

Absence of Interaction between Doppel and GFAP, Grb2, PrP^C Proteins in Human Tumor Astrocytic Cells

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Abstract. *Background:* The doppel protein (Dpl) is a newly recognized cellular prion protein (PrP^C)-like molecule encoded by a novel gene locus, *PRND*, located on the same chromosomal region of the PrP^C coding gene. Recently, Dpl was shown to be aberrantly expressed in astrocytic tumor specimens and in astrocytoma-derived cell lines, showing a peculiar cytoplasmic localization. Here, Dpl interactions with some of the prion-interacting proteins were studied. In particular, whether the tumor astrocytic environment is suitable for doppel interaction with GFAP and Grb2 proteins, as well as with the PrP^C protein itself, was investigated. *Materials and Methods:* In order to verify our hypothesis, an innovative mammalian two-hybrid system and co-immunoprecipitation assays were employed. *Results:* The results reported the absence of protein interactions. Our findings provided evidence that, in our astrocytoma cell-based model, Dpl does not share with PrP^C the ability to interact with GFAP and Grb2. *Conclusion:* Identifying Dpl ligands may provide new insights into the involvement of Dpl in astrocytoma tumor progression.

Astrocytomas are fairly common tumors of neuroectodermal origin that typically show a high degree of tumor malignancy. The specific pathological features of astrocytomas comprise a high degree of neoplastic cell proliferation and invasiveness within the brain peritumoral tissues and, in addition, prominent angiogenesis in the neoplastic tissue (1). The WHO grading system classifies astrocytomas into four grades (I-IV) based on the degree of malignancy, as determined by histopathological criteria (2). These different grades of tumors can be accurately distinguished from each other by a relatively small number

of genes, predominantly involved in such critical processes as cellular proliferation, proteosomal function, energy metabolism and signal transduction (3). A more detailed knowledge of the mechanism underlying tumor progression will allow cancer treatment by specifically targeting deregulated pathways, and lead to the rational design of future treatment modalities, that will be tailored according to the biology of each individual tumor (4). Recently, we described a novel putative tumor marker, doppel protein, the expression of which is altered in astrocytic and hematological tumors (5, 6).

Doppel protein (Dpl, downstream prion protein-like) is encoded by the *PRND* gene (7). *PRND* and its close upstream gene, *PRNP*, coding for the cellular prion protein PrP^C (8), form a small gene family (9, 10). The Dpl protein shows approximately 25% identity with the C-terminal portion of PrP^C and lacks the N-terminally-located octameric repeats and the hydrophobic region. Both the proteins are GPI-anchored membrane polypeptides (11). PrP^C is highly expressed in the central nervous system (CNS) and its protease-resistant form, PrP^{Sc}, is implicated in the pathogenesis of the prion diseases (12). The cellular functions of Dpl and PrP^C are still unknown. NMR studies of PrP^C (13) and Dpl (14) revealed a high structural similarity, raising the possibility that Dpl may be capable of replacing PrP^C *in vivo*. Current data suggest that Dpl is probably not required for the pathogenesis of prion diseases (15) and is most unlikely to be converted into a PrP^{Sc}-like isoform (16). However, Dpl can cause Purkinje cell death and ataxia, when ectopically expressed in *Ngsk*, *Rcm0* and *Zrch II Prnp^{0/0}* transgenic mice (reviewed in (17)) and the neuropathology is prevented by the introduction of a full-length PrP^C (18). These results have been explained by a common mechanism, involving competition between PrP^C and Dpl (19). Recently, a lot of data has been published regarding the function of Dpl: during mouse embryogenesis, Dpl is expressed in the endothelial cells of the CNS, which argues for a possible function of the protein in brain development (20); in adulthood, Dpl is expressed at high

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levels mainly in the testis, and an involvement of the protein in male fertility has been recently proposed in human and mouse (21, 22).

Several efforts have been made in order to investigate the function of PrP^C, by studying interacting proteins, with many candidates being found, although several appear to be located in cellular compartments that are not compatible to sustain PrP^C interaction (23). Conversely, to date, only one PrP^C-interacting protein, namely the 37kDa/67kDa laminin receptor (LRP), has been analyzed for Dpl interaction. In a recent study, Hundt and Weiss described the failure of Dpl::LRP proteins interaction, using a yeast two-hybrid system (24). Additionally, the authors reported the absence of interaction between Dpl::Dpl and Dpl::PrP^C proteins. Differently, Yin and colleagues described a Dpl::LRP proteins interaction through a yeast two-hybrid system, suggesting the existence of a molecular Dpl-PrP^C antagonism (25).

