Biol. Chem., Vol. 384, pp. 243–246, February 2003

Short Communication

Different Isoforms of the Non-Integrin Laminin Receptor Are Present in Mouse Brain and Bind PrP

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The prion protein (PrP) plays a central role in prion diseases, and identifying its cellular receptor appears to be of crucial interest. We previously showed in the yeast two-hybrid system that PrP interacts with the 37 kDa precursor (LRP) of the high affinity 67 kDa laminin receptor (LR), which acts as the cellular receptor of PrP in cellular models. However, among the various isoforms of the receptor that have been identified so far, those which are present in the central nervous system and which bind PrP are still unknown. In this study, we have purified mouse brain fractions enriched in the laminin receptor and have performed overlay assays in order to identify those isoforms that interact with the prion protein. We demonstrate (i) the presence, in mouse brain, of several isoforms of the LRP/LR corresponding to different maturation states of the receptor (44, 60, 67 and 220 kDa) and (ii) the binding of all of these isoforms to PrP. Our data strongly support a physiological role of the laminin receptor/PrP interaction in the brain and highlight its relevance for transmissible spongiform encephalopathies.
Key words: Brain / Cellular receptor / Laminin / LRP/LR / Prion protein.

A fundamental event in the pathogenesis of human and animal prion diseases is the conformational modification of a normal host-encoded protein (PrP^c) from a soluble form to an aggregated, partially protease-resistant form termed PrPsc enriched in β-sheeted structures (for review see Prusiner, 1998). The misfolded isoform (PrP^{sc}) of the prion protein accumulates in the central nervous system and in other areas, such as the lymphoreticular system, during the development of the disease. PrP^{sc} is thought to be a major component of the causative agent of transmissible spongiform encephalopathies (TSE), also called prion diseases. Proteinase K resistance is a hall mark of PrP^{sc} (Prusiner *et al.*, 1984) and PrP mutants causing familial prion diseases (Gauczynski *et al.*, 2002). Molecules interacting with PrP (for review see Gauczynski *et al.*, 2001a) that could play a role in the replication of the infectious particle, as well as the precise location where the conversion from PrP^c to PrP^{sc} take place, have to be identified (Caughey and Raymond, 1991; Telling *et al.*, 1995). It has been shown that the normal isoform, PrP^c, plays a central role in prion diseases: (i) PrP knockout mice are resistant to prion infection (Büeler *et al.*, 1993); (ii) when transgenic mice expressing different levels of PrP^c are infected with the agent of prion diseases, the duration of the incubation period is inversely proportional to the level of PrP^c expressed; (iii) the PrP gene of the host controls the species barrier (Scott *et al.*, 1989; Prusiner, 1993) and (iv) PrP^c expression is necessary for prion-induced neurodegeneration (Brandner *et al.*, 1996). Thus, characterization of the cellular receptor for the prion protein appears to be of crucial interest for understanding the mechanisms of prion replication, CNS invasion, and neurodegeneration characteristically linked to prion diseases. In the yeast two-hybrid system, we have identified the 37 kDa precursor (LRP) of the 67 kDa laminin receptor (LR) as a protein which interacts directly with PrP^c (Rieger *et al.*, 1997). Co-expression of both LRP and PrP in insect and mammalian cells has confirmed this interaction (Rieger *et al.*, 1997). Furthermore, the level of LRP, which has been described previously as a receptor for the Sindbis virus on mammalian cells (Wang *et al.*, 1992), is increased in organs that support prion replication and PrP^{sc} accumulation in experimental scrapie or bovine spongiform encephalopathy (BSE)-infected animals (Rieger *et al.*, 1997). In cellular models including primary cultures and neuronal cell lines, we demonstrated that LRP acts as the cellular receptor for PrP^c, mediating the binding and internalization of recombinant PrP^c (Gauczynski *et al.*, 2001b; Hundt *et al.*, 2001). We identified interaction domains of the cellular prion protein with LRP and proposed a model for the interaction complex of PrP with LRP/LR. In this model, heparan sulfate proteoglycans (HSPGs) would act as co-factors/co-receptors for the binding and internalization process of PrP (Hundt *et al.*, 2001) and may account for the LRP/LR polymorphism. Additional heparan sulfate binding sites have been identified in the cellular prion protein (Warner *et al.*, 2002).

