}/\text{ml}$ of rec. GST-fusion proteins per ml of culture medium. Cells were then washed three times with PBS and prepared for immunofluorescence microscopy, which was performed as described (Gauczynski *et al.*, 2001b).

PrP-binding assay in cell culture followed by western blotting

CHO/CHO-S745 cells (8×10^5) (either non-transfected or transfected with rec. SFV RNAs) were seeded on 6-well plates and incubated at 37°C . Twenty-four hours post-transfection, cells were incubated in medium containing 5 $\mu\text{g}/\text{ml}$ of rec. GST–huPrP23–230 for 18 h at 37°C . Together with the rec. protein, cells were co-incubated with 40 $\mu\text{g}/\text{ml}$ of HSPGs (when indicated). Cells were then washed several times with PBS and scraped off in PBS. After centrifugation the pellets were resuspended in lysis buffer (25 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM CaCl_2 , 3 mM MgCl_2 , 1% NP-40). After addition of Laemmli buffer, samples were separated by SDS–PAGE and blotted on PVDF membrane. Western blotting was performed with the monoclonal anti-PrP antibody 3B5 or the pAb LRP W3 and peroxidase-coupled secondary antibodies.

Mapping of LRP and PrP-binding sites in the yeast two-hybrid system

Constructions of plasmids pSH2-1 and pJG4-5 were described previously (Rieger *et al.*, 1997). For mapping the LRP–PrP interaction site on PrP, the following C-terminal truncated constructs of PrP were generated: pSH2-1-GST::huPrP23–93, pSH2-1-GST::huPrP23–118, pSH2-1-GST::huPrP23–127, pSH2-1-GST::huPrP23–131, pSH2-1-GST::huPrP23–143, pSH2-1-GST::huPrP23–154, pSH2-1-GST::huPrP23–181 and pSH2-1-GST::huPrP180–230. The PrP fragments were amplified by PCR using oligodeoxyribonucleotides coding for different PrP sequences flanked by a *BamHI* (5') and a *Sall* (3') restriction site. The fragments were cloned via *BamHI* and *Sall* into the vector pSH2-1-GST. All PrP constructs were confirmed by sequencing. The different bait plasmids, the prey plasmid pJG4-5-LRP44–295 and the reporter plasmid pSH18-34 (*lacZ*) were co-transformed into EGY48 cells and transformants were tested in β -galactosidase assays. Construction of the plasmid pSH2-1-GST::huPrP23–230 was described previously (Rieger *et al.*, 1997). For mapping the PrP interaction site on LRP, the C-terminal truncated constructs of LRP pJG4-5-LRP44–101 and pJG4-5-LRP44–160 were designed. The LRP fragments were amplified by PCR using oligodeoxyribonucleotides coding for different LRP sequences flanked by *EcoRI* (5') and *Sall* (3'). The fragments were cloned via *EcoRI* and *XhoI* restriction sites into the vector pJG4-5. The resulting constructs were confirmed by dideoxysequencing. The different bait plasmids, the prey plasmids and the reporter plasmid pSH18-34 (*lacZ*) were co-transformed into EGY48 cells and transformants were tested in a β -galactosidase assays.

LRP–LRP and gal-3–PrP interaction studies in the yeast two-hybrid system

The constructs pSH2-1-GST, pJG4-5-GST, pSH2-1-GST::huPrP and pJG4-5-LRP44–295 were described previously (Rieger *et al.*, 1997). The LRP44–295 encoding cDNA was PCR amplified from pJG4-5-LRP44–295. The PCR product flanked by *EcoRI* (5') and *Sall* (3') restriction sites, respectively, was cloned into the vector pSH2-1 via both restriction sites resulting in pSH2-1-LRP44–295, which was confirmed by dideoxy sequencing. Yeast transformations and dotting were carried out as described above. Total RNA was isolated from 293 cells by RNeasy kit (Qiagen). Gal-3 cDNA was amplified by RT–PCR and subcloned into pSH2-1 via *EcoRI* (5') and *Sall* (3') restriction sites resulting in pSH2-1-Gal-3, which was confirmed by dideoxy sequencing. The huPrP23–230 encoding cDNA was excised from pSH2-1-GST::huPrP23–230 and subcloned into pJG4-5 via *EcoRI* and *Sall*. The resulting plasmid pJG4-5-GST::huPrP23–230 was confirmed by dideoxy sequencing. Yeast transformations and dotting were carried out as described above.

Analysis of native LRP::FLAG by size-exclusion chromatography

The Superose 12 PC 3.2/30 column (Amersham Pharmacia) was calibrated with the LMW calibration kit in 20 mM HEPES pH 7.4. Purified LRP-FLAG (2.5 μg) expressed in the SFV system was loaded in a total volume of 25 μl . Chromatography was performed at a flow rate of 30 $\mu\text{l}/\text{min}$. The eluted LRP was detected with a UV-M II monitor at 280 nm.

In vitro interaction of PrP and LRP

Rec. FLAG::huPrP23–230 was expressed in the Baculovirus system (C.Hundt *et al.*, manuscript in preparation) according to rec. FLAG::haPrP23–231 (Rieger *et al.*, 1997) and immobilized on anti-FLAG M1 beads. Twenty microlitres of beads (1:1 slurry; 500 ng of FLAG::huPrP), were incubated in TBS supplemented with 2 mM MgCl_2 with 500 ng of rec. GST and 1 μg of GST::LRP (Rieger *et al.*, 1997),

respectively (molar ratio: 1:1) in the absence and presence of 1.5 µg HSPGs (Sigma). Homogeneity of GST::LRP, GST and FLAG::huPrP23–231 was proven on silver-stained SDS–PA gels. After 1 h at room temperature, the supernatant was removed, beads washed four times with TBS, boiled in SDS-sample buffer and analysed by western blotting developed with mAb GST.

Antibodies

pAb LRP W3 (Rieger *et al.*, 1997) was purified by protein A–Sephacryl chromatography. mAb GST (Santa Cruz Biotechnology), mAb VLA6 (Immunotech.), mAb 3B5 (G.Hunsmann), mAb SAF70 (aa 140–180 of PrP) and pAb JB007 (CEA, France), secondary fluorescein isothiocyanate (FITC), Cy3 (indocarbocyanine) and Texas Red-conjugated antibodies (used at 1:100 dilutions; Jackson Laboratories/Southern Biotechnology) were used.

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