In the astrocytic tumors that we have recently studied, Dpl is ectopically expressed in the cytoplasm and this finding prompted us to test whether some of the known prion-binding cytoplasmic proteins could interact with Dpl too. To that end, Dpl interaction was investigated, in our tumor scenario, with two known prion cytoplasmic-interacting proteins, namely the growth factor receptor-bound protein 2 (Grb2) and the glial fibrillary acidic protein (GFAP), as well as with the PrP^C protein itself. To test the interactions of the proteins in astrocytomas, D384 MG cells were employed, a clone derived from a human astrocytoma cell line (26). To study Dpl-interacting candidate proteins, a two-hybrid system in mammalian cells and the co-immunoprecipitation assay were used.

Materials and Methods

Generation of plasmids. All plasmids used for the experiments were included in the Mammalian Matchmaker Two-Hybrid Assay Kit (Clontech, Palo Alto, CA, USA). The coding sequence (CDS) of the human Dpl gene encoding 26-152 fragment and the human PrP^C gene encoding 23-230 fragment were amplified by PCR from commercial genomic human DNA (Clontech) using appropriate primers (Table I), including *Eco*RI and *Xba*I restriction sites, and ligated in frame into the pMGAL4DNA-BD and pVP16-AD vectors. *GRB2* CDS was amplified from the pOTB7 plasmid clone (RZPD Center, Berlin, Germany), while *GFAP* CDS was amplified from commercial human adult brain cDNA (Clontech). For co-immunoprecipitation assay, the DNA fragments of the CDS were sub-cloned into the pCMV-HA and pCMV-Myc plasmids, using primers that incorporated *Eco*RI and *Not*I restriction sites (Table I). Each construct was sequenced to verify the correct in-frame cloning.

Cell culture and transfection. D384 MG human astrocytoma cells (a kind gift of Prof. M. Ceroni) was used for the two-hybrid and for the co-immunoprecipitation assays. Cells were maintained at 37°C, 5% CO₂, in Dulbecco's Modified Eagle's Medium (D-MEM)/F-12

Table I. Oligonucleotides.

Mammalian two-hybrid:	
Name	Sequence (5'-3')
Dpl-U	CCGGAATTCCGGACGAGGGGCATCAAGCACA
Dpl-L	TGCTCTAGAGCAGCCCCTCTCCAACCAAAAC
PrP-U	CCGGAATTCCGGAAGAAGCGCCCGAAGCCT
PrP-L	TGCTCTAGAGCAGATCCTCTCTGGTAATAGG
GFAP-U	CCGGAATTCCGGATGGAGAGGAGACGCATCAC
GFAP-L	TGCTCTAGAGCATCACATCACATCCTTGTGCTC
Grb2-U	CCGGAATTCCGGATGGAAGCCATCGCCAAATATG
Grb2-L	TGCTCTAGAGCATTAGACGTTCCGGTTCACGG
Co-immunoprecipitation:	
Name	Sequence (5'-3')
Dpl-1U	GGAATTCCACGAGGGGCATCAAGCACA
Dpl-1L	ATTGCGGCCGCTTAGCCCCTCTCCAACCAAAAC
PrP-1U	GGAATTCCCAAGAAGCGCCCGAAGCCT
PrP-1L	ATTGCGGCCGCGATCCTCTCTGGTAATAGG
GFAP-1U	GGAATTCCCATGGAGAGGAGACGCATCAC
GFAP-1L	ATTGCGGCCGCTTATCACATCACATCCTTGTGCTC
Grb2-1U	GGAATTCCCATGGAAGCCATCGCCAAATATG
Grb2-1L	ATTGCGGCCGCTTAGACGTTCCGGTTCACGG

supplied with 10% fetal calf serum, L-glutamine (1%), 100 U/ml penicillin and 0.1 mg/ml streptomycin. HeLa cells, used for the co-immunoprecipitation assays, were maintained at 37°C, 5% CO₂, in D-MEM supplied with 10% fetal calf serum, L-glutamine (1%), 100 U/ml penicillin and 0.1 mg/ml streptomycin. The cell culture media and supplements were provided by Invitrogen (Paisley, UK). Cell transfections were done in 24-multiwell plates with Lipofectamine 2000 (Invitrogen). The transfection conditions were optimized as follows: treatments were made at 95% confluence using, for single well, 1 µg of each plasmid with 2 µl of lipofectamine reagent per µg DNA in HeLa cells, and 3 µg of each plasmid with 1 µl of lipofectamine reagent per µg DNA in D384 MG cells. Transfection efficiencies were evaluated with a ONPG-based β-Gal assay; briefly, 1 µg of pSV-βGal vector (Promega, Madison, WI, USA) was co-transfected with the two-hybrid plasmids, the cells were lysed and 100 µg of each total protein extract was added to an ONPG cocktail solution (77 mM sodium phosphate buffer, 0.88 mg/ml ONPG, 10 mM magnesium chloride, 45 mM β-mercaptoethanol) and absorbance at 405 nm was measured with a microplate reader (Titertek Multiskan).