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The polypeptide predicted from the non-integrin laminin receptor cDNA sequence consists of 295 amino acids and the *in vitro* translation of selectively hybridized mRNA produced a protein with an apparent molecular mass of 37 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Rao *et al.*, 1989). However, other isoforms of the laminin receptor, displaying higher molecular masses, have also been described (Castronovo *et al.*, 1991; Landowski *et al.*, 1995; Menard *et al.*, 1997; Buto *et al.*, 1998). One of these isoforms, the 67 kDa high affinity laminin receptor (LR: presumably the mature form of the receptor), is thought to arise from the heterodimerization of its precursor molecule, the 37 kDa LRP, with a still unidentified molecule (Buto *et al.*, 1998). The 67 kDa LR is believed to be the functional isoform of the receptor regarding its ability to mediate strong attachement of cells to laminin (Lesot *et al.*, 1983; Malinoff and Wicha, 1983; Rao *et al.*, 1983), to be overexpressed on cancer cell sufaces, and to promote the invasive and metastatic capacity of these cells (Menard *et al.*, 1997). Thus, it is of crucial importance to investigate whether this isoform is normally expressed in mouse brain and whether this isoform binds PrP.

We prepared brain protein fractions from uninfected mouse brain homogenates (abbreviated here as AS50: 50% ammonium sulfate fraction) according to Martins *et al.* (1997). Using antibodies recognizing specifically the LRP (W3) and the LR (ab711), we investigated which isoforms of the laminin receptor could be detected in the AS50 brain fraction. With these polyclonal antibodies, we identified 4 different isoforms of the receptor, migrating at 44 kDa (Figure 1, lane 2), 60 and 67 kDa (Figure 1, lane 1), and 220 kDa (Figure 1, lane 1). The 44 kDa isoform, which has been observed previously in cellular extract such as A431 human epidermoid carcinoma cells, corresponds to the precursor receptor LRP (Rao *et al.*, 1989; Buto *et al.*, 1998). The 60 and 67 kDa doublet bands have also been detected in A431 cellular extract and other cancer tissues (Castronovo, 1993; Buto *et al.*, 1998). The 60 kDa peptide was described as a differentially phosphorylated isoform of the mature 67 kDa Laminin receptor (Buto *et al.*, 1998). The last isoform, the 220 kDa protein, presumably corresponds to an oligomeric form of the LR. The specificity of these antibodies was confirmed since no bands were detected using the secondary antibody alone (Figure 1, lane 3). It is interesting to note the different spectrum of recognition of the laminin receptor with the two antibodies used. In the first case, Ab711, a polyclonal antibody directed against amino acids 263 – 282 of the C-terminal domain of human laminin receptor (Wewer *et al.*, 1987) recognizes only the higher molecular mass isoforms of the receptor while the antibody W3 raised against full-length LRP (Rieger *et al.*, 1997) recognizes only LRP. This suggests that the corresponding epitopes are exposed differentially depending on whether the receptor is in a precursor or mature state, and whether it is heterodimerized or oligomerized.

We next wanted to identify which laminin receptor isoform could interact with PrP. To this end we performed overlay assays according to Martins *et al.* (1997) using human GST-PrP fusion protein (GST-PrP) and the AS50 brain fraction. The integrity of the recombinant protein was first verified by western blot (Figure 2a, lane 1). For the overlay, the proteins present in the AS50 fraction were separated by gel electrophoresis and overlaid with recombinant GST::PrP. Then, using a polyclonal antibody directed against GST, we examined the binding of GST::PrP to several proteins displaying molecular masses of approximately 44 kDa and 60/67 kDa (Figure 2b,

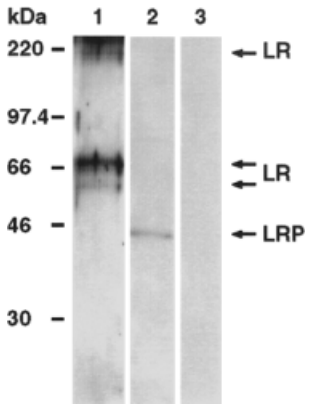

Fig. 1 Antibodies Directed against the Non-Integrin Laminin Receptor Recognize Proteins with Molecular Masses of 44, 60, 66, and 220 kDa.