Mammalian two-hybrid assay and CAT assay. The two-hybrid assay was performed with the Mammalian Matchmaker Two-Hybrid Assay Kit (Clontech). "Bait" and "prey" plasmids, pMGAL4DNA-BD and pVP16-AD, were co-transfected into the D384 MG cells along with a reporter plasmid, pCAT5. Transfection efficiencies were evaluated by means of the ONPG-based β-Gal assay described above. Cell lysates were produced 24 h after transfection by rinsing the cells twice with ice-cold PBS, and incubating in lysis buffer (Roche, Mannheim, Germany) for 30 min at room temperature. Cell debris was removed by centrifugation at 13,000 rpm for 10 min at 4°C and the supernatants were supplemented with Complete

Mini Protease Inhibitor Cocktail (Roche). The protein content was measured by a standard Bradford assay. Equal amounts of proteins (150 µg) for each sample were analyzed by CAT (chloramphenicol acetyl transferase) assay (Roche), according to the manufacturer's instruction. CAT readouts were measured with a microplate reader (Titertek Multiskan) and normalized to the ONPG-based β-Gal assay values.

Co-immunoprecipitation. Total protein extracts were obtained by rinsing the cells twice with ice-cold PBS followed by the addition of ice-cold lysis buffer (1% Triton X-100, 75 mM NaCl, 50 mM Tris-HCl pH 8.0) and incubated for 30 min in ice. Cell debris removal and recovery of the supernatants were as described above. Lysates corresponding to 50-100 µg of total proteins were eventually incubated with 1.5 µg of the relevant antibody, as indicated in Figure 1, for 3 h at 4 °C with gentle agitation. Forty µl packed pre-washed protein G Sepharose beads (Immunoprecipitation Starter Pack, Amersham Biosciences, Freiburg, Germany) were added to each protein extract and incubated for 1 h with gentle agitation. The beads were washed five times with ice-cold lysis buffer, and the conjugated proteins were eluted by incubation at 95 °C for 5 min in 20 µl of Laemmli sample buffer (2% SDS (w/v), 6% glycerol (v/v), 150 mM β-mercaptoethanol, 0.2% bromophenol blue (w/v), 62.5 mM Tris-HCl pH 6.8) and subjected to SDS-PAGE.

Immunoblot analysis. The samples were boiled for 5 min in Laemmli sample buffer and electrophoresed by SDS-PAGE (15% gel). The proteins were transferred onto a nitro-cellulose membrane (Hybond-C Extra, Amersham Biosciences). The membranes were blocked with 2% non-fat milk in PBS containing 0.1% Tween 20 (Amersham Biosciences). The commercial monoclonal anti-c-Myc (Clontech) and rabbit polyclonal anti-human Dpl antibodies (provided by Prof. Valentini) were used at 1:10000 dilution. The blots were treated with peroxidase-coupled secondary antibodies (1:10000 dilution) and the protein signals were revealed by the ECL Advance Western Blotting Detection Kit (Amersham Biosciences).

Results

The mammalian two-hybrid system was employed to verify the interaction between Dpl and GFAP, Grb2 and PrP^C proteins in human astrocytoma-derived cells. First, the construct Dpl26-152 was used as "bait", and the *PRND* coding sequence cloned in the pMGAL4DNA-BD vector (Clontech). Conversely, GFAP, Grb2 or PrP^C23-230 were assayed as "preys" and cloned in the pVP16-AD vector (Clontech). All the constructs were then reversed by inverting the bait-prey modules in the plasmids. Each construct was sequenced and the corresponding protein expression was verified, after transfection, by Western blot (data not shown).