A mouse brain fraction partially purified by ammonium sulfate precipitation (20 µg) was electrophoretically separated and analyzed by Western blotting using two polyclonal antibodies recognizing either the 37 kDa laminin receptor precursor or the mature 67 kDa laminin receptor. The following polyclonal antibodies were employed in this study: Ab711, directed against the peptide P20A (PTEDWSAQPATEDWSAAPTA) amino acids 263 – 282 from the C-terminal domain of human laminin receptor cDNA, and W3, raised against full-length LRP protein.

Methods: purification of murine laminin receptor by successive ammonium sulfate precipitations. A 20% murine brain homogenate was prepared as previously described (Martins *et al.*, 1997). Briefly, mouse brains were homogenized in a 20% ratio in 50 mM Tris-HCl pH 7.4, 0.2% sodium deoxycholate, 0.5% Triton X-100, 1 mM aproptinin, 1 mM leupeptin, 1 mM PMSF, and 1 mM benzamidine and then centrifuged at 12 000 *g* for 30 min. The supernatant was then subjected to successive precipitations with 30% and 50% ammonium sulfate salt. The 50% fraction precipitate (AS50), was then dissolved in 20 mM Tris-HCl, pH 7.4, and 120 mM NaCl. Protein samples were separated on 12% SDS-PAGE gels and transferred to nitrocellulose (Schleicher & Schuell; Dassel, Germany). The nitrocellulose blots were then blocked in Blotto [phosphate-buffered saline (PBS) containing 5% dry skim milk powder and 0.1% Tween 20] for 1 h and then rinsed with PBS-Tween. Blots were then exposed for 1 h to anti-laminin receptor polyclonal antibodies Ab711 (Abcam Ltd., Cambridge, UK) and W3 (Rieger *et al.*, 1997). The blots were washed three times and then exposed to anti-rabbit peroxidase-coupled secondary antibodies (Southern Biotechnology, Birmingham, USA; diluted 1:10 000). Peroxidase reactions were detected using enhanced chemiluminescence according to protocols provided by the manufacturer (Amersham).

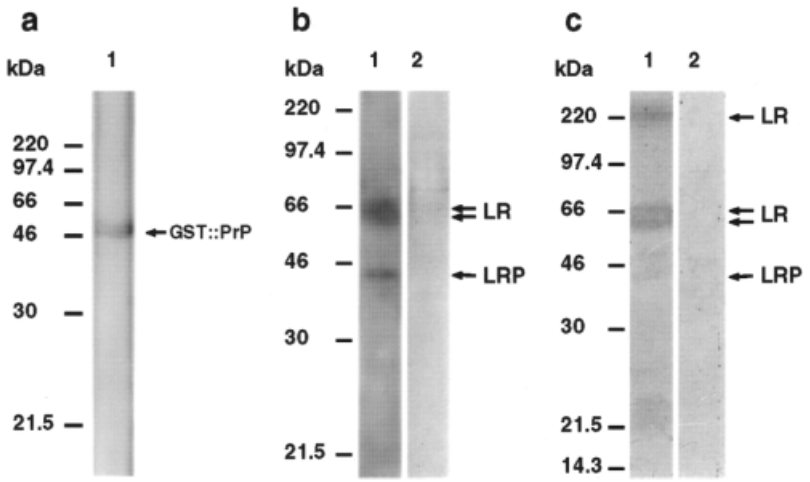

Fig. 2 All Proteins Recognized by Anti-Laminin Receptor Antibodies in the AS50 Brain Fraction Bind PrP.
(a) Verification of the integrity of the recombinant GST::PrP band probed with the PrP-specific antibody 3F4 (lane1). (b) Proteins from the AS50 brain extract were separated by Western blot and overlaid either with recombinant GST::PrP (lane 1) or with control solution (lane 2). The binding of the GST::PrP was visualized with a polyclonal antibody directed against GST. (c) Overlay of [^{35}S]-radiolabeled GST::PrP (lane 1) and GST (lane 2) on immobilized proteins of the AS50 brain fraction.
Methods: all overlay assays were performed on nitrocellulose blots with proteins separated by SDS-PAGE. Blots were incubated with 4 µg/ml of recombinant GST::PrP in PBS containing 0.05% Tween 20 for 3 h at room temperature. After three washes in PBS-Tween (0.05%), the immunoblots were further incubated with an anti-GST polyclonal antibody for 1 h. The reactive bands were visualized with anti-rabbit peroxidase-coupled antibodies. Immunoblots were developed by enhanced chemiluminescence. Overlay assays accomplished with [^{35}S]-radiolabeled proteins were performed using the same methodology without antibodies, and revealed by exposing X-ray films to the blots.
Purification and expression of heterologous proteins (GST::PrP and GST): recombinant GST::huPrP23-230 was synthesized in Sf9 cells by infection with the recombinant baculovirus AcSG2T::huPrP23-230 and radiolabeled in the presence of [^{35}S]-methionine as described for the GST::haPrP23-231 (Weiss *et al.*, 1995). Radiolabeling of GST was performed as for GST::huPrP23-230. Both proteins were purified to homogeneity as previously described in Weiss *et al.* (1995).

lane 1, arrows). The molecular mass of the bands detected corresponded exactly to those detected with LRP/LR antibodies (compare with Figure 1). A mock overlay revealed no unspecific binding of the anti-GST antibody to the AS50 brain fraction (Figure 2b, lane 2). In order to strengthen the finding that PrP binds to proteins exhibiting characteristic molecular masses for the different LR isoforms in SDS-PAGE (Figure 1), we repeated the overlay assay using [^{35}S]-radiolabeled GST::PrP which gives a better resolution of the signal. Validating our previous results, the [^{35}S]-labeled GST::PrP bound to several proteins migrating in the gel at 60 kDa, 67 kDa, 220 kDa, and weakly to the 44 kDa protein (Figure 2c, lane 1), again demonstrating the interaction between PrP and the laminin receptor. As a control, the specific interaction of GST::PrP to the proteins of the AS50 fraction was verified using [^{35}S]-radiolabeled GST (Figure 2c, lane 2).

In this study, we identified those isoforms of the high affinity laminin receptor that are expressed in the murine central nervous system and showed that all these isoforms interact with PrP. We demonstrated the specific binding of both non-radiolabeled and radiolabeled GST::PrP to the 44, 60/67, and 220 kDa isoforms. The 60/67 kDa isoform, referred to as the mature isoform, is considered to be the functional entity. Therefore, these results are suggestive of an effective role of the PrP/67 kDa LR interaction in the metabolism of PrP^{c} and presumably its pathological counterpart PrP^{sc}. Hence, further investigations of the laminin receptor as a potential therapeutic target for TSE pathologies have to be considered. Moreover, a parallel can be established between our present demonstration and a previously published study demonstrating the binding of PrP to a 60/66 kDa protein found in the AS50 murine brain fraction (Martins *et al.*, 1997). In their study, Martins *et al.* exploited a concept called complementary hydropathy, by which peptides encoded by complementary DNA strands bind to each other, and can be used to produce peptides that mimic the binding site of a receptor. Surprisingly, both receptor candidates, *i.e.* the laminin receptor and the protein isolated by complementary hydropathy (Martins *et al.*, 1997) are found in the 50% ammonium sulfate brain extract, share the same electrophoresis pattern with a doublet band at 60/67 kDa, and exhibit the same PrP binding properties in overlay assays. The 66 kDa band of the protein isolated by complementary hydropathy was recently identified as the murine stress-inducible protein 1 (STI1) (Zanata *et al.*, 2002).

Our study confirms that the non-integrin 67 kDa laminin receptor is present in murine brain and that it binds PrP. This fact along with our previous study (Gauczynski *et al.*, 2001b) demonstrating that the laminin receptor acts as the cell surface receptor internalizing

PrP supports the crucial role of this receptor as the cell surface receptor for the prion protein in the brain. In order to better comprehend the pathogenesis of prion diseases and to allow new approaches in therapeutics, the physiological role of the interaction of PrP with the various isoforms of the receptor (44, 60/67 and 220 kDa) will have to be examined by further biochemical and cell biological studies.

Acknowledgments

This study was supported by grants BIOMED PL-97-6054 and QLRT-2000-02085 of the European Union. S. Weiss acknowledges the support by grants 01-KO-0106, 0312876 (BMBF), LMU 3 and 4 (Bavarian Prion Research Foundation) and thanks R. Grosschedl and E.-L. Winnacker for valuable advice and continuous support.

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Received January 31, 2002; accepted December 2, 2002