"Bait" and "prey" plasmid constructs pairs were co-transfected into the D384 MG cells. Protein interactions were determined through the measurement of the reporter gene expression CAT, the absorbance values were normalized with the cellular transfection efficiencies

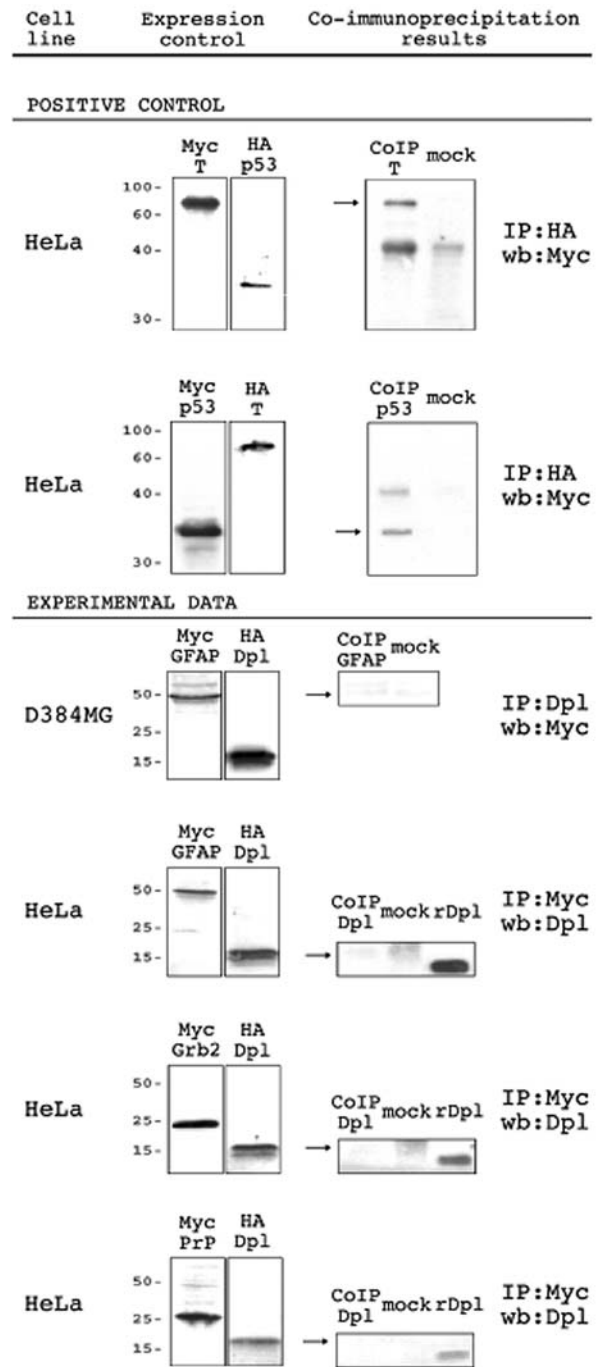


Figure 1. Expression of the transfected constructs (left column) and co-immunoprecipitation results (right column) in the D384 MG and HeLa cells. Molecular weights (kDa) are reported. Arrows indicate the expected co-immunoprecipitation resulted fragments.

resulting from the β-Gal colorimetric assay. To test the efficiency and sensitivity of the mammalian two-hybrid interaction assay, positive and negative controls, provided by the manufacturer, were used; in addition, the previously

Table II. Mammalian two-hybrid results (D384 MG cells).

pM GAL4 DNA-BD*	pVP16 AD*	Interaction
Dpl(26-152)	GFAP	+/-
Dpl(26-152)	Grb2	-
Dpl(26-152)	PrP ^C (23-230)	-
PrP ^C (23-230)	GFAP	++
PrP ^C (23-230)	Grb2	++
p53	SV40 T antigen	++++ ^(a)
p53	CP (viral coat protein)	-(b)

^(a)positive control

^(b)negative control

*Inverted configurations of bait and prey gave consistent results.

reported interactions of PrP^C with GFAP and Grb2 protein (27, 28) were also tested. As shown in Table II, a faint indication of interaction between Dpl26-152::GFAP proteins and stronger interactions between PrP^C23-230::Grb2 and PrP^C23-230::GFAP proteins were found, compared to the controls; no interaction was revealed by the Dpl26-152::Grb2 and Dpl26-152::PrP^C23-230 protein combinations.

We sought to confirm the finding regarding the Dpl26-152::GFAP protein interaction using a co-immunoprecipitation assay in the D384 MG cells; HeLa cells were employed as an additional cellular control. Co-immunoprecipitation was also used to confirm once more the absence of interaction of Dpl26-152 with Grb2 and PrP^C23-230 proteins, respectively. The genes were cloned into the pCMV-HA and pCMV-Myc vectors (Clontech), using the described oligonucleotides reported in Table I. The corresponding proteins were co-immunoprecipitated and visualized in Western blot using specific antibodies, namely human anti-c-Myc and anti-Dpl, as described in Figure 1. Control experiments were set up in order to verify the specificity and sensitivity of the co-immunoprecipitation assay. Briefly, the coding region of the murine p53 72-390 fragment and of the SV40 large T antigen 87-711 fragment were cloned into the pCMV-Myc and pCMV-HA vectors and co-transfected into HeLa cells; protein extracts were subjected to the co-immunoprecipitation assay. Control experiments, as expected, highlighted p53::SV40 large T antigen proteins interaction (Figure 1); differently, interaction between Dpl26-152 and GFAP proteins was absent (Figure 1). As illustrated in Figure 1, the two proteins did not co-immunoprecipitate, either in the D384 MG or in HeLa cells. In addition, Figure 1 shows the results of Dpl26-152::Grb2 and Dpl26-152::PrP^C23-230 co-immunoprecipitations in HeLa cells; even in this case, no interaction between the investigated proteins was found.

Discussion

Recently, we described a significant expression of the doppel protein in brain tumors, suggesting a possible role of this protein in tumor progression. Dpl has been found abnormally expressed in the cytoplasm of astrocytoma specimens and in astrocytoma-derived cell lines (5). Similar evidence was reported in different neoplasms such as those of hematological origins (6).

In this study, we aimed to address possible Dpl functions within astrocytoma tumors, assaying protein interactions with two described PrP^C-interacting proteins, GFAP and Grb2 (27, 28), using the human astrocytoma-derived cell line clone, D384 MG, as an experimental model. To this purpose the Mammalian Matchmaker Two-Hybrid Assay Kit (Clontech) was used, based on the standard two-hybrid approach proposed by Fields and Song (29), which enables the determination of an interaction between two candidate proteins in mammalian cells. This innovative assay allows the verification of protein interactions resulting from a wide screening, such as a two-hybrid assay. Astrocytoma D384 MG cells were chosen because of their low endogenous Dpl expression level (5) that could interfere with the assay; besides, among available glial tumor cells, D384 MG was selected because of its high transfection efficiency (data not shown). Among the numerous PrP^C-interacting proteins that have been discovered, GFAP and Grb2 share a common cytoplasmic localization with Dpl in astrocytic tumors. Grb2 is a cytosolic, signal transduction adaptor protein involved in the epidermal growth factor-controlled signal transduction pathway that takes part in the regulation of the cytoskeleton and DNA synthesis during cell differentiation and development (30, 31). GFAP is an intermediate filament protein, specific for the cytoskeleton of the astrocytes and its function is mostly structural, *i.e.* to maintain the astrocyte cell shape; furthermore, GFAP is considered a reliable marker of differentiation for normal astrocytes and for tumor of the astrocytic lineage (32).

However, our results excluded a direct and detectable interaction between these proteins: a faint measurable interaction between Dpl and GFAP proteins was described using the mammalian two-hybrid system, but not confirmed by the co-immunoprecipitation assay. Considering the latter assay more specific, this result could reflect an absence of interaction between the two proteins.

In addition, despite the structural similarities between cellular prion protein and Dpl, the absence of Dpl::PrP^C proteins interaction was also confirmed, in agreement with previous studies performed in mice testis (33), in Madin-Darby Canine Kidney cells (34) and using a yeast two-hybrid system (24). Yet, in different cells like neuroblastoma SK-N-BE, Massimino and colleagues suggested a possible interaction between Dpl and PrP^C in similar membrane

microdomains (35). The interaction between Dpl and PrP^C is still an open question that requires much effort to elucidate, as the results concerning these studies are probably dependent on the specific cell or tissue investigated.

Among the central challenges of brain tumors are the identification of markers for improved diagnosis and classification of tumors, and the definition of targets for more-effective therapeutic measures. Proteomics approaches to tumor markers identification hold the promise of identifying specific protein modifications in tumor tissues to assist in individualizing treatments for certain cancers. Our recent data describe Dpl expression in some types of cancer; particularly, Dpl in astrocytic tumors may play a role in tumor progression. It is conceivable that the function of Dpl in these tumors and its role in tumor progression might be further investigated through a more extensive screening for Dpl potential interacting candidates in the tumor specimens by means of a yeast two-hybrid assay (work in progress).

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